THE ROLE OF EXTRACELLULAR ATP IN THE CONTROL OF LOCAL BLOOD FLOW

By

Thomas Forrester, M.B.Ch.B., Ph.D.

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Submitted in candidature for the degree of Doctor of Medicine to The University of Glasgow

Research performed in the Institute of Physiology, University of Glasgow, the Wellcome Surgical Research Institute, University of Glasgow, Indiana University Cardiopulmonary Laboratory and the Department of Physiology, Saint Louis University Medical Centre.

July 1981

Dedicated to my Family
for their patience and
support, on both sides
of the Atlantic

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ACKNOWLEDGEMENTS

It would not be possible to acknowledge individually the many fruitful discussions that I have experienced on the topic of extracellular ATP, but they can be generally grouped into 'early,' 'challenging' and 'late affirmatory.' It is a privilege and great pleasure to thank Professor Ian. A. Boyd, my first research supervisor, who was initially confronted with some strange results from me but managed, at a difficult stage, to keep me going on a scientific basis. Other early discussions must include, of course, Professor R.C. Garry, whose great knowledge of the history of physiology enabled him to cite many examples of serendipity which were consoling to me. Professor John S. Gillespie gave me an early insight into the rigors of presenting new and controversial material; his advice will always be remembered.

Challenging discussions from Professor Henry Barcroft, Professor John T. Shepherd, Professor G. Burnstock and Dr. Robert Berne provided much useful criticism and comment that was of inestimable value for later review work. I would like to include here another group of challenging discussions, and these are probably the most fruitful of all in any laboratory. It was my privilege to supervise Dr. Peter Fletcher, Dr. Osman Hassan, Dr. Carole Williams and Dr. Mark Clemens for their dissertations. Throughout those periods of planning and experimentation there were many discussions, mostly spontaneous, but always rigorous. I acknowledge their enquiring minds, doubting everything, accepting very little on face value, and eventually helping to forge parts of the story as presented in this volume.

'Late affirmatory' discussions with other workers in related fields conveyed the pleasant feelings of results confirmed. I thank Dr. Maurice Israel for organizing a symposium on extracellular nucleotides under the auspices of the Gif Lectures in Neurobiology, held at Gif-sur-Yvette, France. His efforts brought together for the first time the many people in varying disciplines who were studying extra-

cellular nucleotide action. The frequent exchanges that took place in Gif were of great value to all who were able to attend these lectures.

I am grateful to many other people for their support and collaboration during different times in the period 1968 - 81 and such support has been acknowledged specifically in the published work. In the same way the financial support throughout these projects is acknowledged where appropriate.

SUMMARY

The functions of intracellular adenosine triphosphate (ATP) have been intensively studied for fifty years. However the present collection of papers assembles evidence supporting a physiological role for nucleotides extracellularly. The fact that the extracellular actions of ATP are produced by concentrations of exogenous ATP so much lower than those found on the other side of the cell membrane would indicate that the membrane itself is intimately involved. The action of nucleotides on vascular smooth muscle is one which has been known for as long as the history of these compounds. ATP is a powerful dilator of blood vessels in skeletal and cardiac muscle. Evidence is provided here that ATP is one of the major contributors to the process of vasodilatation in these tissues when an extra supply of oxygen is required.

ATP has been detected in the solution bathing an active frog sartorius muscle, in the perfusate from perfused frog hindlimb during contraction, in the perfusate from resting and active cat soleus muscle and in the venous effluent from exercising human forearm muscle. The rate of degradation of ATP in human plasma was assessed and a comparison was made of the amounts of ATP released from human forearm muscle contracting at 5% of the maximum voluntary contraction and the amounts necessary to be infused to cause the same increase in blood flow. It was calculated that 7.5 - 10.5 ug/min (14 - 20 nmole/min) were released, comparing favourably with the 16 ug/min infused by Duff, Patterson & Shepherd (1954) to produce the same increase in blood flow. It was concluded that ATP was released in enough quantities to satisfy the vasodilator requirements of human forearm muscle contracting at 5% of maximum voluntary contraction.

Release of ATP into the perfusate from heart muscle in response to hypoxia was first demonstrated by Paddle & Burnstock (1974). Using a working rat heart preparation, the appearance of ATP in the coronary sinus effluent was detected in response

to 90 seconds of hypoxia. Since the percentage recovery of ATP perfused through the coronary circulatory bed was approximately 1%, it was concluded that ATP played a significant role in the vasodilatation seen in response to myocardial hypoxia. It was not certain whether the ATP came from nerves, vascular smooth muscle or myocardial cells. The use of isolated adult rat heart cells determined one source of the ATP released in response to hypoxia. Release of ATP was increased fourfold within 30 seconds of rendering the cells hypoxic. When the cells were restored to oxygenated conditions the release of ATP reverted to control levels. The amounts of ATP released were calculated to fall well within the range necessary for production of near maximum vasodilatation of the coronary vascular bed.

In view of the probable major role that ATP plays in the control of local blood flow in skeletal and cardiac muscle, the effects of ATP, AMP, adenosine, pyrophosphate and phosphate on the cerebral vasculature were explored. Perivascular application of ATP to cat pial arterioles caused dilatation at a threshold concentration of 10^{-11}M. This amount compares with the amounts of adenine nucleotide detected by Pull & McElwain (1972) being released from electrically stimulated brain slices. It was thus concluded that ATP is released in amounts which are more than adequate to produce near maximum local dilatation in brain tissue.

Intracarotid administration of ATP produced surprising results in both cat and baboon. In both cases the cerebral blood flow was approximately doubled when ATP was infused into the carotid artery at a rate of 10^{-6}mole/min.. Taking into account the carotid flow in each case, the calculated threshold concentration in cats was 4×10^{-9}M and in baboons 4×10^{-8}M. No significant effects were seen with AMP, pyrophosphate or inorganic phosphate. The threshold response to intracarotid adenosine lay between 4×10^{-7}M and 4×10^{-6}M in the baboon. Intracarotid ATP increased the oxygen consumption of the baboon brain parenchyma. This effect was attributed in part to an elevation of cellular cyclic AMP levels in the brain. Osmotic disruption of the blood-brain barrier with urea did not affect the vasodilatory or metabolic response to intracarotid ATP.

This work has defined and substantiated the extracellular role of ATP as a regulator of local blood flow in exercising skeletal and cardiac muscle. Upon critical review of the literature the role of adenosine is most probably in the modulation of post-exercise hyperaemia. The significance of circulating nucleotides and their derivatives is emphasized by the dramatic results obtained with intracarotid infusions of ATP. If adenine nucleotide levels in the circulation can be raised, and ATP itself can have an effect beyond the blood-brain barrier on the brain metabolism, then the concept of nucleotides having an 'hormonal' function in exercise must be considered. The results presented throughout this work provide complimentary evidence for the existence of purine receptors as proposed by Sattin & Rall (1970) and Burnstock (1978).

INTRODUCTION

This collection of published work represents the development of some ideas concerning the physiological significance of extracellular nucleotides. The unusual finding that a substance released from contracting frog skeletal muscle could actually have a stimulatory action when perfused through an isolated frog heart has led to many interesting experiments and conclusions. The identification of the substance as adenosine triphosphate (ATP) is briefly dealt with in the first abstract and covered in detail in the second paper. The identification and assay of ATP released from active frog skeletal muscle formed a substantial part of a dissertation presented as a thesis for Doctor of Philosophy to the University of Glasgow (1967). The remainder of the material covers the period 1968 - 81 during which time many new ideas and concepts were developed concerning the actions of extracellular nucleotides.

Seventeen of the twenty-one papers presented were published in The Journal of Physiology, ten of those being in abstract form. Abstracts which are published in The Journal receive a rather unique form of peer review. The Physiological Society precirculates among its members abstracts submitted for presentation at a scientific meeting of the Society. A ten minute oral presentation is followed by a five minute period of discussion. A vote is then taken from the membership as to the suitability of the abstract for publication in The Journal.

Three papers are of a review nature (papers 18, 19 & 21). The first of those was the result of an invited lecture given at Gif-sur-Yvette, France, under the auspices of the Gif Lectures in Neurobiology. The second was the result of an invitation to participate in a Tutorial Lecture Series given by the American Physiological Society in October, 1978. The third review paper, 'Adenosine or Adenosine Triphosphate?' was the response to participate in the satellite symposium 'Mechanisms of Vasodilatation' held in Antwerp as part of the

XXVIIIth International Congress of Physiological Sciences held in Budapest, July, 1980.

Commentary - paper 1.

This short abstract introduces the original methods of approach to the problem of identification of a substance released from contracting frog sartorius muscle into a surrounding bathing solution. The pronounced stimulatory effect on the frog heart is shown with the effect on extract of firefly tails included in the insert. The identification of the substance as ATP seemed secure since firefly extract responds to ATP but hardly at all to ADP, while the frog heart responds in a specific manner to adenosine triphosphate but in a very different fashion to the other purine and pyrimidine nucleotides (see Fig. 5 next paper for response to ITP and UTP).

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Release of adenosine triphosphate from active skeletal muscle

By T. FORRESTER. *Institute of Physiology, University of Glasgow and the Boyd Medical Research Institute, Glasgow*

In a previous communication (Boyd & Forrester, 1965) it was shown that active frog sartorius muscle liberates a substance which has a pronounced stimulatory effect on the perfused frog heart preparation of Boyd & Eadie (1961).

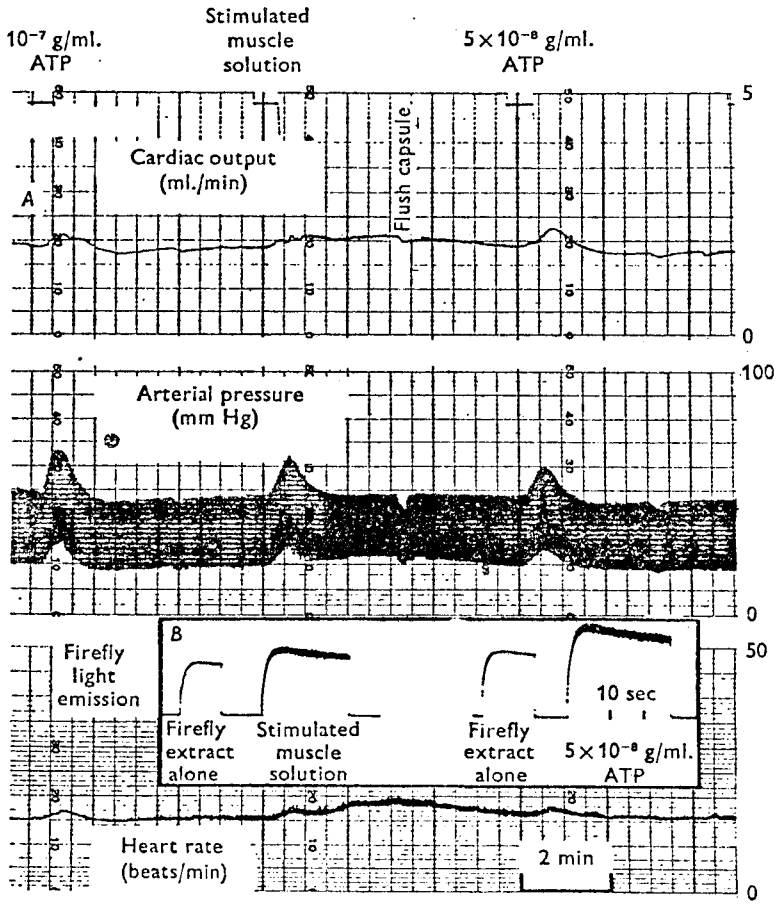

Fig. 1(*a*). The response of a frog heart to perfusion with ATP and with Ringer's solution in which a twitching sartorius muscle was bathed. (*b*) The emission of light from firefly tail extract when exposed to ATP and the stimulated muscle solution used in (*a*) above.

[P.T.O.

It has now been established that this stimulatory effect was not due to changes in the potassium or the calcium concentrations in the Ringer's solution bathing the twitching sartorius muscle, nor was it due to release of catechol amines from the muscle, since the effect was not blocked by adrenergic blocking agents.

It appears that the stimulatory effect on the heart is produced by adenosine triphosphate (ATP) released from active muscle. The evidence is as follows:

1. The action of ATP on the frog heart is qualitatively similar to that of the stimulated muscle solution (Fig. 1*a*).

2. In a chromatography procedure the stimulatory substance is eluted through a Sephadex column in the same fractions as adenosine triphosphate.

3. The enzyme 'apyrase' catalyses the reaction ATP–AMP (Kalckar, 1943). Adenosine monophosphate does not stimulate the heart. When a solution of ATP is incubated with apyrase, its stimulatory action on the heart is greatly modified. When a stimulated muscle solution is incubated with apyrase, its stimulatory action is also modified in the same manner.

4. Using a modification of the luciferase method of assaying the ATP. (Strehler & McElroy, 1957) it has been shown that the stimulated muscle solution causes light to be emitted from a firefly tail extract (Fig. 1*b*). The time course of the emitted light from the test solution was similar to that of a known solution of ATP in a similar concentration.

From these results it is concluded that ATP is released from active frog skeletal muscle *in vitro*.

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Commentary - paper 2.

This is the full account of the measurement of ATP released from muscle. At this juncture a valid criticism was that only damaged cells could act as the source. The only argument presented here against the possibility of damage was the fact that the potassium levels in the bathing solution remained at control levels throughout a period of 30 minutes soak (see Fig. 18a, Ph.D. Thesis). Obviously there would not be the 'stirring' effect that would take place when the muscle twitched. Further discussion on damage is addressed in paper 7 (Forrester & Hassan).

It is to be noted that the signals with the firefly test have not the rapid rise time seen in later results (see paper 6, Fig. 2). This does not reflect the rate of reaction of ATP with the luciferase enzyme but is merely a function of the time constant of the powerpack used to deliver the voltage across the photomultiplier tube.

The major issue of the mode of release from muscle has not been raised yet. The suggestion that ATP might be released by way of the transverse tubular system was naive but has not been completely ruled out since that time. The last short paragraph indicates for the first time in this work the possibility that ATP might be involved with local control of blood flow.

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With 10 *text-figures*
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THE RELEASE OF ADENOSINE TRIPHOSPHATE FROM FROG SKELETAL MUSCLE *IN VITRO*

By I. A. BOYD and T. FORRESTER

From the Institute of Physiology, University of Glasgow and the Boyd Medical Research Institute, Glasgow

(*Received* 20 *May* 1968)

SUMMARY

1. Active frog sartorius muscle *in vitro* liberates a substance into the bathing solution which has a pronounced stimulatory action on the frog heart.

2. The stimulatory effect is not due to an increase in the K^+ concentration of the bathing solution, nor is it due to the liberation of catecholamines.

3. In a molecular sieve chromatography procedure the stimulatory substance can be eluted in a single fraction which shows a maximum absorption of U.V. light at a wave-length of 265 nm, indicative of the presence of substances containing a purine ring.

4. Low concentrations (10^{-7}–10^{-8} g/ml.) of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and uridine triphosphate (UTP) have a marked stimulatory action on the frog heart. The action of ATP and ADP on the heart is qualitatively very similar to that of the muscle bathing solution, while the action of UTP is distinctly different. The triphosphates of inosine, cytidine and guanosine stimulate the heart when in high concentration only. Adenosine and adenosine monophosphate do not stimulate the heart.

5. Incubation of the muscle bathing solution and of solutions of ATP with the enzyme apyrase for the same time produces a similar marked reduction in the stimulatory action of both on the heart. Apyrase catalyses the break-down of nucleotide triphosphates to monophosphates.

6. The elution behaviour of the stimulatory substance determined by molecular sieve chromatography is the same as that for ATP.

7. The muscle bathing solution causes light to be emitted from firefly lantern extract, the pattern of light emission being similar to that produced by nucleotide triphosphates.

8. The concentrations of ATP having the same quantitative action on

the frog heart and on firefly extract as a given muscle bathing solution are almost identical, whereas the matching concentrations of ADP and UTP in the two methods of assay are widely different.

9. It is concluded that ATP is released from active frog skeletal muscle *in vitro*. This release may play an important part in the reactive hyperaemia of muscular exercise since ATP has a powerful vasodilator action.

INTRODUCTION

During an attempt to assay acetylcholine (ACh) released from frog skeletal muscle on the sensitive perfused frog heart preparation of Boyd & Eadie (1961) it was found that the bathing solution in which a sartorius muscle had twitched repeatedly for some time produced a pronounced augmentation of the heart beat (Boyd & Forrester, 1965). Clearly some stimulatory substance or substances were liberated during muscle activity in sufficient quantity to obscure the inhibitory action on the heart of any ACh present in the muscle bathing solution. This paper describes experiments which establish that the principal stimulatory substance in the muscle bathing solution was adenosine triphosphate (ATP), and that ATP is released from active skeletal muscle *in vitro* in amounts which can be measured accurately by both physiological and biochemical assay techniques.

The problem of identification of the stimulatory substance was approached in several ways. In the first place the changes in the pH and ionic composition of the bathing Ringer fluid following activity of the muscle were measured and tests conducted to show that these changes could not account for the stimulatory action of the muscle bathing solution on the frog heart. Secondly, the muscle bathing solution was tested by various techniques for the presence of substances known to be concerned in the activity of skeletal muscle. Thirdly, the qualitative action of many of these substances on the perfused frog heart was compared with that of the muscle bathing solution. Simultaneous measurement of heart rate, arterial pressure and cardiac output made it possible to differentiate the time course of action of any substance on the pace-maker and on the cardiac muscle itself (Boyd & Pathak, 1965). Thus, although a variety of substances stimulate the frog heart, the over-all qualitative action of any one substance is accurately reproduced in repeated tests and is almost specific for that substance.

By these means the stimulatory substance was shown to be one of the nucleotide triphosphates or diphosphates. Final proof that it was, in fact, ATP was obtained by demonstration of its almost complete destruction by a specific enzyme, by measurement of its elution behaviour in

molecular sieve chromatography, by the pattern of light emission produced from firefly lantern extract, and by comparative quantitative assay on frog heart and firefly lantern extract.

METHODS

Single sartorius nerve–muscle preparations from either *Rana temporaria* or *R. pipiens* were used. The muscle was washed for 10 min in Ringer solution of the following composition: NaCl 109 mM; $NaHCO_3$ 4·3 mM; KCl 3·8 mM; $CaCl_2$ 1·6 mM; NaH_2PO_4 0·22 mM; glucose 5 mM; neostigmine 10^{-6} g/ml. The neostigmine was included to prevent hydrolysis of ACh which was being collected for assay in the same solution. The muscle was then immersed in 2 ml. of Ringer solution, previously aerated with a 95 % O_2, 5 % CO_2 gas mixture, in a glass container. The nerve was suspended in air above the Ringer solution and lay across two platinum wire electrodes within the container which was corked so that the nerve remained moist. The muscle lay unsupported in the Ringer solution so that damage to the muscle due to tearing from any points of attachment was avoided. The muscle was made to twitch repetitively by the application of 4 V square pulses, supramaximal for the muscle twitch, to the nerve at a frequency of 2 pulses/sec for a period of 30 min. Thereafter, the muscle was left to soak for a further 5 min in the same bathing solution which was then analysed in a number of ways.

Frog heart perfusion system. The heart was perfused continuously *in situ* through the posterior vena cava with Ringer solution identical to that used for bathing the sartorius muscle. The perfusion system was that of Boyd & Eadie (1961) in which the heart worked against a constant resistance and the arterial pressure, cardiac output and heart rate were simultaneously recorded on a moving chart. Since small changes in perfusion pressure may produce significant changes in cardiac performance, the perfusion (venous) pressure was monitored continuously. A perfusion system driven by constant air pressure was developed so that 1 ml. samples of any solution to be tested could be perfused through the heart in place of the standard Ringer solution without any appreciable alteration in venous pressure either at the start, during, or at the end of the test.

Measurement of K^+ and Ca^{2+} concentration. Measurements of K^+ concentration were made using an EEL flame photometer. Measurements of Ca^{2+} concentration were made using the complexiometric titration method of Wilkinson (1957) in which the amount of Ca^{2+} removed from the test solution by chelation with diaminoethanetetra-acetic acid (EDTA) is measured.

Measurement of protein liberated from muscle. The method described by Lowry, Rosebrough, Farr & Randall (1951) was used. The principle depends on the fact that when protein containing tyrosine or tryptophan is added to an alkaline solution containing Cu^{2+} a blue colour develops. The change in colour intensity was estimated at 625 nm using a Zeiss PMQ II spectrophotometer.

Molecular sieve chromatography. Glass chromatography columns were packed with the cross-linked dextran gel Sephadex G-25 (Pharmacia, Sweden) as described by Boyd & Forrester (1966). When 2 ml. of a solution of blue dextran (Dextran 2000, Pharmacia) was placed on top of a column all of the dextran was eluted into a volume of exactly 3 ml. Any newly packed column not meeting this requirement was discarded.

In each experiment the column was equilibrated with the same Ringer solution as was to be used for bathing the sartorius muscle and for perfusing the frog heart. A 2 ml. sample of muscle bathing solution was obtained in the usual way and two drops of a concentrated blue dextran solution were added to it. After thorough mixing this solution was pipetted carefully on to the top of the column and its progress through the column was followed visually. Eluate was collected from the column from the moment when the blue dextran first appeared in it. The first 3 ml. fraction containing the dextran was discarded, and ten

subsequent 2 ml. fractions of eluate were collected (1 ml. fractions in a few experiments). Each of these fractions was later perfused through a frog heart to determine which of them contained the unknown stimulatory substance ('X'), and also the relative amounts of 'X' in each fraction shown by the percentage increase in mean arterial pressure or cardiac output produced. Thus, the elution behaviour of substance 'X' was determined (Fig. 7).

An identical procedure was carried out in which a known concentration of ATP (disodium dihydrogen salt of adenosine-5-triphosphoric acid, British Drug Houses), made up in the same Ringer solution as that used to bathe the sartorius muscle, was eluted through the same column and the fractions collected were tested on the same frog heart. The concentration of ATP used was usually 5×10^{-7} g/ml. since this produced about the same degree of stimulation of the heart as a sample of the muscle bathing solution not eluted through the column. Thus, the elution behaviour of ATP in very low concentration was determined.

This molecular sieve chromatography procedure was also used to remove the protein present in the muscle bathing solution. All the protein was recovered in the initial 3 ml. dextran fraction and one or two fractions of protein-free eluate were obtained before any of substance 'X' appeared.

Apyrase incubation procedure. The enzyme apyrase, prepared from potatoes, converts nucleotide triphosphates to monophosphates. Two enzymes, in fact, are contained in apyrase: adenosine-5'-triphosphatase and adenosine-5'-diphosphatase (Liebecq, Lallemand & Degueldre-Guillaume, 1963). Purified apyrase crystals (Sigma Co.) in 2 mg amounts were added to either 1 ml. or 2 ml. samples of ATP in concentrations varying from 10^{-8} to 10^{-6} g/ml. This quantity of apyrase was far in excess of that needed theoretically to convert even the largest amount of ATP in any of the samples to adenosine monophosphate (AMP). Apyrase crystals in 2 mg amounts were added to either 1 ml. or 2 ml. samples of the muscle bathing solution. Incubation of all solutions with apyrase took place in a water-bath at 30° C for times varying from 5 to 15 min. The samples were then cooled to room temperature and perfused through the frog heart.

Firefly luminescence procedure. A modification of the method described by Strehler & Totter (1952) was used. A photomultiplier tube with a guiding lens system and a reflecting cone was mounted in a light tight container. The tube power supply was adjusted to 1300 V since this voltage gave a maximum signal-to-noise ratio. The photomultiplier tube output which was proportional to the intensity of the light received, was fed into a pen recorder with an input impedance of 30 kΩ (Devices Sales Ltd.).

In each experiment firefly lantern extract (type FLE 50, Sigma Co.) was reconstituted and 0·25 ml. pipetted into a narrow 0·5 ml. glass cuvette. The cuvette was placed firmly on the guiding lens with a thin layer of optical glue (Silicone Fluid 200, Midland Silicones Ltd.) between it and the lens. The pen recorder drive was switched on and then 0·25 ml. of Ringer solution was pipetted into the cuvette. The reflecting cone and light tight cover were replaced, and the tube power supply was switched on. A control recording of the intensity and time course of the emission of light from the firefly lantern extract alone was obtained, after which the power supply was switched off. A test recording was then obtained by repeating the whole procedure using 0·25 ml. of muscle bathing solution or of a known concentration of one of the nucleotide triphosphates or diphosphates, in place of the Ringer solution. The light signal from the firefly extract alone was small and constant whereas the light emission produced by a test solution was greater in magnitude and had a characteristic time course of decay (Fig. 8).

In earlier experiments it was not appreciated that exposure of the photomultiplier tube to room illumination could result in a continued output from the tube for a very long period. Even though the complete test procedure was carried out in a darkroom, whenever the tube power supply was switched on an output was obtained in the absence of firefly extract ('dark current'; Fig. 8*a*). This dark current signal was constant in amplitude and free of the oscillations characteristic of light emission from firefly lantern extract. Under these

conditions the dark current had to be subtracted from the test signal before a true value for the light emission from the firefly lantern extract was obtained. The photomultiplier tube was stored in the dark for a month and in subsequent experiments, carried out under a red darkroom safety light, the dark current was zero (Fig. 8*b–e*).

The time elapsing between the moment of addition of the test solution to the firefly lantern extract and the moment when the tube supply voltage attained its maximum value was about 10 sec. Thus, the initial 'flash' of light emitted was not recorded. Nevertheless, light signals of appreciable magnitude and characteristic time course were obtained using both muscle bathing solutions and known concentrations of nucleotide triphosphates or diphosphates.

RESULTS

Frog sartorius nerve–muscle preparations were suspended in 2 ml. of frog Ringer solution and made to twitch repetitively by application of supramaximal stimuli to the nerve at a frequency of 2/sec for ½ hr. The muscle twitch was well maintained throughout the period of stimulation in every experiment. Muscle bathing solutions obtained from fifty-two active frog sartorius muscles were perfused through a total of thirty-seven frog hearts. In every case a marked augmentation of the force of cardiac contraction was produced (e.g. Figs. 1, 2, 5, 6*b*, 9; test 'X').

Each muscle bathing solution was analysed using the various procedures described in Methods. The term 'muscle bathing solution' as used below implies one in which an active muscle was suspended. In a few experiments a 2 ml. sample of Ringer solution, in which an inactive muscle was suspended for ½ hr, was analysed. Such solutions are specifically referred to as having been obtained under resting conditions.

Changes in pH and ionic composition of the muscle bathing solution

The pH of the Ringer solution in which the muscle was bathed was initially 7·2. After the period of muscle activity the pH varied from 7·2 to 7·9. It was shown previously by Boyd & Pathak (1965) that alterations of pH within this range do not affect the frog heart.

Changes in the K^+ concentration of the bathing solution were expected as a result of the ion fluxes during propagation of muscle action potentials. Further, some K^+ efflux from damaged muscle fibres was a possibility. No significant change in K^+ concentration was found in solutions in which resting frog sartorius muscles were bathed for ½ hr. An average increase of 21% was found in the K^+ concentration of solutions bathing seventeen sartorius muscles following the standard period of activity described in Methods. The maximum increase in any one experiment was 41%. Six frog hearts were perfused with Ringer solution containing up to twice the normal amount of K^+ and in no case was a stimulatory effect similar to that produced by the muscle bathing solution found. One experiment is illustrated in Fig. 1. This heart was continuously perfused with Ringer

solution containing 2·9 mM-K^+. Perfusion for ½ min with a muscle bathing solution containing 3·6 mM-K^+ resulted in typical augmentation of the force of cardiac contraction. Perfusion for 1 min with Ringer solution containing 3·7 mM-K^+ produced no change in cardiac activity. Thus, efflux of K^+ from active muscle was excluded as a cause of the observed stimulatory effect.

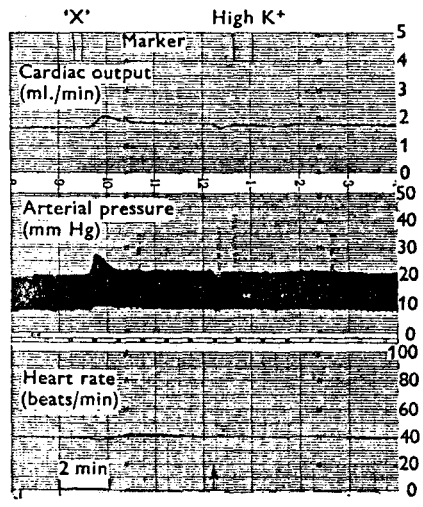

Fig. 1

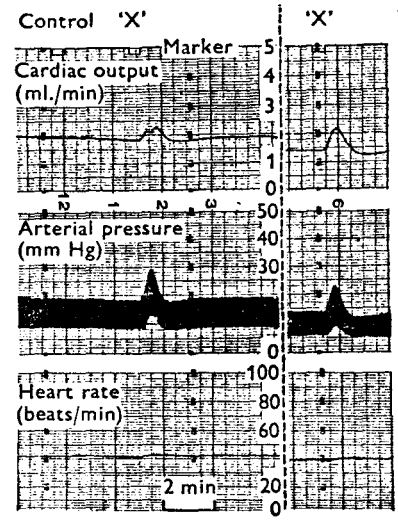

Fig. 2

Fig. 1. Comparison of the action on a perfused frog heart of a 'muscle bathing solution' containing unknown stimulatory substance ('X') with that of Ringer solution containing a high K^+ concentration (3·6 mM). Heart continuously perfused *in situ* with Ringer solution containing 2·9 mM-K^+. Cardiac output, arterial pressure and heart rate recorded simultaneously. K^+ concentration of muscle bathing solution, 3·7 mM. ↑, fall in perfusion pressure during flushing of residual test solution from venous pressure transducer (Boyd & Eadie, 1961). Note that increase in K^+ concentration alone did not stimulate the heart.

Fig. 2. Comparison of the stimulatory action of the same muscle bathing solution ('X') on a perfused frog heart before and after adrenergic blockade. Vertical interrupted line, 2 hr period during which heart was perfused with pronethalol hydrochloride (10^{-6} g/ml.) and ergotamine tartrate (10^{-6} g/ml.) in Ringer solution. Action of 'X' was not affected by the blocking agents. Dotted trace indicates actual cardiac output during failure of drop counting mechanism.

Both influx and efflux of small amounts of Ca^{2+} take place during muscle activity; McLean & Hastings (1934) reported that small changes in Ca^{2+} concentration could affect the frog heart. The Ca^{2+} concentration of the Ringer solution in the present work varied from 0·95 to 1·13 mM, while that of fourteen muscle bathing solutions varied from 0·92 to 1·22 mM; the mean value was 1·08 mM in both sets of measurements. A change

in Ca^{2+} concentration from 0·9 to 1·4 mM in the Ringer solution perfusing three frog hearts produced no stimulation of the heart comparable with that produced by the muscle bathing solution, so that a change in Ca^{2+} concentration in the muscle bathing solution was not the cause of its stimulatory action on the heart.

Substances concerned in muscle activity

Protein. The protein content of the muscle bathing solutions was estimated by the method of Lowry *et al.* (1951). The total protein liberated from sixteen muscles during the standard period of muscle activity varied from 80 to 360 μg (mean 220 μg), whereas the amount liberated from twenty resting muscles during the same period ranged from 55 to 180 μg (mean 125 μg). Fifteen resting muscles which were shaken vigorously in 2 ml. of Ringer solution for $\frac{1}{2}$ hr liberated protein in amounts varying from 115 to 245 μg (mean 180 μg), suggesting that the twitching muscle liberates protein which is extracellular in origin.

When four muscle bathing solutions were eluted through a molecular sieve chromatography column as described in Methods all the protein was recovered in the first 3 ml. fraction collected in every case. One such protein-containing fraction was perfused through a frog heart and no stimulation was produced. Protein was thus excluded as a cause of the stimulatory effect.

Catecholamines. Adrenaline released from sympathetic nerve terminals in frog muscle would stimulate the frog heart. The release of catecholamines in sufficient amount to produce the observed degree of stimulation was highly unlikely; nevertheless, the possibility was excluded by the use of adrenergic blocking agents. In the experiment illustrated in Fig. 2 the stimulatory action on the heart of a muscle bathing solution was unaltered by pronethalol and ergotamine in amounts sufficient to block the action of noradrenaline (10^{-6} g/ml.). Similar results were obtained in five other experiments using adrenaline.

Purine ring compounds. Ten muscle bathing solutions were examined using U.V. spectrophotometry to see if there were any particular wavelengths at which there was maximal absorption of light. The protein present in the solution obscured any peaks that might have been present (Fig. 3*a*).

As described above, however, all the protein could be removed in the first fraction of eluate collected from a molecular sieve chromatography column. Further, almost all the stimulatory substance was recovered in a volume of 14 ml. or less eluted through the column some time later. Three muscle bathing solutions were eluted through a chromatography column, and samples of eluate which had a pronounced stimulatory action on the

heart were examined by U.V. spectrophotometry. In each case a maximum absorption of U.V. light occurred at a wave-length of about 265 nm (Fig. 3*b*). This was indicative of the presence of substances containing a purine ring, such as ATP, which show maximum absorption of U.V. light at this wave-length when dissolved in Ringer solution (Fig. 3*c*).

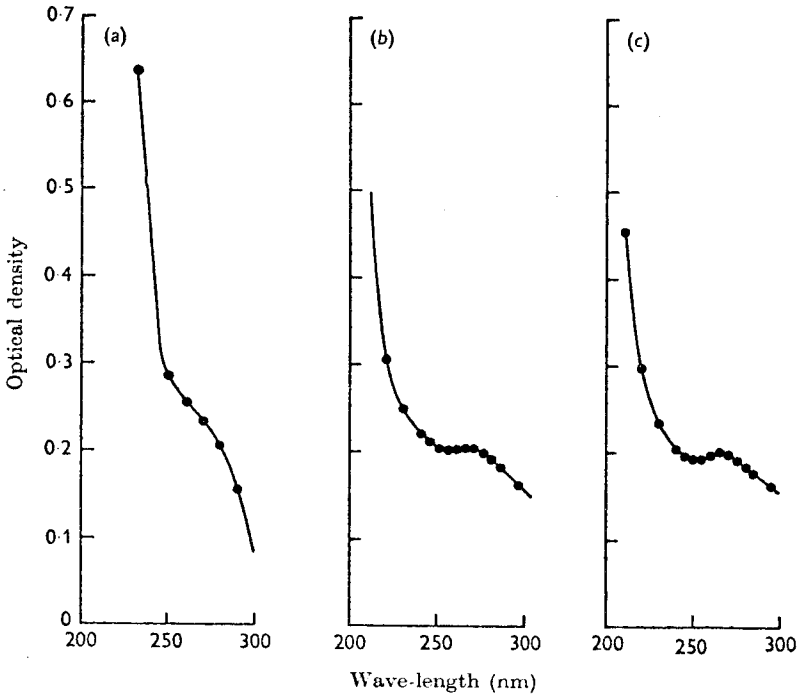

Fig. 3. Comparison of ultraviolet absorption spectra of muscle bathing solutions and ATP.

a. Muscle bathing solution after standard period of indirect stimulation of muscle. Absorption peaks obscured by presence of protein.

b. Similar muscle bathing solution after removal of protein by column chromatography. Peak of absorption at 265 nm wave-length in sample of eluate having pronounced stimulatory action on the heart.

c. Peak of absorption at 265 nm in solution of ATP (10^{-6} g/ml.) in Ringer solution.

Although the absorption peak for the stimulatory column fraction was not very pronounced, presumably because the stimulatory substance was present in low concentration only, this test suggested that it would be profitable to study the action of purine ring compounds on the frog heart in detail.

Qualitative action of purine ring compounds on the frog heart

Experiments in the present work verified that adenosine does not stimulate the frog heart, as was shown previously by Drury & Szent-Gyorgi (1929), by Ostern & Parnas (1932), and by Flössner (1934); nor do the other nucleosides inosine, cytidine, uridine or guanosine. The triphosphates of adenosine and all the other nucleosides listed above, however, do stimulate the heart although there are qualitative and quantitative differences in their actions (Fig. 5). The stimulatory action of ATP on

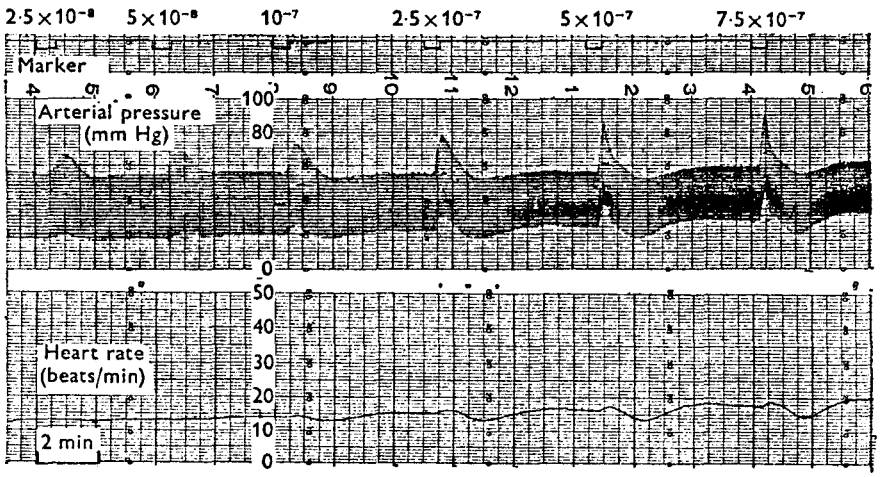

Fig. 4. The response of a perfused frog heart to progressively increasing concentrations of ATP in Ringer solution. ATP concentration in g/ml. indicated above corresponding marker step. Note that low concentrations of ATP have a pure inotropic action while higher concentrations have a triphasic chronotropic action in addition.

the heart was first described by Ostern & Parnas (1932) while Buday, Carr & Miya (1961) and Versprille (1963) have shown that ATP has an immediate inotropic effect on the frog heart.

In the present work it was found that ATP had a pure positive inotropic action when in concentrations of 10^{-9}–10^{-8} g/ml., and occasionally up to 5×10^{-8} g/ml., the increase in the force of contraction producing a rise in mean arterial pressure and cardiac output without any change in heart rate (Fig. 4). In greater concentration (10^{-7}–10^{-6} g/ml.) the maximum inotropic effect of ATP was achieved rapidly with a consequent sharp peak in the arterial pressure recording, and a chronotropic action was also evident. The action of ATP on heart rate was typically triphasic, a small rise in rate coincident with the positive inotropic effect being followed by a fall in rate which in turn was followed by a more prolonged rise in rate

which persisted after perfusion with ATP was discontinued and which increased with concentration. Adenosine diphosphate (ADP) had a qualitative and quantitative action on the heart very similar to ATP. Adenosine monophosphate (AMP) did not stimulate the heart.

If the action of the muscle bathing solution on the heart shown in Figs. 1 and 2 is compared with that of low concentrations of ATP in Fig. 4, it

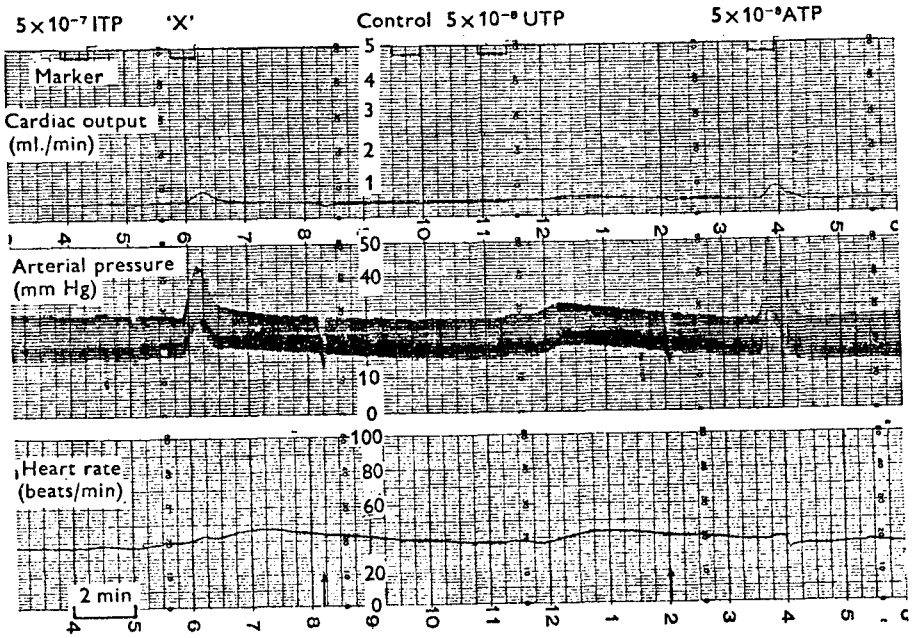

Fig. 5. Part of an assay of a muscle bathing solution ('X') on the perfused frog heart against the triphosphates of inosine (ITP), uridine (UTP) and adenosine (ATP). The action of 'X' on cardiac output and arterial pressure was less than that of ATP (5×10^{-8} g/ml.) but much greater than, and qualitatively different from, that of UTP (5×10^{-8} g/ml.); ITP (5×10^{-7} g/ml.) did not stimulate the heart. See also assay of same muscle bathing solution on firefly lantern extract (Fig. 8*d*).

will be seen that stimulation of comparable time course is produced. Furthermore, the inotropic and chronotropic actions of ATP and of the muscle bathing solution on the same heart in Figs. 6 and 9 are very similar.

On the other hand, the action of UTP in low concentration on the heart differs qualitatively from that of ATP or of the muscle bathing solution; the concentration of UTP having the same inotropic action as a given concentration of ATP has a much more pronounced and long-lasting chronotropic action. The triphosphates of inosine, cytidine and guanosine have no action on the heart in concentrations 10 times greater than that at which ATP and UTP have a marked effect (Fig. 5), and stimulate the

heart only when in concentrations of the order of 10^{-5} g/ml. Even then their action differs qualitatively from that of ATP or of muscle bathing solutions.

This evidence strongly suggested that the stimulatory substance was either ATP or ADP, but for its final identification a specific test to distinguish triphosphates from diphosphates was required.

Abolition of activity by a specific enzyme

The enzyme 'apyrase' decomposes nucleotide triphosphates in two stages, converting them first to diphosphates and then to monophosphates. In the present work it was found that the monophosphates of adenosine, inosine and cytidine, even in a concentration of the order of 10^{-5} g/ml., did not stimulate the frog heart. An incubation period of 10 min was found to be the minimum necessary to complete the conversion of ATP to AMP, judged by the abolition of the stimulatory action of ATP on the frog heart (Fig. 6*a*). This is in agreement with the results of Traverso-Cori & Cori (1962) who showed by biochemical techniques that AMP is not produced from ATP until after 10 min incubation with apyrase.

Seven experiments were carried out in which the solutions bathing different active sartorius muscles were each perfused through a different frog heart. In each case the action of the muscle bathing solution, before and after incubation with apyrase, was compared with that of solutions of ATP similarly treated. In every experiment the initial inotropic action of both ATP and muscle bathing solution was abolished by apyrase (Fig. 6). Control samples of Ringer solution incubated with apyrase had no action on the heart, while a final test with pure ATP showed that the apyrase itself had not modified the sensitivity of the heart. Thus, it was established that the unknown stimulatory substance was either a triphosphate or a diphosphate.

In an attempt to distinguish between triphosphates and diphosphates, the action on the heart of solutions of ADP, before and after incubation with apyrase, was studied in two of the above experiments. It was anticipated that the time of incubation necessary to produce abolition of the action of ADP would prove to be significantly less than that for ATP. However, because of the variability in the required incubation time in different experiments, it was not possible in practice to distinguish ATP from ADP by the apyrase test.

Measurement of elution behaviour in column chromatography

When any muscle bathing solution was eluted through a Sephadex column prepared as described in Methods, a constant elution pattern for the stimulatory substance was obtained even when different columns were

used. Successive 2 ml. samples of eluate were perfused through the frog heart, and their action compared with that of a sample of the original muscle bathing solution. The first few millilitres of eluate had no stimulatory action, whereas five or six subsequent 2 ml. samples had a stimulatory

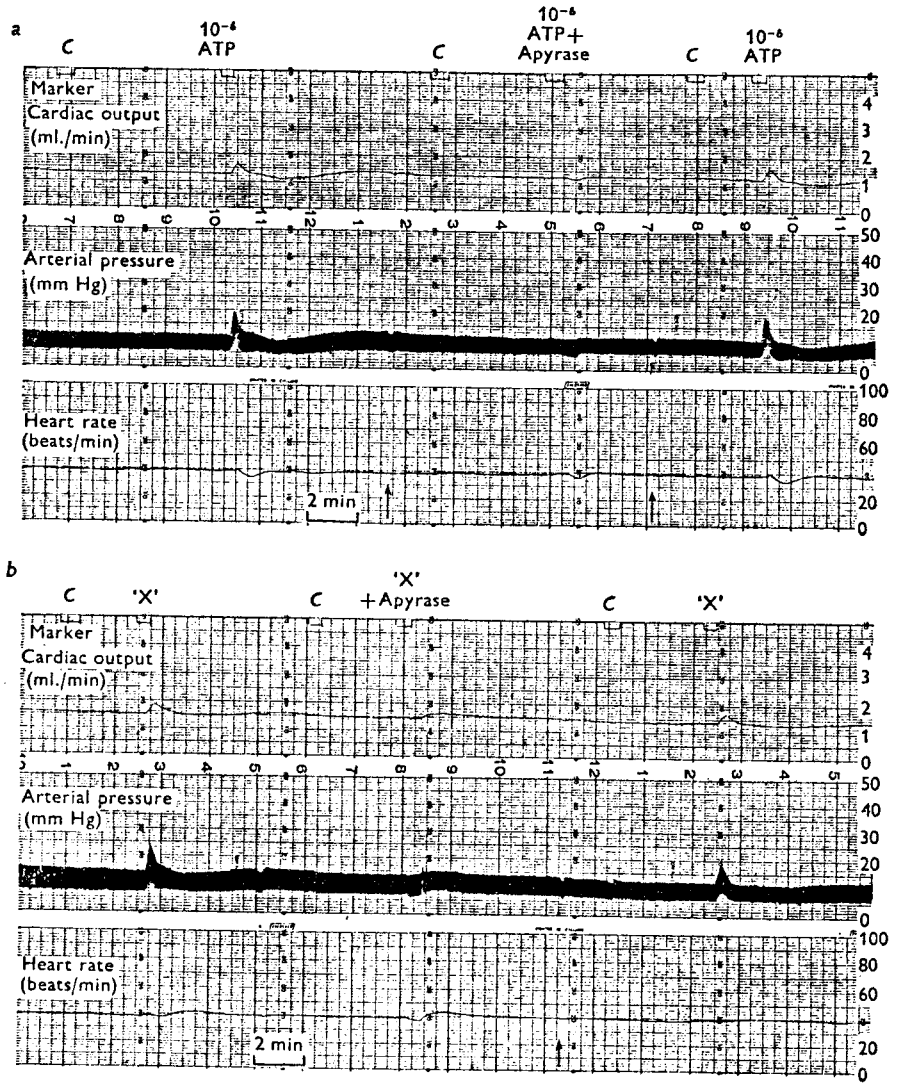

Fig. 6. Comparison of the action on the same frog heart of ATP (10^{-6} g/ml.) (Fig. 6*a*) and a muscle bathing solution ('X', Fig. 6*b*) before and after 15 min incubation of each solution with apyrase (1 mg/ml.) at 30° C. Apyrase abolished the initial inotropic action of both ATP and 'X'. Later tests showed that apyrase itself did not alter the sensitivity of the heart to either ATP or 'X'. *C*, control perfusion with Ringer solution.

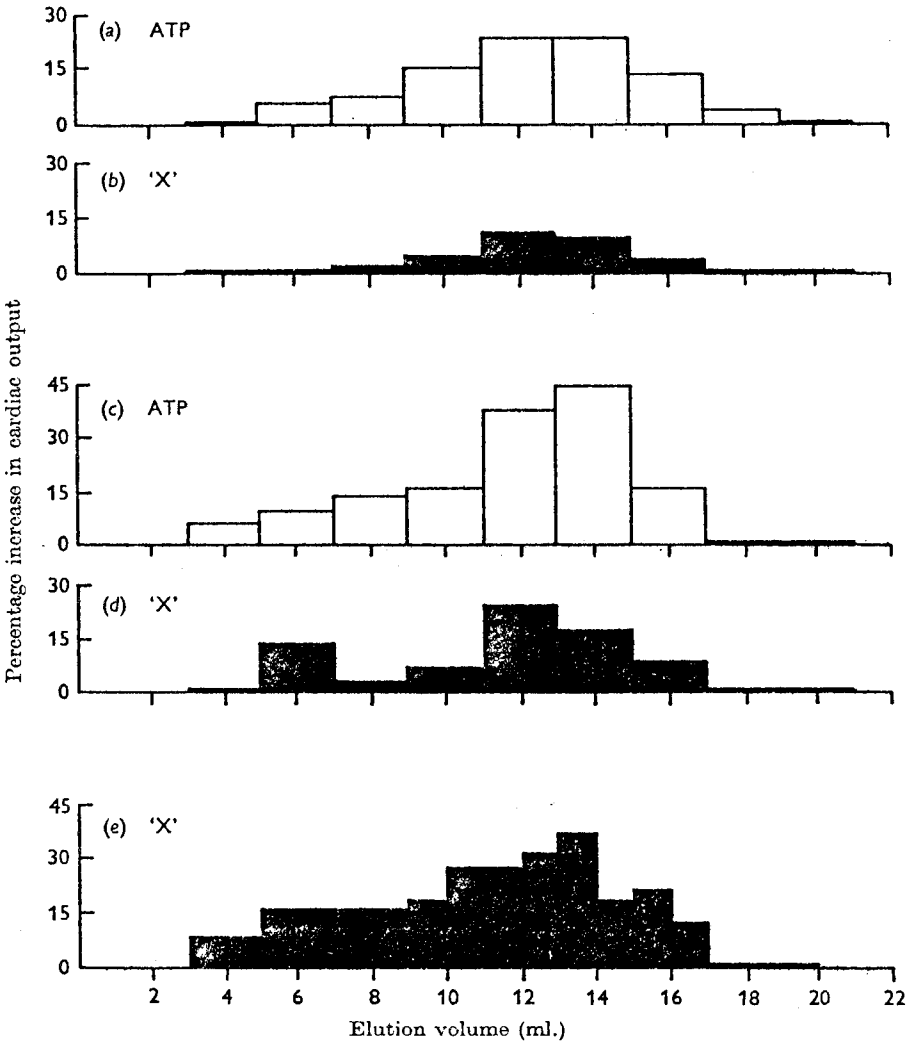

Fig. 7. Comparison of the elution behaviour of ATP and the unknown stimulatory substance ('X') in a molecular sieve chromatography column. The histograms show the stimulatory action of successive samples of eluate on the perfused frog heart (ATP, unshaded histograms; 'X', shaded histograms).

a. 2 ml. of ATP (5×10^{-7} g/ml.) eluted through a column; 2 ml. samples of eluate were tested.

b. 2 ml. of a muscle bathing solution eluted through same column as in *a*.

c. 2 ml. of ATP (5×10^{-7} g/ml.) eluted through a different column.

d. 2 ml. of a second muscle bathing solution eluted through same column as in *c*.

e. 2 ml. of another muscle bathing solution eluted through a third column; 1 ml. samples of eluate were tested.

action on the heart (Fig. 7*b*, *d*). The degree of stimulation is expressed in Fig. 7 as the percentage increase in cardiac output produced by each sample. In every one of four experiments the peak in the elution pattern of the stimulatory substance occurred about 13 ml. after the start of collection of eluate. Almost all the stimulatory substance was recovered in about 12 ml. of eluate, first appearing about 5 ml. after the start of collection. The elution pattern was independent of the volume of the fractions collected for testing on the heart (Fig. 7*e*).

A 2 ml. sample of ATP in a concentration producing approximately the same degree of stimulation of the heart as the muscle bathing solution (usually ATP, 5×10^{-7} g/ml.), was eluted through the same column as each of the corresponding muscle bathing solutions. Elution patterns for ATP in two experiments are shown in Fig. 7*a*, *c*. In every experiment the elution behaviour of ATP was almost identical to that of the stimulatory substance. These results provide further evidence that the stimulatory substance was ATP or a closely related substance. Owing to the complexity of these experiments determination of the elution behaviour of the other nucleotide triphosphates was not attempted.

Emission of light from firefly lantern extract

When ATP is added to an extract of fireflies a flash of light appears immediately and lasts for a considerable time, the intensity and duration of the flash depending on the concentration of ATP present (McElroy, 1947). In a later study Strehler & McElroy (1957) found that the reaction was highly specific for ATP, no light signal being produced by the triphosphates of inosine, uridine and guanosine. With the firefly lantern extract used in the present work, however, equal concentrations of the triphosphates of adenosine, inosine, uridine, cytidine and guanosine produced light signals which were almost exactly the same in magnitude and time course. A comparison between the light produced by 5×10^{-8} g/ml. of both ATP and UTP is shown in Fig. 8*d*. A given concentration of ADP produced a much smaller light signal than the same concentration of ATP (Fig. 8*e*). With the technique employed in the present work small differences in concentration of any triphosphate could be discriminated easily (Fig. 8*c*).

Muscle bathing solutions from ten sartorius muscles were tested on firefly extract. In nine of these tests an emission of light was produced which could be matched in magnitude and time course with that produced by a particular concentration of ATP or other nucleotide triphosphate (Fig. 8*b*, *d*). In one experiment the emission of light produced by a muscle bathing solution was abolished after incubation of the solution with apyrase (Fig. 8*a*).

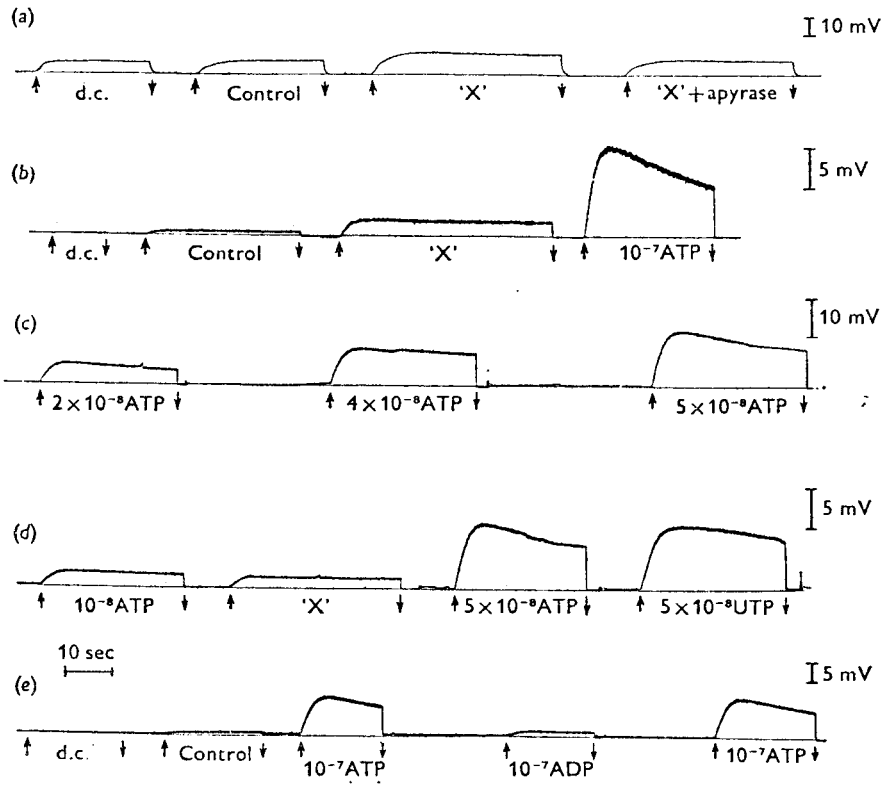

Fig. 8. Emission of light from firefly lantern extract by the unknown stimulatory substance ('X') and by nucleotide triphosphates. ↑, 1300 V power supply to photomultiplier tube switched on; ↓, power switched off. Dark current (d.c.) appreciable in record *a*, but zero in records *b* to *e*. For each test 0·25 ml. of muscle bathing solution ('X'), or nucleotide triphosphate solution, added to 0·25 ml. firefly extract. Control, 0·25 ml. saline added to 0·25 ml. firefly extract.

a. Emission of light by a muscle bathing solution ('X') exceeded that of control and the difference was abolished by incubation of 'X' with apyrase for 15 min at 30° C.

b. Comparison of light emission produced by a second muscle bathing solution with that produced by ATP (10^{-7} g/ml.).

c. Finely graded emission of light produced by progressively increasing concentrations of ATP (g/ml.).

d. Assay of another muscle bathing solution against ATP and UTP. Response to 'X' equivalent to ATP (10^{-8} g/ml.) and less than that produced by 5×10^{-8} g/ml. of any nucleotide triphosphate. For assay of same muscle bathing solution on frog heart see Fig. 5.

e. Comparison of light emission produced by similar concentrations of ATP and ADP. ADP is approximately 10 times less potent than ATP.

Assay of stimulatory substance

As a result of the procedures described above it was concluded that the stimulatory substance was either ATP, ADP or UTP. By comparing the concentrations of each of these substances which produced the same magnitude of response as a given muscle bathing solution in tests using both frog heart and firefly lantern extract it was established that the stimulatory substance was, in fact, ATP.

A typical assay of a muscle bathing solution against ATP and UTP using the firefly technique is illustrated in Fig. 8*d*. A test with the muscle bathing solution between tests with two known concentrations of ATP showed that the unknown stimulatory substance ('X') caused light emission equivalent to ATP in a concentration of 10^{-8} g/ml. and much less than that produced by ATP or UTP in concentrations of 5×10^{-8} g/ml. In nine similar experiments the concentration of nucleotide triphosphate which matched the action of different muscle bathing solutions varied from 10^{-8} to 5×10^{-8} g/ml.

A typical assay of a muscle bathing solution against ATP using the frog heart technique is illustrated in Fig. 9. The unknown stimulatory substance ('X') had an inotropic action on the heart slightly less than that produced in an immediately preceding test with ATP ($2{\cdot}5 \times 10^{-7}$ g/ml.) and slightly greater than that in a later test with ATP (10^{-7} g/ml.). An assay in a second experiment is represented graphically in Fig. 10. The concentration–response relation for the action of ATP on this heart is represented by crosses, while an assay at a different time in the experiment is indicated by circles. A test with a muscle bathing solution (filled circle) was preceded and followed by tests with ATP (open circles) and the stimulatory substance had an action equivalent to that of ATP (9×10^{-8} g/ml.). In a total of eighteen experiments on different hearts the action of the stimulatory substance was equivalent to concentrations of ATP varying from 10^{-8} to 10^{-6} g/ml., the matching concentration in most cases being between 10^{-8} and 5×10^{-8} g/ml. as in the assays using the firefly technique.

In contrast, the concentration of UTP having the equivalent initial inotropic action on the heart as a muscle bathing solution was always greater than 10^{-7} g/ml. Since this concentration of UTP always produced a far greater emission of light from firefly lantern extract than was ever obtained with any muscle bathing solution, the stimulatory substance could not have been UTP.

In three experiments it proved possible to assay the same muscle bathing solution against ATP using both heart and firefly techniques. In one experiment the equivalent concentration of ATP was 9×10^{-8} g/ml. in the

heart assay (Fig. 10) and the same muscle bathing solution when tested 2 hr later by the firefly technique matched a concentration of ATP of 5×10^{-8} g/ml. (Forrester, 1966, fig. 1). In a second experiment the equivalent concentration of ATP in the heart assay was clearly less than that produced by 5×10^{-8} g/ml. and about the same as would have been produced

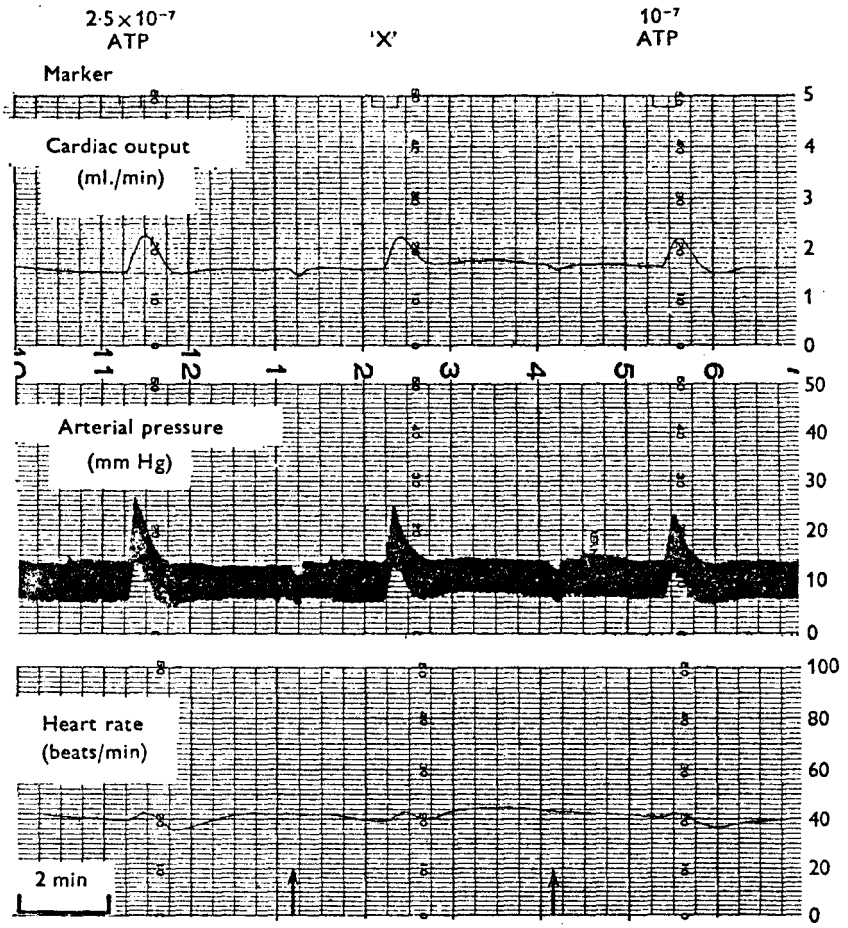

Fig. 9. Assay of a muscle bathing solution ('X') against ATP on a perfused frog heart. Action of 'X' on both pace-maker and on ventricular muscle was equivalent to that of a concentration of ATP between 10^{-7} and $2{\cdot}5 \times 10^{-7}$ g/ml.

by 10^{-8} g/ml. (Fig. 5); the matching concentration of ATP in the firefly assay was 10^{-8} g/ml., also (Fig. 8*d*). In this second experiment the possibility that the stimulatory substance was UTP was definitely excluded. The matching concentration of UTP in the firefly assay was clearly much less than 5×10^{-8} g/ml. (Fig. 8*d*), whereas the matching concentration in the heart assay was obviously much greater than 5×10^{-8} g/ml. (Fig. 5).

The emission of light from firefly lantern extract by ADP was only one tenth of that produced by the same concentration of ATP (Fig. 8*e*). Since equivalent concentrations of ADP and ATP have the same action on the frog heart, in neither of the experiments described above could the stimulatory substance have been ADP. Thus it was concluded that the stimulatory substance was ATP.

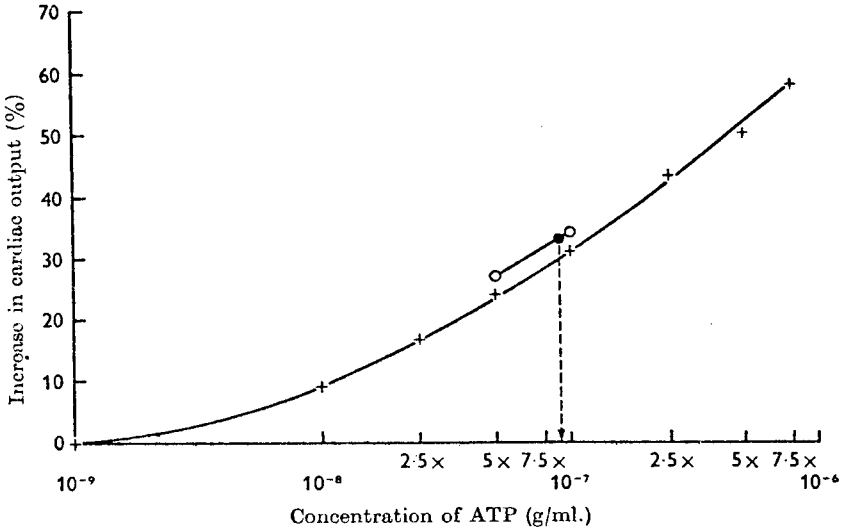

Fig. 10. Graphical representation of an assay of a muscle bathing solution ('X') against ATP on the perfused frog heart. Crosses, concentration–response relation for the action of ATP on cardiac output (derived from Fig. 4). Circles, response in a later test with a muscle bathing solution (filled circle) preceded and followed by tests with two concentrations of ATP (open circles) in a similar experiment to Fig. 9. Assay result, 'X' equivalent to a concentration of ATP of 9×10^{-8} g/ml.

The output of ATP from eighteen sartorius muscles during the standard period of nerve stimulation varied from 2×10^{-8} to 10^{-6} g, the mean output being 4×10^{-7} g. Since the average wet weight of four sartorius muscles was 50 mg, the mean output of ATP per gram of wet muscle was approximately 8 μg. About 30% of the weight of wet muscle is contributed by extracellular fluid (Dydyńska & Wilkie, 1963), so that the mean output of ATP per gram of dry muscle was 11·4 μg (0·018 μmole). The amount of ATP situated intracellularly has been estimated at 2–4 μmole/g dry muscle (Maréchal, 1964) so that there was a loss of roughly 1–2% of the intracellular ATP into the muscle bathing solution during the standard period of stimulation.

DISCUSSION

Since adenine nucleotides are present in abundance intracellularly (Glick, 1946), it is possible that damaged muscle cells release significant amounts of ATP into the surrounding bathing solution. Damaged cells may also liberate K^+ into the bathing solution. The fact that there was no rise in the K^+ level in resting muscle bathing solutions shows that the muscle fibres for all practical purposes were undamaged. In any case the contents of any grossly damaged cell would have been completely removed during the washing procedure which preceded the period of stimulation. In one experiment a resting muscle was deliberately damaged with a resultant increase in the K^+ concentration in the bathing solution of almost 100%.

Caspersson & Thorell (1942) were able to locate the position of the adenylic acids in living skeletal muscle from various species. They noted that these substances were most concentrated nearest to the I-band of the sarcomere presenting a pattern of sharply differentiated light and dark bands traversing the muscle fibres. After vigorous exercise of the muscle the sharp differentiation was 'more or less smudged out', as would be the case if the adenylic acids had been extruded from the muscle fibres. Huxley (1964) and Page (1964) have shown independently that particles as large as ferritin and colloidal gold (diameters 11 and 20 nm, respectively) can penetrate to the central element of the triad formation (T-system) when externally applied to frog muscle fibres. These studies provided the first direct evidence of continuity between the lumen of the transverse tubules and the extracellular space in frog skeletal muscle. Endo (1966) confirmed this when he found that various fluorescent dyes could diffuse freely in and out of frog skeletal muscle; the dyes accumulated in a pattern of bands each of which corresponded in position to one of the T-tubules. Hill (1959), while studying the problem of the location of adenine nucleotides in frog skeletal muscle, showed that about 70% of incorporated labelled material (principally ATP) lay within the I-band of the sarcomere; he noted that this nucleotide seemed to have 'a transverse freedom of movement within the muscle fibre'. He suggested that the facility for this transverse diffusion could be provided by the sarcoplasmic reticulum. It may be that in the present work the ATP found in the muscle bathing solution was released by way of the transverse tubular system of the sarcoplasmic reticulum during activity of the sartorius muscle.

The release of ATP from contracting muscle may play an important role in the production of hyperaemia in skeletal muscle during exercise, since it has long been known that ATP is a powerful vasodilator substance (Fleisch & Weger, 1937; Folkow, 1949).

We are grateful to Dr H. M. Keir for advice and guidance about some of the biochemical techniques employed. We are indebted to Professor S. M. Hilton for the loan of some Pabst nucleotides. We wish to thank the Trustees of the Boyd Medical Research Institute for providing laboratory facilities. Technical assistance was provided under an M.R.C. grant.

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Commentary - paper 3.

This short note was the result of an experiment of a quite controversial nature. The extensive ATPase activity of whole blood and blood plasma had been well known since the work of Jorgensen (1956). Contemporary argument was that ATP could not possibly exist in blood plasma because of these enzymes. However the converse argument was adopted by the author - if there are so many extracellular ATPases in the blood, then perhaps they are there for a purpose, specifically to degrade any ATP that might enter this fluid compartment. The process could be either deamination or dephosphorylation, both of which would effectively abolish the vasodilatory property of ATP.

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The identification of adenosine triphosphate in fresh human plasma

By T. FORRESTER.* *Institute of Physiology, University of Glasgow*

When diluted fresh human plasma is perfused through a frog heart a pronounced stimulatory effect is produced which is almost identical to that produced on the frog heart by low concentrations of ATP.

Samples of whole blood were obtained from the antecubital vein in twenty-two subjects. Each subject was instructed to keep the forearm musculature as relaxed as possible. The samples were mixed with 0·3% EDTA, centrifuged at 4° C for 25 min and the supernatant plasma diluted 1:10, 1:20 or 1:25 with Locke solution. Each solution was then immediately tested on the perfused frog heart system of Boyd & Eadie (1961), and on firefly lantern extract (Type FLE 50, Sigma) using a modification of the technique of Strehler & McElroy (1957). Continuous perfusion of the frog heart with Locke solution was possible without alteration of performance.

The procedures for the identification of ATP were the same as those used in the detection of ATP released from active frog sartorius muscle (Forrester, 1966; Boyd & Forrester, 1968). Diluted plasma from every subject caused light to be emitted from firefly extract, a test which is reputed to be highly specific for ATP (Strehler & McElroy, 1957). Diluted plasma from six subjects had the same stimulatory effect on frog heart as that of low concentrations of ATP; this effect was abolished in every case after incubation of the solution with the enzyme 'apyrase'. Using a molecular sieve chromatography technique (Boyd & Forrester, 1966), the elution behaviour of the substance in the plasma stimulating the frog heart was the same as that for ATP.

Having established that ATP can exist in fresh human plasma in spite of the fact that plasma contains enzymes capable of degrading ATP (Ireland & Mills, 1964), a quantitative estimation was undertaken. Plasma from ten subjects was tested on firefly extract, two of these solutions being simultaneously assayed on a frog heart. The mean concentration of ATP in the plasma was 0·57 μg/ml. (S.D. $\pm$ 0·24). Similar values for the concentration of ATP were obtained for the two samples assayed on frog heart and firefly extract. That the parallel assay gave closely matching results is further evidence for the stimulatory substance in the plasma being ATP, since the frog heart technique can distinguish between ATP and other triphosphates, while the firefly extract gives only a slight response to equivalent concentrations of diphosphates (Boyd & Forrester, 1968).

* Address for one year: Department of Biophysics, University College London, W.C.1.

[P.T.O.

The source of the ATP in plasma has not yet been fully determined but it has been demonstrated recently that the concentration of ATP in the venous effluent from active forearm muscles rises markedly above the resting levels (T. Forrester & A. R. Lind, unpublished).

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Commentary - paper 4.

With the identification of ATP in human plasma the natural experiment to follow was the measurement of ATP in the venous effluent from exercising muscle. The standardization of muscular exercise *in vivo* (voluntary) is not an easy procedure, but at this time Professor Henry Barcroft drew the author's attention to the work of A.R. Lind and his colleagues who had perfected a way of measuring the blood flow through the human forearm at various standard levels of isometric exercise. A combination of the two techniques, ATP measurement in human plasma and measurement of muscle blood flow during standard exercise of human forearm muscle resulted in this paper and the full account in paper 5.

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Adenosine triphosphate in the venous effluent and its relationship to exercise[1]

T. FORRESTER AND A. R. LIND
Indiana University Cardiopulmonary Laboratory, Wright-Patterson Air Force Base, Ohio

SUSTAINED OR STATIC CONTRACTIONS bring about a remarkable rise of both systolic and diastolic blood pressure and, in consequence, mean blood pressure (15–18). For example, it is not uncommon to find a mean blood pressure in excess of 140 mm Hg at the end of a handgrip contraction held to the point of fatigue. The concomitant increase in the heart rate is modest, and values rarely exceed 120 beats/min. On release of the muscular tension, both the heart rate and the blood pressure immediately begin to fall, and usually return to resting values in about a minute. The other well-defined cardiovascular response to sustained contractions is the local increase of blood flow in the active muscles. Unless the tension is very high, the forearm blood flow increases during the handgrip contraction (15). After the tension is released, the flow increases again to a peak value and then returns relatively slowly to resting values over a period of several minutes (15, 16). The dimension and duration of the postexercise hyperemia depend to a large extent on the preceding muscular tension and its duration. Thus, both the systemic circulatory responses, represented by changes of blood pressure and the heart rate, and the local blood flow to the muscles behave in a similar manner *during* the sustained contraction, but afterwards the systemic responses are in a different direction and have a different time sequence from those found in the blood flow to the muscles.

The mechanisms controlling these different cardiovascular functions remain unknown. Little attention has been paid to defining the mechanism for the rise of blood pressure during sustained contractions; the suggestion that it may be due to an increase in potassium efflux (5, 17) must remain speculative at present. It is nearly a hundred years since Gaskell (11) suggested that some substance released from a contracting muscle was responsible for the local vasodilatation. Since then, many attempts have been made to define the substance responsible, but with no success (1, 12), although a recent description of the effects of increased potassium in the presence of a low O_2 tension may be important (21).

Adenosine triphosphate (ATP) has long been known as a potent vasodilator (6) and is also known to have an important role to play in the contractility of cardiac muscle (19). Earlier attempts to demonstrate ATP in the effluent of active muscle proved fruitless and led to the rejection of this as the substance responsible for exercise hyperemia (8, 13). However, the recent description of procedures by which very small amounts of ATP can be identified and measured (3, 10, 14, 22) and the subsequent demonstration of the release of ATP from isolated, active frog muscle (3, 9, 10) led us to consider the possibility that ATP may be released also from the intact active human muscle. If that were so, it is conceivable that ATP might be responsible for either *a*) the pressor response to sustained contractions, or *b*) the vasodilatation that occurs during and after exercise. Since the changes of the blood pressure and heart rate are in the opposite direction (and with quite different time courses before reaching control values) after a sustained contraction, it is probable that these two hypotheses are mutually exclusive.

METHODS

Four normal, healthy men volunteered to act as subjects. They performed contractions on an accurate handgrip strain-gauge dynamometer (4). At the start of each experiment each subject made two brief maximal voluntary contractions (MVC), the higher of which was taken as the MVC. The later test contractions were set at 10% and 20% MVC, each held for 4 min. The subjects were recumbent throughout each experiment which involved two periods of 18 min each, with a 10-min interval between them. Each period was identical, except for the tension exerted during contractions. Two minutes were allowed for control values to be obtained, followed by 4 min of contraction and a recovery period of 12

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min. Blood samples were obtained from an indwelling venous Teflon catheter introduced into a vein in the antecubital fossa and passed about 5 cm retrogradely to lie in a vessel draining the deep venous plexus in the muscles; samples of blood, each of 5 ml, were obtained once before each contraction, three times during the contraction (15 sec, 2 and 3 min after onset), and four times during the recovery starting 10 sec, 2, 7, and 12 min after the contraction ended. Blood pressures were measured every minute (and every half-minute during contractions) by auscultation on the other arm. Forearm blood flows were measured in a second session to avoid applying pneumatic cuffs to the test forearm with possible resulting hematological trauma. In this second session, the laboratory conditions were identical to those in the session when blood samples were collected; it has been shown that in these circumstances the blood flows during and after sustained contraction at known proportions of the MVC are accurately repeatable (16). The Whitney mercury-in-rubber strain-gauge plethysmograph (23) was used to measure forearm blood flows before, during, for 2 min immediately after exercise, and for 2 min at the times when venous samples were collected later in the recovery period. Heart rates were counted from a continuously recording ECG.

The concentration of ATP in the plasma was measured by a modification of the Strehler and McElroy (22) firefly luminescence technique; the procedure has been described in detail elsewhere (3, 10). Briefly, the test solution is introduced to a glass cuvette, containing reconstituted firefly lantern extract, in a light-tight instrument. If ATP is present, the resultant flash of light is detected by a photomultiplier tube, the output voltage of which is measured. It has been shown that the intensity of the light produced from firefly lantern extract is proportional to the concentration of ATP applied to it (3, 10).

The blood samples were dripped directly into centrifuge tubes containing ethylenediaminetetraacetic acid to prevent clotting and to protect the cells. On one or two occasions the blood was collected into nonwettable syringes before transference to the centrifuge tubes. After centrifuging gently at 1,500 rpm for 20 min, the plasma was diluted by 1:10 with saline to minimize any opacity due to protein content; 0.25 ml of this diluted plasma was applied to 0.25 ml of firefly extract in a glass cuvette, the light detected by a photomultiplier tube, and the voltage change was recorded on a Sanborn ECG pen recorder. The three procedures to identify small quantities of ATP which have been described in detail elsewhere (3, 10) were also used in this investigation. *a*) Selected test solutions of 2 ml plasma were incubated with 1 mg potato apyrase for 15 min at 30 C; this enzyme selectively inactivates ADP and ATP so that before-and-after samples can be tested with the firefly-luminescence technique to determine the existence of these nucleotides in the test solution. *b*) A sample of plasma was compared with a known solution of ATP using a Sephadex column to assess the wavelength of the eluted samples. *c*) Some test solutions were applied to the perfused frog heart preparation (2) and were compared directly with ATP solutions; the behavior of the frog heart preparation to solutions of ATP is characteristic and identifiable. These procedures verify the identity of the test material.

RESULTS

The average concentration of ATP in the resting venous blood of four subjects, before the contraction, was 0.50 μg/ml (0.24–1.6 μg/ml). During each contraction at 10% and 20% MVC that concentration rose. A further rise to a peak value was seen immediately following the contraction. Thereafter the concentration fell slowly toward resting levels in the recovery period. Although there was considerable variation in the concentration of ATP in the plasma of the different subjects, the pattern of response was similar for each. The following results give some impression of the variability of individual values; at 10% MVC, the highest average value during the contraction was 0.9 μg/ml (0.4–2.0 μg/ml) while after the contraction, it was 2.8 μg/ml (0.7–5.9 μg/ml). At 20% MVC, the corresponding values were 1.3 μg/ml (1.2–2.4 μg/ml) and 1.5 μg/ml (0.5–3.1 μg/ml).

Since the blood flow also increases during and after the contractions, the concentration of ATP in the venous effluent does not give a clear idea of the total output of ATP from the active muscles. Accordingly, the output of ATP was calculated as the product of the concentration and the flow and is shown before, during, and after each contraction in Fig. 1, along with the blood pressure and forearm blood flow. The bottom section of the

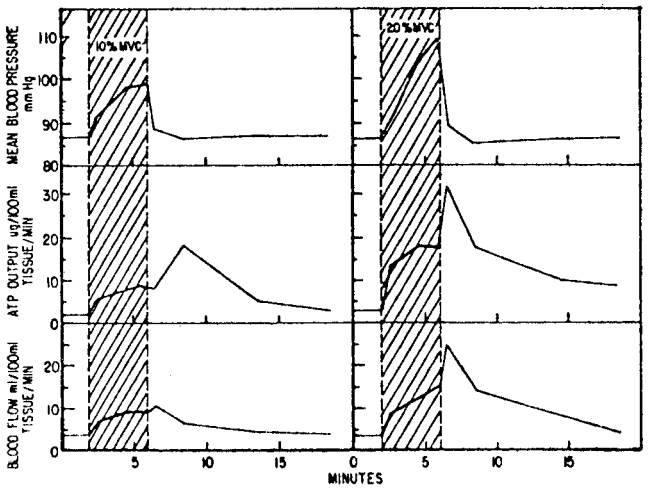

FIG. 1. The average forearm blood flows, ATP output, and mean blood pressure before, during and after sustained handgrip contractions held for 4 min at 10% MVC and 20% MVC. Note that while all three measurements increase during the exercise, when the tension is released the blood pressure falls at once and quickly returns to control levels, whereas the forearm blood flow and ATP output increase immediately and then return slowly toward control values.

figure shows the average blood flows for all four subjects at 10% MVC and for three of them at 20% MVC; blood samples were unobtainable in adequate quantities for the fourth subject at 20% MVC, probably due to venospasm around the catheter, and his results were discarded. The top section of the figure shows the mean blood pressure (taken to be the diastolic pressure plus one-third pulse pressure) averaged for the subjects. The average ATP output in the venous effluent is shown in the middle section of Fig. 1 to enable easy comparison to be made with the blood pressure and the forearm blood flow. Although there was some individual variation in the absolute changes of all those findings, the pattern for each subject was similar to the average values shown.

The patterns of the forearm blood flow and the ATP output were similar, increasing during the contraction, showing a further rise to peak values immediately after the contraction and subsiding to control values slowly. By contrast, the mean blood pressure, which also increased *during* the contraction, fell sharply when the tension was released and approximated to control values within a minute of the end of the contraction. Heart rates, which for the sake of clarity are not shown in Fig. 1, behaved in the same fashion as the blood pressure. The average resting value was 74 beats/min. At the end of contractions at 10% and 20% MVC, the values were 81 beats/min and 92 beats/min, respectively; in both cases they returned to control values within a minute of the release of the tension.

DISCUSSION

The results of this study have shown that ATP is found in the venous effluent of resting human forearm muscles. Increases in both the concentration and amounts of ATP are found during and after sustained, static exercise. The increased output of ATP found in the venous effluent *during* the contraction mimics the responses of the blood pressure, heart rate, and local blood flow. But after the muscular tension was released, the blood pressure and the heart rate fell immediately and reached control values approximately 1 min later, while the ATP output increased to a peak value before falling slowly to control values over a period of several minutes. The pattern and the time course of the changes in ATP output *after* the contraction mimicked only the changes in forearm blood flow. It seems reasonable to postulate that the changes in ATP output play no part in the pressor response to sustained contractions. It is hard to see how increases in the ATP output, both during and after the static effort, could result in an increased blood pressure only during the contraction. Since the patterns of response of the forearm blood flow and the ATP output were similar, it is equally reasonable to postulate that the ATP is responsible for the hyperemia of exercise. It is possible that other vasodilator mechanisms are available during and after muscular activity, such as an increased potassium efflux in the presence of a reduced O_2 tension (21), but the increased output of ATP, known to be a potent vasodilator, is sufficient by itself to account for exercise hyperemia.

When Duff, Patterson, and Shepherd (7) infused ATP into the human brachial artery, the forearm blood flow increased. Increments in blood flow of these subjects of about 1 ml, 2 ml, 6 ml, 11.5 ml, and 19.5 ml/100 ml tissue per min were obtained when the amounts of ATP infused (altered from the original data to represent amounts of infusate/100 ml tissue per min) were approximately 0.1 μg, 0.5 μg, 1.5 μg, 6.5 μg, and 25.5 μg/100 ml tissue per min. By comparison, the assessment of all the results from our study yields ATP outputs of about 0.5 μg, 2.5 μg, 7 μg, 17 μg, and 30 μg/100 ml tissue per min for similar increments of blood flow. These two sets of data are remarkably close, particularly since the conditions of the two investigations were quite different. In one case the arm was resting while in the other the muscles were active. In one case the ATP was being infused while in the other it was being measured in the venous effluent of the muscles. In both series of tests the changing amounts of ATP locally in dilating vessels cannot be assessed accurately—problems of mixing may well be responsible for some of the differences seen. Certainly, there are quite sufficient amounts of ATP available in the active forearm to account for the hyperemia of exercise.

During the sustained contractions, the blood flow increases against the mechanical compression of the contracting fibers, presumably due in part to the increase of perfusion pressure and in part to the active vasodilatation resulting from increased quantities of ATP in the circulation. After the tension is released, the mechanical compression of the blood vessels by the active muscle fibers is also released and at that time the perfusion pressure falls. While it is not possible to determine the net effect of these two counteracting effects, it is clear that further active vasodilatation can take place as a result of the further increase of ATP output after the exercise ends.

The authors express their appreciation to Professor Henry Barcroft for his continued interest in this study. We also acknowledge the excellent technical services of Mrs. A. Duhon, Miss M. Greer and Messrs. G. Schumpert and M. Souder.

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Discussion

DR. LAHIRI: The ATP concentration in the venous blood appears at a time when lactate should also be in high concentration. Is it possible that some ATP is not available for metabolic purposes in this condition, and how do you exclude the possibility that red cells are not giving up ATP to the venous blood?

DR. LIND: We have measured the lactate efflux at the levels of 10% and 20% of maximal voluntary contraction (MVC) and the patterns of the lactate and ATP responses are different. During and after 10% MVC, the changes in lactate efflux are practically nil. Even with 20% MVC held for 5 min the changes during contraction are very small and those afterward only moderate and short-lived.

As to shedding of ATP from formed elements of the venous blood, we have no direct evidence, but would guess that rather than red cells, platelets might be involved.

DR. FORRESTER: We have mixed ATP quantitatively with red cells in vitro with results indicating the red cells to be fairly stable as far as release of ATP is concerned.

DR. LANDOWNE: You indicate that there is numerically less ATP in arterial blood during the control period than in venous blood. If this is a real difference (i.e., if the ATP assays are sufficiently precise to enable such reliable estimates of A-V differences), it would imply that there is a release of ATP during the control period as well as during contraction. Moreover, the magnitude of the A-V difference in concentration during the control period is as great as in any other pair of samples. Therefore, I submit that the calculated difference in amount depends on the difference in flow. That is to say that, arithmetically, the flow determines these differences.

DR. LIND: Well, it depends on whether the flow determines it or it determines the flow and we submit that the ATP is determining the flow.

DR. REEVES: Have you calculated how much hemolysis would account for your background noise (i.e., the ATP concentration in arterial plasma), just from the known ATP concentration in the erythrocytes?

DR. FORRESTER: This is a most pertinent point and in fact hemolysis, destruction of blood platelets, the fact that the blood is obtained from an arterial cannula with a lot of turbulence of the whole blood is all working against there being a small amount of ATP in arterial samples. These small amounts may well be due to excessive turbulence during collection of the blood.

Commentary - paper 5.

Once again a valid criticism of this work was associated with cellular damage. The cells in question were the formed elements of the blood, particularly the blood platelets, which contain millimolar concentrations of ATP. It was concluded from this paper that some of the ATP detected was indeed coming from damaged platelets, but the remainder was added to the blood on its passage through the active forearm musculature.

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IDENTIFICATION OF ADENOSINE TRIPHOSPHATE IN HUMAN PLASMA AND THE CONCENTRATION IN THE VENOUS EFFLUENT OF FOREARM MUSCLES BEFORE, DURING AND AFTER SUSTAINED CONTRACTIONS

BY T. FORRESTER AND A. R. LIND

From the Institute of Physiology, University of Glasgow, and Indiana University Cardiopulmonary Laboratory, 6570 Aerospace Medical Research Laboratories, Environmental Medical Division, Wright–Patterson Air Force Base, Ohio, 45433, U.S.A.

(*Received* 27 *January* 1969)

SUMMARY

1. When diluted human plasma is perfused through a frog heart, a marked augmentation of the heartbeat is produced which is very similar in action to that of low concentrations of adenosine triphosphate (ATP) on the heart.

2. It was established that the substance in the plasma responsible for the heart stimulation was ATP. The following tests were used: (*a*) the diluted plasma emitted light from firefly lantern extract characteristic of the light signal produced by a solution of ATP; (*b*) the stimulatory effect on the frog heart and luminescent effect upon the firefly extract were abolished by incubation of the plasma solution with the enzyme apyrase, which converts ATP to adenosine monophosphate (AMP); AMP does not stimulate the heart or cause light to be emitted from firefly extract; (*c*) the stimulatory substance in the plasma was eluted through a column of Sephadex G-25 in the same pattern as ATP; and (*d*) simultaneous assay of plasma solutions on frog heart and firefly extract produced the same quantitative result as that produced by a solution of ATP.

3. The amount of ATP in plasma from the venous blood of resting subjects ranged from 0·19 to 0·95 μg/ml. (mean 0·63 μg/ml., S.D. $\pm$ 0·25); up to half of the ATP detected could be attributed to blood platelet damage. Simultaneous arterial and venous samples of blood from four subjects at rest had mean concentrations of 0·19 μg/ml. (0·07–0·26 μg/ml.) and 0·70 μg/ml. (0·57–0·84 μg/ml.) respectively.

4. The concentration of ATP in the venous effluent from exercising forearm muscles was measured. The venous concentration consistently increased over the resting values in response to exercise while in one subject little change occurred in the arterial blood concentration during the exercise. It was concluded that the ATP was added to the blood in its passage through the muscle bed.

5. The origin of the ATP, including erythrocytes, blood platelets and active skeletal muscle, is discussed.

INTRODUCTION

In order to demonstrate the release of ATP from active frog skeletal muscle *in vitro*, techniques were developed which could identify and measure ATP in as low a concentration as 10^{-9} g/ml. (Forrester, 1966, 1967; Boyd & Forrester, 1968). In view of the proposal that ATP might be liberated into the blood stream from active skeletal muscle *in vivo* (Forrester, 1967), these very sensitive techniques were applied to peripheral human blood, despite the fact that human plasma contains enzymes capable of destroying ATP within a short period of time (Jørgensen, 1956; Ireland & Mills, 1964; Holmsen, Stormorken & Goote, 1965).

The first part of this paper describes the identification and assay of ATP in fresh human plasma obtained from the antecubital vein; the second part deals with the investigation of ATP in venous blood draining the forearm muscles before, during and after sustained forearm exercise. A preliminary communication of this work has been given to the Physiological Society (Forrester, 1969).

METHODS

Samples of venous blood were obtained from the antecubital vein using only a brief period of manual constriction around the upper arm in order to effect enough distension of the vein for a 'clean' venepuncture. In four subjects samples were obtained from a plastic cannula inserted retrogradely into one of the deep veins of the forearm; no occlusion of the upper arm was thus necessary to obtain samples from these subjects. In every case the subject was instructed to keep the forearm musculature as relaxed as possible just before and during the sampling. Venous blood was slowly drawn off into a plastic 'non-wettable' syringe and 4 ml. then transferred to a plastic tube containing 0·3 % ethylenediamine-tetra-acetic acid (EDTA) and immediately put on ice for 5–10 min. After centrifugation at 1500 rev/min for 25 min at 4° C, the supernatant plasma was then diluted with either 0·9 % pyrogen-free saline solution (w/v) or Locke solution of composition 154 mM-NaCl, 5·6 mM-KCl, 2·2 mM-$CaCl_2$ and 6·0 mM-$NaHCO_3$.

The methods of identification have been previously described by Boyd & Forrester (1968). The frog-heart perfusion system (Boyd & Eadie, 1961), molecular sieve chromatography (Boyd & Forrester, 1966) and apyrase tests were used without modification. In the firefly technique (see Strehler & McElroy, 1957) the same photomultiplier tube (14-stage E.M.I. 6262 with a conventional S11 photocathode) was

used as before, since the 'dark current' was virtually zero, permitting a large signal-to-noise ratio at high voltages.

Continuous perfusion of the frog heart with Locke solution was possible without alteration of performance (Forrester, 1967). The samples diluted with saline were assayed on firefly extract only. Solutions of ATP (disodium dihydrogen salt of adenosine-5′-triphosphoric acid, British Drug Houses) were made up in either pyrogen-free saline solution for assay on firefly extract or Locke solution for assay on frog heart.

Glassware was siliconized using 'Repelcote' (Hopkin and Williams, Ltd).

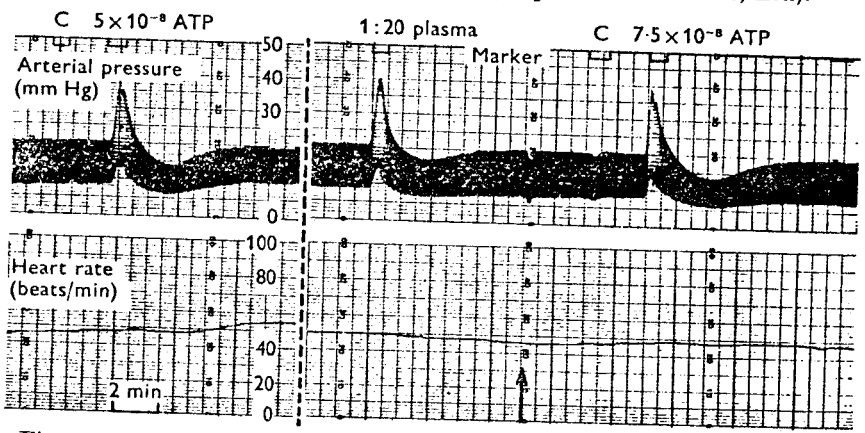

Fig. 1. Comparison of the action on a perfused frog heart of solutions of ATP and a diluted solution of fresh human plasma. Heart continuously perfused with Locke solution. C, control injection of Locke solution; vertical interrupted line, period of 25 min; ↑, fall in perfusion pressure during flushing of residual plasma solution from venous pressure capsule. Note the great similarity of action of both plasma and ATP solutions (g/ml.) on the blood pressure. There is little effect on the heart rate.

RESULTS

Pilot experiments showed that when suitably diluted fresh human plasma was perfused through a frog heart a pronounced stimulatory effect was produced. Figure 1 shows a comparison of the action of a diluted plasma solution and solutions of ATP on a frog heart. The effect of a 1:20 plasma solution has been 'bracketed' between the effects of ATP, 5×10^{-8} and $7{\cdot}5 \times 10^{-8}$ g/ml. There is clearly a great similarity of action between the test solution and the two solutions of ATP on the blood pressure. There is in all three cases an immediate positive inotropic effect on the myocardium, with little effect on the heart rate. Plasma from eight subjects was found to have the same qualitative action as ATP on the frog heart.

Since it is possible to discriminate the action of ATP from the actions of other nucleotide triphosphates on the frog heart (Boyd & Forrester, 1968), these preliminary results prompted the application of other tests for the identification of ATP.

Action of dilute plasma on firefly lantern extract. Extracts of firefly lanterns (*Photinus pyralis*) emit a slowly decaying flash of light when mixed with solutions of ATP, the time course of the signal decay being proportional to the concentration of ATP applied (McElroy, 1947). This reaction is reputed to be highly specific for ATP (Strehler & McElroy, 1957), although it was found in a previous investigation that the firefly extract

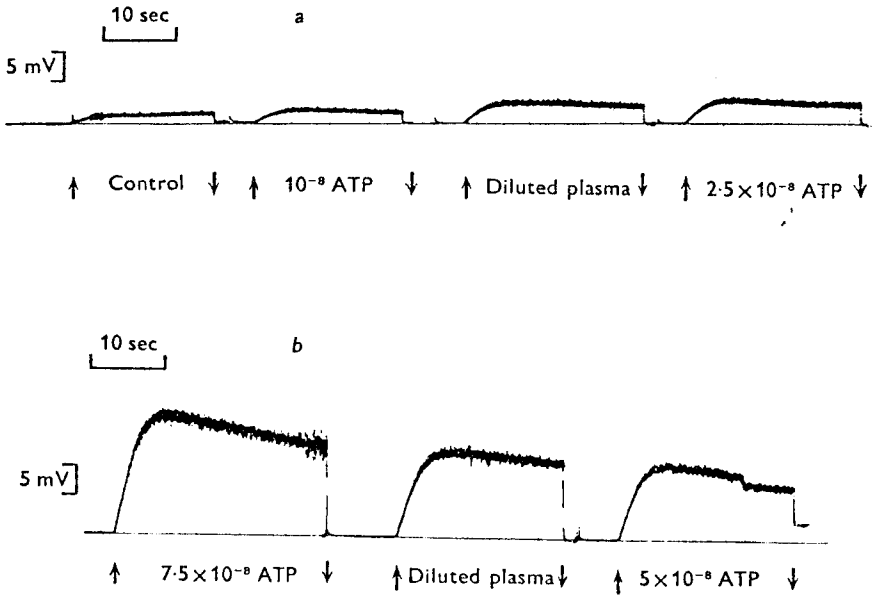

Fig. 2. Light emission from firefly extract in response to solutions of ATP (g/ml.) Upward deflexion indicates a light signal; ↑, voltage across photomultiplier tube switched on; ↓, voltage switched off. (*a*) Control, firefly extract + Locke solution; plasma sample, diluted 1:10 with Locke solution, has an effect almost equivalent to that of ATP $2{\cdot}5 \times 10^{-8}$ g/ml. (compare Fig. 5, last test). (*b*) A sample of plasma diluted 1:5 from another subject has its effect 'bracketed' between the effects of ATP 5×10^{-8} g/ml. and $7{\cdot}5 \times 10^{-8}$ g/ml. Note the similar time course of signal decay with plasma solutions and matching ATP solutions.

used (type FLE 50, Sigma Co.) also responds to low concentrations of other triphosphates (Boyd & Forrester, 1968). Samples of plasma from twenty-one subjects caused light to be emitted from firefly extract. In every case there was a light signal produced which could be matched in time course and intensity with that produced by small amounts of ATP. Figure 2 shows the effect of two plasma solutions, diluted 1:10 (*a*) and 1:5 (*b*) with saline, on extract of firefly. The light signals produced by the plasma solutions match those produced by the solutions of ATP.

Action of a specific enzyme on dilute plasma. The enzyme 'apyrase'

(adenosine-5′-diphosphatase, purified crystals, Sigma Co.) first converts ATP to ADP and 10 min later converts ADP to AMP. It has been demonstrated that the enzyme shows some specificity towards ATP (Krishnan, 1949), but it has been found in practice that it is not possible to distinguish ATP from ADP using apyrase in conjunction with the present methods (Boyd & Forrester, 1968). Figure 3 shows the effect of perfusing a dilute plasma solution, before and after incubation with apyrase, through a frog heart. Adenosine monophosphate does not stimulate the heart (Forrester, 1967). The stimulating effect of the plasma solution has been largely abolished after incubation and this is taken to indicate that the apyrase has had a dephosphorylating effect on the substance in the plasma which stimulates the frog heart.

Six plasma samples were tested in this way and in each case the positive inotropic effect was almost abolished by 10 min incubation with apyrase.

Column chromatography. In a molecular sieve chromatography procedure (Boyd & Forrester, 1966), it was established that the substance in the plasma which caused light to be emitted from firefly extract was eluted through a column of Sephadex G-25 in the same pattern as ATP. Undiluted plasma samples from five subjects were each tested in the following way. A 2 ml. plasma sample was mixed with blue dextran (a high molecular weight substance which acts as a visual indicator) and eluted through a column previously equilibrated with pyrogen-free saline solution. The column has the property of separating the protein and other molecules of large molecular weight from substances of smaller molecular weight. In general, the smaller the molecule, the more slowly it is eluted through the column. Consecutive fractions of eluate were collected from the column and then tested on firefly extract. It was found that the peak value of light emission from these fractions occurred at the same locus as that of ATP. Figure 4 shows the result of one such experiment. In this case a sample of plasma was obtained from a subject who was undergoing some mild forearm exercise; 2 ml. of the plasma sample was eluted through the column. Fractions of eluate were tested on firefly extract and the fraction which caused most light to be emitted was incubated with apyrase and then tested once again on the firefly. Figure 4(*a*) shows the pattern of light emission produced by the consecutive fractions. The hatched area shows the diminished light emission of that fraction after incubation with apyrase. Figure 4(*b*) shows the result of eluting a solution of ATP, 10^{-6} g/ml. through the same column 24 hr later. The greatest light signal is again produced by the fourth 2 ml. fraction.

It was not determined whether any destruction of ATP took place during its passage through the column, although this seems likely, since it was found on all five occasions when a solution of pure ATP was eluted

through a column after a sample of plasma had previously passed through it, that only a very small amount of ATP could be detected. Perhaps the column retains unknown substances from the plasma which have a dele-

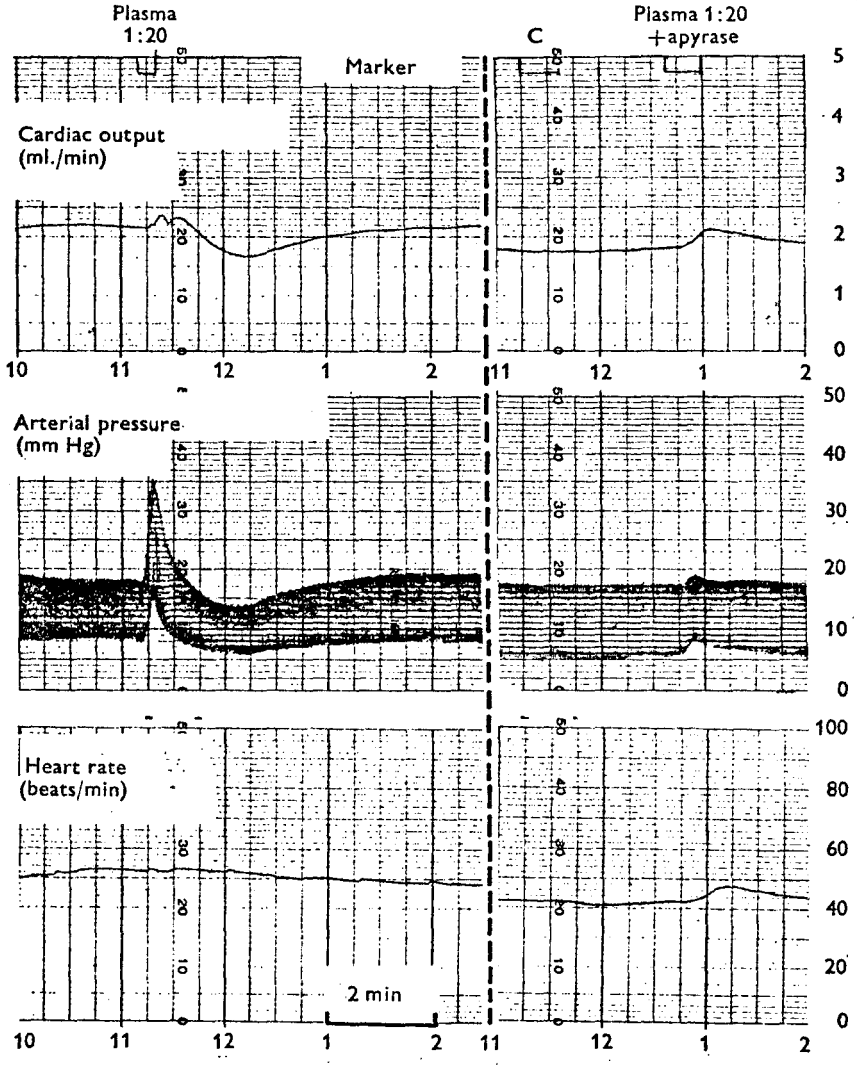

Fig. 3. The effect of a diluted plasma solution before and after incubation with apyrase on a perfused frog heart. Vertical interrupted line, interval of 20 min. The plasma sample, diluted 1:20, gives the usual marked positive inotropic effect; the cardiac output increased so rapidly at this point that the drop-counting mechanism failed. There is little effect on the heart rate. After incubation with apyrase the same solution has a much diminished inotropic effect. The increase in cardiac output has been caused by an increase in the heart rate.

terious effect on ATP. This phenomenon prevented accurate calculations of percentage regain of ATP from the column.

From these results—namely that the dilute plasma solution stimulates the frog heart in a manner similar to that of ATP, causes light to be emitted from firefly lantern extract, is eluted through a column of Sephadex G-25 in the same pattern as ATP and has its effect on both frog heart and firefly extract markedly reduced by incubation with apyrase—it was concluded that the substance in the plasma which stimulated the frog heart was ATP.

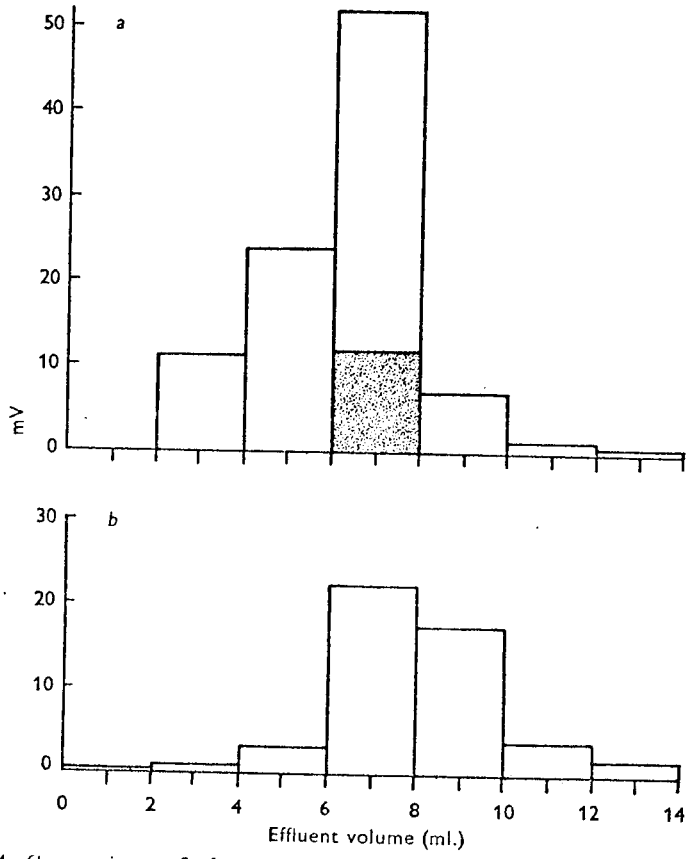

Fig. 4. Comparison of the behaviour of the substance in the plasma causing light emission from firefly extract and ATP in a column of Sephadex G-25. (*a*) Elution pattern from a plasma sample obtained from an exercising forearm. Peak light emission occurs in the fourth 2 ml. fraction. Hatched portion represents the light emitted from that fraction after incubation with apyrase. (*b*) Elution pattern obtained after passing a solution of ATP, 10^{-6} g/ml., through the same column 24 hr later. Again the peak light emission occurs in the fourth 2 ml. fraction.

Assay of plasma levels of ATP

An attempt to estimate the ATP in plasma was made, it having been established that small amounts of ATP could indeed exist in human plasma. Anticoagulated plasma samples from a total of thirteen subjects (9 ♂, 4 ♀) were assayed on firefly extract; three of those were assayed simultaneously on frog heart and firefly extract. The firefly procedure can distinguish between the actions of ATP and ADP but not between low concentrations

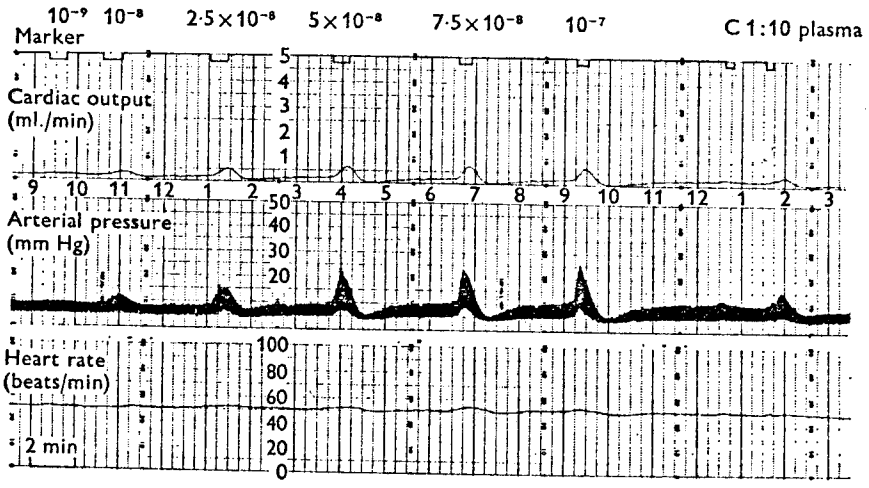

Fig. 5. Assay of a diluted plasma solution on a frog heart. From left to right, first six tests show the effects of graded concentrations of ATP in g/ml.; last test shows that the effect of a plasma solution diluted 1:10 is almost equivalent to that of ATP $2{\cdot}5 \times 10^{-8}$ g/ml. The same plasma solution was also assayed on firefly extract, with the same result (Fig. 2*a*).

of the various triphosphates; the frog-heart assay cannot distinguish between ATP and ADP but can distinguish between the actions of different triphosphates (Boyd & Forrester, 1968, figs. 5 and 8).

In each firefly assay a dose–response curve of light produced by the extract in response to ATP was obtained. The plasma samples were measured for light emission and an equivalent concentration of ATP assessed from the calibration curve (Fig. 6). In the three cases of simultaneous assay on the frog heart and firefly extract a dose–response curve of percentage output increase against concentration of ATP was derived on each heart and the test sample had its effect on cardiac output equated to a concentration of ATP. Figures 2(*a*) and 5 show the result of a parallel assay of a plasma solution. Figure 2(*a*) shows the effect of the solution, diluted 1:10, upon firefly extract. The effect of the solution is almost equivalent to that of a solution of ATP, $2{\cdot}5 \times 10^{-8}$ g/ml., giving the same

time course of signal decay. In Fig. 5 the initial tests on the heart show the gradually increasing effects of graduated concentrations of ATP on the cardiac output and blood pressure. The last test (on the right of the Figure) shows the effect of the same sample of diluted plasma; once again the effect lies between the effects of ATP, 10^{-8} g/ml. and $2{\cdot}5 \times 10^{-8}$ g/ml. The results obtained from the three parallel assays were not significantly different

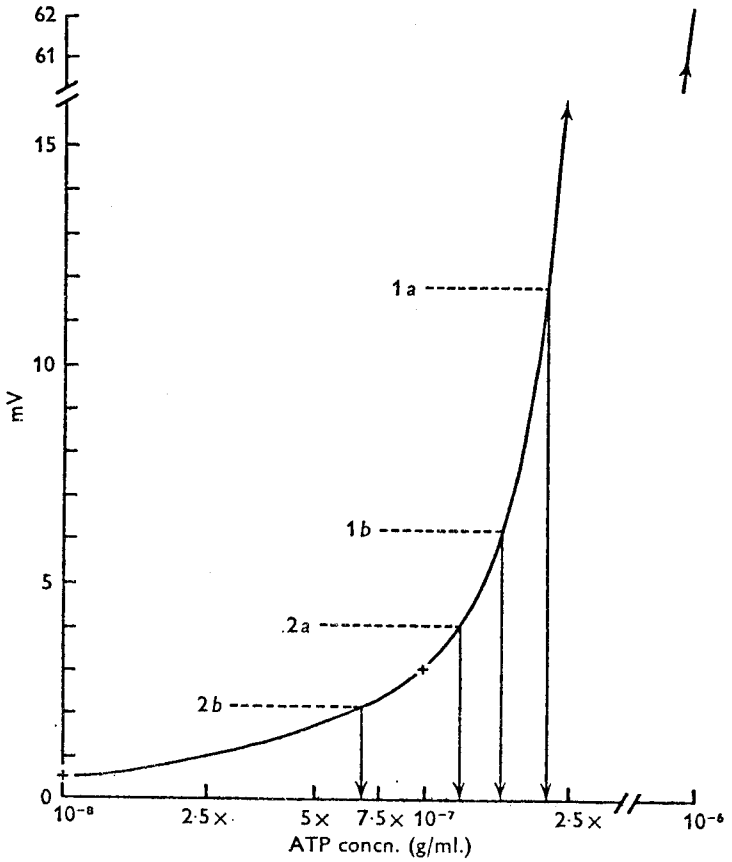

Fig. 6. Effect of time and 'non-wettable' glassware on ATP levels in plasma. Plasma sample from one subject divided into four parts, each diluted 1:5 with 0·9 % saline solution (w/v). Crosses, part of a dose–response relation of the action of ATP on firefly extract. Note interrupted axes in order to accommodate large light signal produced by ATP 10^{-6} g/ml. 1*a*, sample assayed immediately after centrifugation, using non-siliconized ware; assay result, 1·0 μg/ml. 1*b*, sample 1*a* assayed 10 min later; assay result, 0·85 μg/ml. 2*a*, sample assayed immediately after centrifugation, siliconized glassware used; assay result, 0·65 μg/ml. 2*b*, assay of sample 2*a* 10 min later; result, 0·35 μg/ml. Clearly in both 'wettable' and 'non-wettable' glassware the samples show a decline of ATP levels after 10 min. Siliconization also reduces the levels of ATP detected in human plasma.

from those obtained from the firefly extract alone, and so it was presumed that in every case the firefly extract gave an indication of the amount of ATP in the plasma solution and not of ADP or any of the other triphosphates.

The mean concentration of ATP measured in all samples of venous blood anticoagulated with EDTA was 0·63 μg/ml. (S.D. $\pm$ 0·25). The mean result for the four females tested was 0·64 μg/ml. (range 0·19–0·95 μg/ml.).

The effect of clotting on ATP levels in plasma. At an early stage in this work the blood samples were allowed to clot during the centrifugation period and the serum, gently expressed from the supernatant 'white' clot,

TABLE 1. Concentrations of ATP (μg/ml.) in samples of serum and plasma from four subjects. Last column shows the concentration of a 5 ml. solution of ATP, 0·5 μg/ml., after applying it to specimens of whole clot from each subject for a period of 1 min

Subject	Serum	Plasma	ATP, 0·5 μg/ml., + clot after 1 min
1	< 0·10*	0·23	0·34
2	< 0·10*	0·22	0·34
3	< 0·10*	0·15	0·35
4	—	0·10	0·50

* Samples of serum assessed on the lower part of calibration curve (see Fig. 6).

was then estimated for ATP. The time interval required to obtain serum samples was no different from that taken to obtain samples of plasma. From these early results there was an indication that the concentration of ATP in serum was lower than that in plasma, and so experiments were carried out to test whether the formation of a clot had any effect upon the levels of ATP in the plasma.

Blood samples were obtained by simple venepuncture from four subjects; each sample was divided into three parts. The first was allowed to clot during centrifugation, the second was routinely anticoagulated and the third portion was left to clot without centrifugation. The third samples had any residual supernatant blood decanted after a period of 20 min, leaving a clot behind in the tube. To this clot was added 5 ml. ATP, 0·5 μg/ml.; this mixture was left to stand for 1 min, after which time the ATP solution was decanted and assayed on firefly extract. The paired samples of serum and plasma were assayed on firefly extract immediately after centrifugation. Table 1 shows the results obtained for the concentrations of ATP in serum and plasma and the effect of applying a clot to ATP, 0·5 μg/ml., for 1 min. From these figures it is clear that, with the same samples and over the same period of time, the levels of ATP are markedly

reduced if the clotting process is allowed to take place. The application of an established clot to a known amount of ATP also diminishes its concentration within the space of 1 min. In three out of the four subjects the concentration was reduced, while in the fourth subject there was no change in the concentration. These experiments thus excluded the possibility that the clotting process might add to the amount of ATP detected in the plasma.

The effect of siliconized glassware on ATP levels in plasma. It is well known that for the collection and preservation of blood platelets the use of 'non-wettable' glassware is essential. Since blood platelets contain high concentrations of ATP (Born, 1958) it became essential to determine the effects on the plasma levels of ATP using glassware rendered non-wettable by siliconization. Samples of blood obtained from three subjects were divided into two portions, one group being handled in siliconized glassware, the other being centrifuged in non-siliconized tubes. In one subject the sample of blood was split into four portions, two for siliconized glassware and two for 'wettable' glassware. Figure 6 illustrates the result of an assay of these samples from the last subject on firefly extract. When the light emission was assessed on a dose–response curve, those samples handled in 'wettable' glassware (Fig. 6, 1*a*, 1*b*) had clearly a higher level of ATP than those which were contained in 'non-wettable' glassware (Fig. 6, 2*a*, 2*b*). In the case of the samples which were assayed immediately after centrifugation, sample 1*a* had a concentration of ATP, 1·0 μg/ml., whereas 2*a* had an ATP concentration of 0·65 μg/ml. Estimations 1*b* and 2*b* are the ATP concentrations of these samples measured 10 min later. It is to be noted that these assays were performed on the steepest part of the calibration curve, there being more than twice the light produced from sample 1*a* than from 2*a*.

The mean concentration of ATP in the samples where non-siliconized glassware was used was 0·64 μg/ml. (range 0·5–1·0 μg/ml.), virtually the same as the over-all assay figure; the mean concentration of ATP in samples contained in siliconized glassware was 0·41 μg/ml. (range 0·24–0·60 μg/ml.). Thus it seems that up to one-half of the ATP detected in the main series of assay results (mean 0·63 μg/ml.) is probably contributed by platelet damage when centrifuging was performed in tubes which had not been siliconized.

Rate of decline of ATP levels in plasma at room temperature. It was found that when a dilute solution of plasma was left at room temperature (18–22° C) for 1 hr after centrifugation, no ATP could be detected by the present methods. Obviously continuous ATPase activity could proceed in the plasma at room temperature.

An assay of two solutions of diluted plasma immediately following

centrifugation (1*a*, 2*a*) and then 10 min afterwards (1*b*, 2*b*) is illustrated in Fig. 6. After 10 min the ATP level in sample 1 declined from 1·0 to 0·85 μg/ml., while the level in sample 2 declined from 0·65 to 0·35 μg/ml. In another sample from the same subject there was a fall from 0·42 to 0·25 μg/ml., using siliconized glassware. An average fall in ATP concentration of 34% in the 10 min period 30–40 min after withdrawal of the blood from the vein would suggest that ATP can decay rapidly in diluted plasma, even at room temperature.

Release of ATP into the venous effluent of forearm muscles during and after sustained contractions

An assessment of the concentration of ATP in the venous effluent from forearms was made before, during and after voluntary sustained contractions of the forearm muscles.

Methods. The combination of three techniques was used for this study: (1) strain-gauge plethysmography (Whitney, 1953), (2) the firefly luminescence procedure, and (3) a method of accurately controlling static muscular contractions which allows measurement of blood flow during, as well as before and after, the muscular activity (Clarke, Hellon & Lind, 1958).

Five young, healthy men volunteered to act as subjects. The sustained contractions were performed on a simple hand-grip dynamometer (Clarke *et al.* 1958) on which the anatomical 'learning' responses were minimal. The maximal voluntary contraction (MVC) was determined for each subject; this was established on the day before the experiment to prevent the possibility that some residual influence of the MVC would persist and affect the venous concentration of ATP. The test contractions were chosen to be 10 and 20% of the MVC. The former provided a non-fatiguing contraction while the latter represented a tension which resulted in muscular fatigue.

The subjects lay on a bed, recumbent, throughout each experiment. At the outset a Teflon catheter was introduced into a vein in the antecubital fossa of the test forearm; the tip of the catheter was passed retrogressively to lie in a vessel draining the deep venous plexus in the muscle. Samples of venous blood were taken from the catheter mainly by allowing the blood to flow directly into centrifuge tubes containing EDTA. About 5 ml. blood was drawn for each sample. In a small number of cases, when the flow through the catheter was very low, samples were withdrawn into a syringe (non-wettable) and transferred at once to the centrifuge tubes. When samples were not being collected, the Teflon catheter was continually infused with pyrogen-free saline at a rate not exceeding 1 ml./min.

Forearm blood flows were measured by the Whitney (1953) mercury-in-rubber strain-gauge plethysmograph. To avoid any possibility of haematological trauma due to the inflation of plethysmographic cuffs, the blood flows were obtained on a separate occasion, under identical conditions. Changes in forearm blood flow in response to specific sustained contractions have been shown to be repeatable for individual subjects (Lind & McNicol, 1967).

Brachial arterial and venous blood samples were obtained from four subjects at rest and the ATP concentrations of these samples were measured.

Each subject made the test contractions of 10 and 20% MVC with his preferred hand; four of the five subjects were right-handed. The procedure was based on a 20

min cycle as follows: 2 min of resting control values, 4 min of the test contraction and 14 min of post-exercise examination. Venous blood samples were withdrawn once or twice during rest, three times during the contraction and four times during the post-exercise period. The samples were put on ice and centrifuged together at the end of each 20 min test. The first contraction for each subject was 10 % MVC; the subjects then underwent the same procedure at a tension of 20 % MVC. On one subject who performed only the 10 % MVC, blood samples were taken simultaneously from a Cournand needle introduced into the brachial artery of the arm to be exercised; after each sample the needle was flushed with a few ml. saline to keep the needle patent.

On two occasions solutions were diluted and frozen for a later parallel assay on firefly extract and on frog heart. The same quantitative result was produced, indicating that the firefly technique was measuring ATP and not ADP or any of the other nucleotides (Boyd & Forrester, 1968, figs. 5, 8*d*).

The concentration of ATP in each sample for each subject is shown in Fig. 7. Successive samples from resting forearms of the same subjects showed a remarkable consistency in the concentration of ATP. The samples taken during and after both contractions showed higher ATP concentrations than the resting values. In three out of the four subjects there was an increase in concentration throughout the 10 % MVC. At 20 % MVC, where, for technical reasons, results were available on only three out of four subjects, the last samples taken during exercise all had a lower concentration than the previous exercise sample.

A noteworthy feature of these results is the consistently different pattern of ATP concentration in the 10 % MVC compared to that of the 20 % MVC during the post-exercise period. After the 10 % MVC there was a marked peak of ATP concentration 2 min after tension was released; this was not the case at 20 % MVC, where the actual concentrations were lower, in general, than they were after the 10 % MVC. There was no obvious relationship between the venous concentration of ATP and the amount of blood flow. The forearm blood flow reaches a steady state at 10 % MVC, which is a contraction that does not result in fatigue, followed by a small post-exercise hyperaemia which was completed some 4 min after the contraction ended. At 20 % MVC, a tension which eventually results in fatigue, there was a continual rise of blood flow through the contraction to values higher than those obtained during the lower tension, followed by a clear post-exercise hyperaemia which persisted for 8 min or more. These findings are similar to those described before (Lind & McNicol, 1967).

It was clear that the concentration of ATP in the venous effluent was increased in association with sustained forearm exercise, but the actual amounts involved were difficult to assess. If ATP entered the blood stream at the site of the muscle bed, the concentration would of course be diluted by the concomitant vasodilatation during the exercise period. If the ATP has as its source a point proximal to the muscle bed, then the

increase in blood flow through the muscle would serve to increase the actual amounts of ATP appearing in the venous effluent per unit time. Thus it became essential to find whether ATP was present in the arterial blood passing to the forearm musculature.

Resting arterial and venous samples of blood were taken from four subjects. The mean arterial concentration of ATP was 0·19 μg/ml. (0·07–0·26 μg/ml.) and in venous blood of the same subjects it was 0·70 μg/ml. (0·57–0·84 μg/ml.), similar to the venous levels found previously (Forrester,

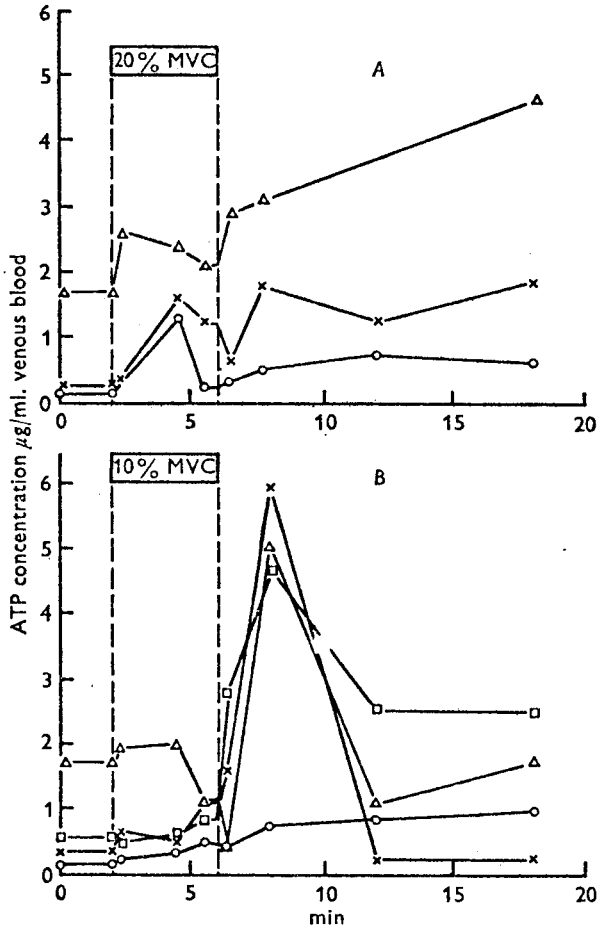

Fig. 7. The ATP concentration (μg/ml.) in the venous effluent before, during and after 20 % (*A*) and 10 % (*B*) maximum voluntary contraction. Different symbols represent the results of different subjects. The shaded bar indicates duration of the sustained forearm contraction. Note the slow rise in concentration of ATP during the post-contraction phase in the 20 % MVC (tension resulting eventually in fatigue) in contrast to the sharp rise and fall obtained in the non-fatiguing 10 % MVC.

1969). Thus it appeared that, under 'resting' conditions, blood coming from the forearm musculature contained more ATP than that travelling to it. One experiment was performed where simultaneous arterial and venous samples were taken from a subject before, during and after a 10 % MVC. Figure 8 (*a*) shows the concentrations of ATP obtained in the venous and arterial blood. Except for one sample, taken immediately after the start of the contraction, the concentration of ATP was clearly higher in

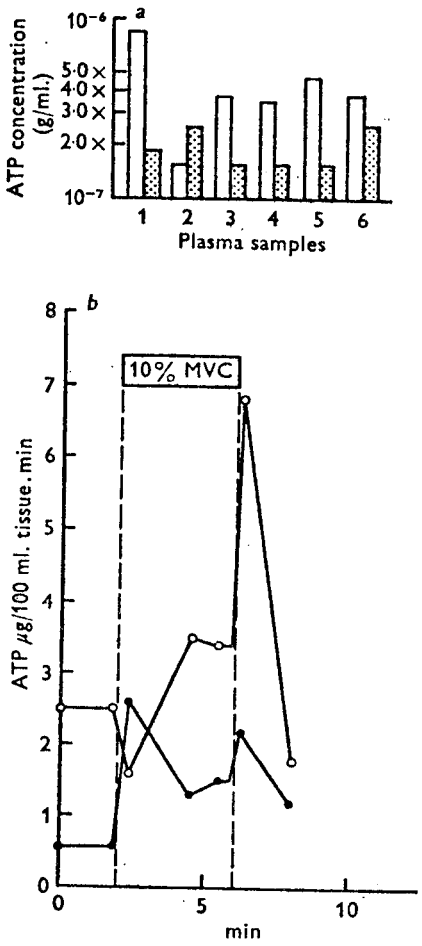

Fig. 8. Concentrations and amounts of ATP in arterial and venous blood of one subject before, during and after a 10 % MVC: (*a*) shows a histogram of concentrations of arterial (▦) and venous (□) samples taken simultaneously; sample 1, resting value; samples 2, 3 and 4 taken at intervals during contraction; samples 5 and 6 obtained after the contraction. (*b*) shows comparison of total amounts of ATP in venous (○) and arterial (●) blood before, during and after the contraction.

the vein than in the artery, even during the period of vasodilatation. The blood flow through the forearm was measured at a 10% MVC and the actual amounts of ATP in arterial and venous blood before, during and after the contraction were expressed in μg/100 ml. tissue.min (Fig. 8*b*). Apart from the first venous sample during the work period, the amount of ATP in both arterial and venous blood increased during work and immediately after work. Two minutes after work the concentration of ATP had returned or nearly returned to normal resting levels. The blood flow during the contraction at this tension increased to a steady-state level approximately three times the resting value and then showed an increase to five times the resting value in the first 10 sec after work. It returned almost to the control value 2 min after the contraction ended.

DISCUSSION

Adenosine triphosphate has been identified in human arterial and venous blood and it has been shown that there is an increased concentration produced in the venous effluent from exercising forearm muscles. The main question arising from these results concerns the source of the ATP. Are the plasma levels of ATP in samples of blood obtained from resting subjects merely an index of damage to the formed elements of the blood?

The erythrocyte contains high concentrations of ATP (Bartlett, Savage, Hughes & Marlow, 1953). In the series of experiments where known amounts of ATP were applied to clotted whole blood there was inevitable mixing of free erythrocytes with the solution of ATP. Any liberation of ATP from these cells would almost certainly have been detected; in fact, the ATP concentration was reduced by this procedure (Table 1).

Blood platelets contain a higher concentration of ATP than other tissues, with the exception of the adrenal medulla and mammalian skeletal muscle (Born, 1958). Experiments in the present work indicate that platelet damage was contributing up to half of the total amount of ATP found in the plasma. Early observations by Zipf (1931) have indicated that whole blood, when exposed to mechanical disturbance, will increase its vasodilator property. Folkow (1952) has also shown that vasodilator agents are released even when slight handling of arterial blood takes place, and that the most important of these substances might be ATP released from the erythrocyte. It now seems likely from the present results that the increase in vasodilator property of shaken blood was due mainly to ATP released from damaged platelets, rather than from erythrocytes.

The increase in concentration of ATP in the venous effluent from exercising forearm muscles may prove to be significant. It is unlikely that muscles performing sustained contractions at the level of 10 and 20% of

the maximum voluntary contraction inflict any damage on the formed elements of the blood, although it is not known whether local effects of contracting muscle can render blood platelets more fragile, thus increasing the amount of ATP released from them. Abood, Koketsu & Miyamoto (1962) have shown *in vitro* that ATP is liberated from both nerve and muscle cells in association with depolarization. It may be that the increase in ATP concentration of blood coming from exercising forearm muscle represents some residual ATP produced by recent activity of muscle and nerve. If indeed ATP is released from active skeletal muscle *in vivo*, the question must be raised as to the part it may play in the hyperaemia occurring in muscles during and following contraction, since ATP is a powerful vasodilator agent.

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We wish to express our appeciation to Professor Henry Barcroft for his continued interest in this study. We also acknowledge the excellent technical services of Mrs Anne Murray of the Physiology Department, Glasgow, and Mrs A. Duhon, Miss M. Greer, Mr G. Schumpert and Mr M. Souder of the Indiana University Cardiopulmonary Laboratory, Wright Patterson Air Force Base, Ohio.

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Commentary - paper 6.

The full report discussed in the previous paper was received with interest in view of the great vasodilator potency of ATP upon muscle blood vessels. The older literature dealing with other extracellular actions of ATP was reconsidered and other workers contemplated the possible role of nucleotides as local vasodilators. A vital question arose: is there enough ATP released locally to produce a vasodilatation to match that seen in heavy muscular exercise? Earlier work by Duff, Patterson & Shepherd (1954) had demonstrated the potent (and painless) nature of the action of ATP on human forearm muscle blood vessels. They had also assessed the blood flows in forearm muscle in response to various infused concentrations of ATP. In other words a dose-response curve was already available for human forearm muscle in terms of ATP. This paper compares the amounts of ATP recovered from the exercising forearm with that amount injected by Duff *et al* for the same blood flow. Evidence was provided that there was indeed a significant amount of ATP released from the forearm musculature to cause vasodilatation in exercise.

The problem of what concentrations occurred locally around the resistance vessels was not yet resolved, nor was the question of the fate of the products of nucleotide degradation yet addressed.

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AN ESTIMATE OF ADENOSINE TRIPHOSPHATE RELEASE INTO THE VENOUS EFFLUENT FROM EXERCISING HUMAN FOREARM MUSCLE

By T. FORRESTER

From the Institute of Physiology, University of Glasgow, Glasgow, W. 2

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SUMMARY

1. Human subjects performed a sustained contraction of the forearm muscles for 4 min in the presence of arterial and venous occlusion.

2. The contraction was maintained at 5% of the maximum voluntary contraction, a tension during which the muscle blood flow might be expected to increase by about three times (Lind & McNicol, 1967).

3. Adenosine triphosphate (ATP) was identified in the venous effluent from occluded exercising forearm, but not in the venous effluent from occluded forearm without exercise.

4. The rate of degradation of ATP was assessed in plasma at 37° C, with an estimate of the percentage loss occurring between sampling and testing. This enabled the rate of appearance of ATP in the blood at the time of exercise to be calculated as approximately 7·5–10·5 μg/min (14–20 n-mole/min). These amounts are compared with 16 μg/min that was infused intra-arterially into human forearm to cause a threefold increase in blood flow (Duff, Patterson & Shepherd, 1954).

5. It is likely that the ATP detected in the venous effluent has active muscle as the source; if so, then the amounts calculated to be released could satisfy the vasodilator requirements of active skeletal muscle.

6. The effects of circulating ATP on respiration and coronary blood flow during exercise is discussed, including the role it may play locally in the production of ischaemic pain.

INTRODUCTION

It is now generally agreed that the hyperaemia in exercising skeletal muscle is mediated by changes in the local chemical environment. Many changes have been put forward as being responsible for this vasodilatation,

including hypoxia (Guyton, Ross, Carrier & Walker, 1964), release of ATP from active muscle (Boyd & Forrester, 1968), increase in potassium concentration (Dawes, 1941; Kjellmer, 1965), increase in osmolarity (Mellander, Johansson, Gray, Jonsson, Lundvall & Ljung, 1967), a combination of hypoxia and increased potassium (Skinner & Powell, 1967) and, more recently, an increase in the concentration of inorganic phosphate (Hilton & Vrbovà, 1970).

A common approach to the problem has been to perfuse the resting skeletal muscle with blood or saline having a composition similar to that of the venous blood obtained from the muscle when active. The increase in blood flow so produced is then compared with the hyperaemia occurring at the time of exercise. In this way the roles of oxygen, hydrogen ion, potassium, osmolarity and magnesium have been considered (Haddy & Scott, 1968; Scott, Rudko, Radawski & Haddy, 1970), but none appear to be satisfactory either on the grounds of potency, or of the amounts detected in the venous effluent, or both. The role of inorganic phosphate has also been investigated in this manner by Barcroft, Foley & McSwiney (1971). They found that during vigorous exercise of the forearm muscles the phosphate in the venous effluent increased in concentration by 20 %. However, an infusion of phosphate which raised the plasma phosphate level in the venous effluent by 400 % had no effect upon the rate of forearm blood flow in the resting forearm.

ATP is well known as a powerful vasodilator of skeletal muscle blood vessels, hyperaemia of a magnitude equivalent to that found in exercise being easily achieved with quite moderate intra-arterial doses (Duff *et al.* 1954). The release of ATP from active skeletal muscle *in vitro* has also been demonstrated (Abood, Koketsu & Miyamoto, 1962; Boyd & Forrester, 1968). Recently it has been shown that there is an increase in the concentration of ATP in the venous effluent from exercising human forearm muscle (Forrester & Lind, 1969); however, a quantitative study was not really possible for several reasons. First, the increase in blood flow through exercising skeletal muscle has the effect of washing out and diluting the ATP to an unknown extent. Secondly, the rate of destruction of small amounts of ATP in the bloodstream was unknown and thirdly, it was realized that a proportion of the ATP detected came from damage to blood platelets after collection of the samples. With refinements in technique it has now been possible to avoid damage to blood platelets, thus eliminating the background levels of ATP; the problem of dilution *in vivo* has been minimized by arresting the blood flow during the period of exercise; the rate of degradation of ATP in plasma at 37° C has been assessed, along with an estimation of the percentage loss occurring between sampling and testing.

The purpose of this study was to obtain an estimate of the original amounts of ATP appearing in the venous effluent at the time of exercise and to compare them with the amounts that Duff *et al.* (1954) infused into the forearm to cause the equivalent of a physiological vasodilatation. A preliminary report of these results has been given to the Physiological Society (Forrester, 1972).

METHODS

Experiments were performed on seven healthy male volunteers aged from 22 to 54 years. Each subject sat comfortably in an upright chair and grasped a vertical wooden bar steadily with the thumb hooked round a fixed vertical post. The arm was kept straight and horizontal. The bar had a 3·5 kg weight suspended from it, making the subject sustain a grip of about 5% of their maximum voluntary contraction (MVC).

Procedure. The subject was asked to grip the bar and at the same time an arterial occlusion cuff was applied above the elbow and inflated to a pressure of 180 mm Hg. Exercise continued for 4 min after which time the cuff was deflated to pressures ranging from just below systolic to zero mm Hg. This allowed blood to enter the forearm, displacing the blood already in the forearm musculature towards a sampling needle (Gillette type 120, external diameter 1·1 mm) situated in an antecubital vein. Samples of blood were taken at intervals before, during and immediately after the occlusion period, the subject still maintaining the sustained forearm contraction.

Each sample of venous effluent (3·6 ml.) was transferred to a centrifuge tube, containing 0·4 ml. of citrate anticoagulant, which was centrifuged at 2000 rev/min for 25 min at 5° C. The supernatant plasma was then tested using the firefly luminescence technique. All blood samples were handled throughout in non-wettable glassware and plastic non-wettable containers. Glassware was siliconized using 'Repelcote' (Hopkin & Williams Ltd.).

Firefly luminescence procedure. A modification of the method devised by Strehler & McElroy (1957) was used and has been previously described (Boyd & Forrester, 1968). The same photomultiplier tube (14-stage E.M.I. 6262 with a conventional S II photocathode) was used as before; the dark current was virtually zero, giving a maximum signal-to-noise ratio at high voltages. Some improvements were made for this investigation. An automatic pipette gun (Schwarz Bioresearch Co.) with non-wettable plastic dispensers was used to apply the sample of plasma to the extract of firefly tails (type FLE 50, Sigma Co.). Thus mixing of the solution became more efficient and the time taken to assay the samples was also greatly reduced. A switch was incorporated between the powerpack and the phototube enabling the voltage used (1300 V) to be applied instantly across the tube. This made it possible to record the time course of the light signal immediately after pipetting in the solution of plasma (compare Fig. 2*b*, Forrester & Lind, 1969 with Fig. 3*b*) and became an important factor in the comparison of responses evoked from firefly extract by different triphosphates. The voltage across the tube was switched on 6 sec after pipetting the plasma sample into the extract of firefly tails.

The response of the extract to ATP, $2{\cdot}5 \times 10^{-8}$, 5×10^{-8} and 10^{-7} g (0·05, 0·1 and 0·2 n-mole)/ml. is shown in Fig. 1*a*. Note that the maximum light signal is at the beginning and that there occurs a steady fall in the light emission. Since the firefly extract alone emits a steady light signal a decay in the light signal had to be present before ATP was judged to be present in a solution. A dose–response curve is obtained by plotting the maximum light signal obtained 6 sec after mixing the solution of

ATP with extract (Fig. 1*b*). Variations of light signal are slight at the lower concentrations and in general the shape of the dose–response curve is remarkably constant. The response to similar doses of adenosine diphosphate is also shown.

Anticoagulation. It was found that exposure of a solution of plasma to solutions of ethylenediamine-tetra-acetic acid (EDTA) caused the appearance of ATP in the plasma. In contrast, a solution of citrate (2% trisodium citrate in normal saline) had no effect on the levels of ATP in plasma. This finding, together with the scrupulous siliconization of glassware, reduced the background levels of ATP in plasma to below the threshold sensitivity of the firefly test.

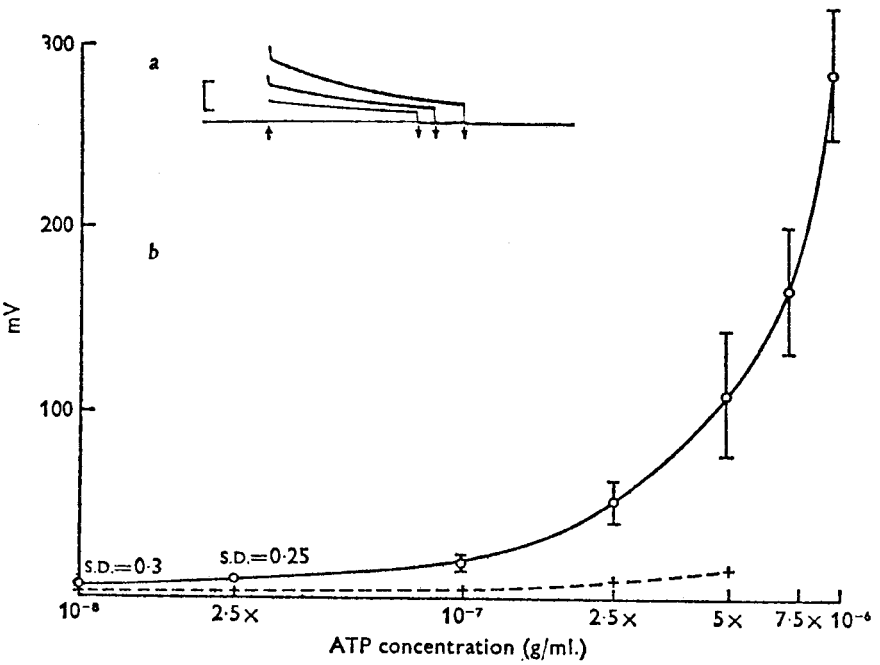

Fig. 1*a*. Light signals from firefly extract in response to ATP, $2{\cdot}5 \times 10^{-8}$, 5×10^{-8} and 10^{-7} g/ml. ↑, voltage switched on; ↓, voltage switched off; vertical bar, 10 mV; shortest trace, 50 sec. *b*, Concentration–response curve of firefly extract to ATP. Ordinate, mV drop in proportion to current in phototube. Abscissa, concentration of ATP applied. Dotted line, response to ADP. Vertical bars are ± one S.D.

Measurement of emission spectrum. The Aminco–Bowman spectrophotofluorometer was used to measure the spectrum of light emitted from the firefly extract in response to various triphosphates. The nucleotides were added by pipette to firefly extract in a cell already mounted in the spectrophotofluorometer. The spectrum was continuously scanned from 200 to 800 nm in a period of 90 sec. High concentrations of nucleotide were used (10^{-4} g/ml. or 182 n-mole/ml.) so that the intensity of the light signal was great enough for detection by the phototube (Aminco–Bowman type IP 28). Scanning was not started until there was minimal decay in the light signal. This technique has been demonstrated to the Physiological Society (Forrester, 1970).

Radioactivity measurements. Adenosine-2-H3-5′-triphosphate, cytidine-5-H3-5′-

triphosphate, guanosine-8-H3-5′-triphosphate, inosine-8-H3-5′-triphosphate and uridine-5-H3-5′-triphosphate were supplied as the ammonium salt by the Radiochemical Centre, Amersham. After elution through the Sephadex column (see below) the fractions were counted using a liquid scintillation spectrometer ('Tri-carb', Packard).

Molecular sieve chromatography. A column of Sephadex (G-25, fine grade, Pharmacia, Sweden) was used and has been described in detail elsewhere (Boyd & Forrester, 1966).

Apyrase test. Potato apyrase (Sigma Co.) was used as previously described, without modification (Boyd & Forrester, 1968).

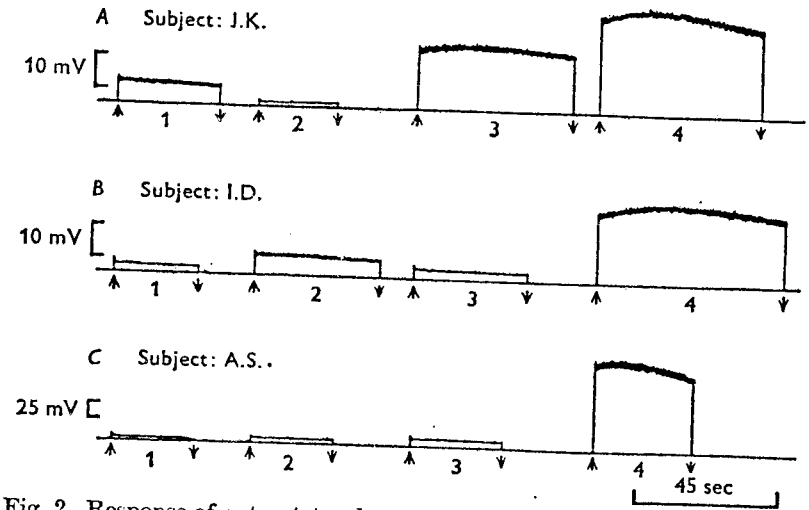

Fig. 2. Response of extract to plasma samples from the forearms of three subjects, *A*, *B* and *C*. Sample 1 taken before onset of exercise; samples 2 and 3 taken during occlusion + exercise; sample 4 taken just after occlusion, exercise continuing. ↑, voltage switched on; ↓, voltage switched off.

RESULTS

In nine experiments on seven subjects sustained exercise of the forearm muscles in the presence of arterial occlusion produced a change in the venous plasma which caused a light signal to be produced when mixed with extract of firefly tails.

Fig. 2 illustrates the response of firefly extract to samples of plasma obtained from three of the subjects before, during and after arterial occlusion, each subject performing a sustained contraction of the forearm muscles throughout. Sample 1 in each case was taken before the onset of exercise. The occlusion cuff was then inflated and exercise commenced. Samples 2 and 3 were collected during the period of occlusion and sample 4 was obtained just after the occlusion cuff was deflated. In every experiment the response to samples taken just after arterial occlusion was always much greater than the response to plasma from the resting forearm. In six

subjects the venous effluent was tested after at least 4 min of arterial occlusion without exercise. In no case did any post-occlusion sample of plasma produce a light signal from firefly extract. Fig. 3 compares the response of firefly extract to samples of plasma from the same forearm after occlusion + exercise (*A*) and then after occlusion alone (*B*). The sampling needle was introduced through the same skin puncture into the same antecubital vein in the second experiment. The cuff was inflated between taking samples 1 and 2; sample 3 was obtained just after cuff deflation. No light signal is evoked from firefly extract by the post-occlusion sample from the resting forearm.

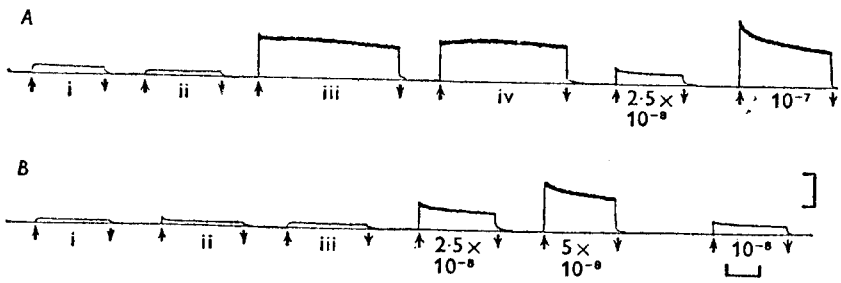

Fig. 3. Response of extract to plasma from a forearm undergoing *A*, occlusion + exercise and *B*, occlusion only.

A (i), before exercise; (ii) 3 min after onset of occlusion and exercise; (iii) 2½ min later, cuff at 80 mm Hg; (iv) 1½ min later, cuff at 60 mm Hg. Responses to ATP (g/ml.) also shown.

B (i) before occlusion; (ii) after 4 min of occlusion; (iii) 20 sec later. Note the greater sensitivity to ATP, $2{\cdot}5 \times 10^{-8}$ g/ml.

Scales: vertical, 10 mV; horizontal, 10 sec. Apply to both traces. ↑, voltage switched on; ↓, voltage switched off.

Identification of ATP

Although ATP was previously identified in plasma from exercising forearm muscle (Forrester & Lind, 1969), nevertheless, further tests of identification were applied in this work in addition to the firefly luminescence test.

Column chromatography. A post-occlusion sample of plasma (Fig. 5, subject S.W., fourth sample) was eluted through a column of Sephadex and the fractions tested on firefly extract. Fig. 4*B* shows the pattern of elution of the substance evoking a light signal from firefly extract. In Fig. 4*C* the elution pattern of a post-occlusion sample of plasma from another subject is shown. In this case the fraction occurring at 9 ml. was incubated with 1 mg of the enzyme apyrase at 30° C for 10 min and then tested again on the extract. Apyrase converts ATP to AMP (Krishnan, 1949) which does not produce a light signal from firefly extract. The extent of the light production after incubation with apyrase is shown by the dark

column at 9 ml. The shaded column shown at 10 ml. is the result of incubating this fraction for 10 min at 30° C without apyrase. The slight reduction in the signal would be expected as a result of the residual ATPase activity in the plasma (see Fig. 6). Fig. 4*A* shows the pattern of elution of a solution of ATP, 1 μg (1·8 n-mole)/ml. The peak signal appears at almost

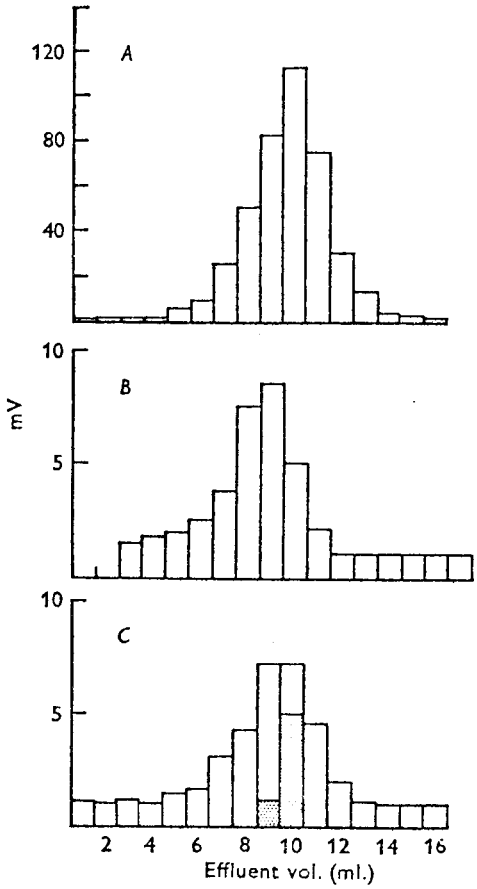

Fig. 4. Elution of ATP and plasma samples through a Sephadex column. Fractions estimated on firefly extract. *A*, ATP, 10^{-6} g/ml. *B*, post-occlusion sample obtained during exercise. *C*, post-occlusion sample from another subject (I.D.); fraction at 9 ml. incubated with apyrase, retested on extract (▨); fraction at 10 ml., incubation control (□).

the same locus as that for the samples of plasma. It is interesting to compare this elution pattern with that obtained using labelled ATP; in both cases the peak lay near to 9·5 ml. Since the firefly extract is relatively insensitive to ADP (see Fig. 1), this would indicate that little break-down of ATP occurs when a pure solution is eluted through the column.

It was possible with the use of labelled nucleotides to obtain the elution patterns of CTP, GTP, ITP and UTP. The only triphosphate having nearly the same elution pattern as ATP was GTP; however, GTP could hardly be the main triphosphate present in the plasma samples since the firefly extract is relatively insensitive to GTP and very large amounts would be required to produce the equivalent amount of light evoked by the plasma samples.

A notable feature of the firefly response to plasma samples was the 'hump' in the time course of light production (Figs. 2 and 3). This is somewhat different from the response seen with doses of pure ATP (Fig. 1). Balfour & Samson (1959) have shown that the firefly luciferin/luciferase enzyme system is not entirely specific for ATP; the extract can emit light when other triphosphates are added to it. The time course of light emission from firefly extract in response to the triphosphates of inosine, xanthosine, cytidine, guanosine and adenosine was measured. In every case there was an immediate response, but in the case of CTP there was further slow development of light, reaching a peak some seconds later. This was also shown to a lesser degree by the other triphosphates. It was found that CTP always had the greatest time-to-peak value.

Balfour & Samson provided evidence for the existence in firefly extract of enzymes catalysing the following reaction: $x\text{TP} + \text{ADP} = x\text{DP} + \text{ATP}$, where x is any nucleotide base. One explanation for the slow development of the light signal seen in the firefly response to plasma from exercising muscle might be that there are small amounts of other triphosphates present in the plasma, together with ADP. The transphosphorylases present in the firefly extract would convert any of those triphosphates to ATP, which in turn would contribute to the light signal, since the extract is much more sensitive to ATP than the other triphosphates.

Emission spectrum. There was a possibility that the other triphosphates caused light emission from firefly extract because another type of luciferin molecule was present in the extract. The light emitted from various luciferins has maxima ranging from 470–570 nm. However, the only luciferin having peak light emission at the maximum of 562 nm in response to ATP is the one found in insects, including the firefly *Photinus pyralis* (Pesce, Rosén & Pasby, 1971). The emission spectra from firefly extract in response to CTP, GTP, ITP, UTP and XTP were measured and they all showed a peak of light emission at 562 nm. It is therefore concluded that the firefly extract used in this work (type FLE 50, Sigma Co.) contains a luciferin which emits light in response to all the triphosphates. In order to discriminate between the various triphosphates the firefly technique has to be used in conjunction with column chromatography.

It is concluded from the above tests using firefly luminescence, column

chromatography and apyrase that ATP is present in the venous plasma from exercising forearm muscle.

Estimation of the amounts of ATP released during forearm exercise

The initial light signal produced from firefly extract by plasma samples was used to assay the amounts of ATP (see Methods). Samples of plasma that gave no decay of light signal when applied to a firefly extract were given an assay value according to the strength of the steady light signal

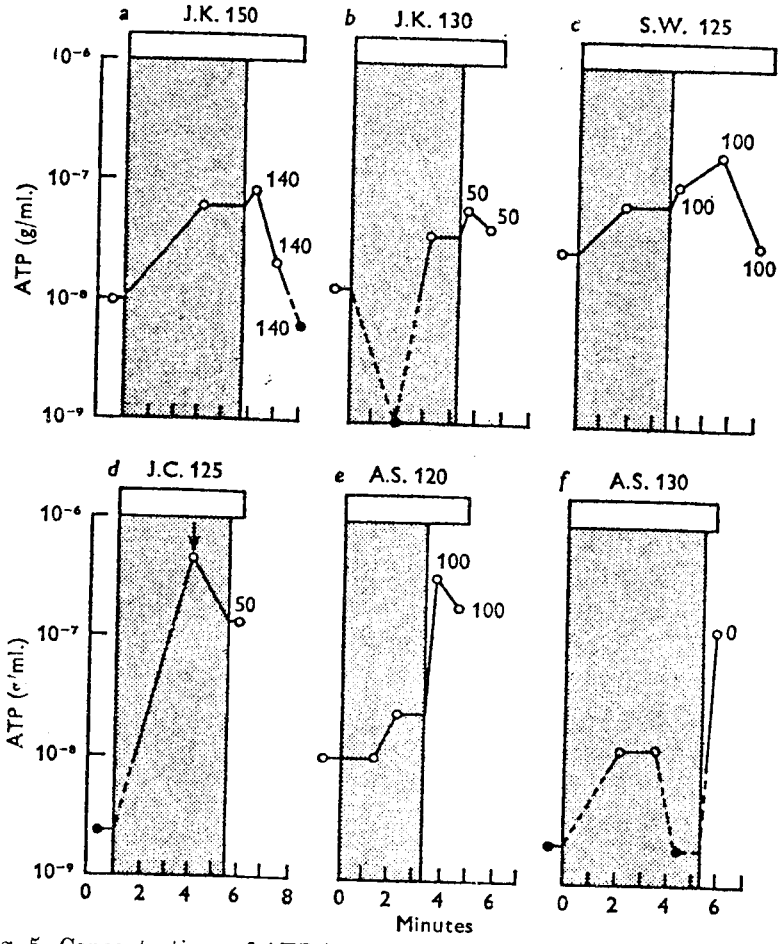

Fig. 5. Concentrations of ATP in venous plasma before, during and after arterial occlusion + exercise. Clear rectangles, duration of exercise; shaded areas, time of arterial occlusion. Resting systolic blood pressure given above each figure. Filled symbols, samples without decay in light signal (see Methods) and therefore below assay threshold (dashed lines). Vertical arrow in *d*, see text. Figures above post-occlusion samples, cuff pressure when sample was taken.

emitted, but were nevertheless deemed to have a value below the threshold for accurate measurement.

Fig. 5 shows the results of six experiments on four of the subjects. In subject J.K. (*a*) the initial systolic blood pressure was 150 mm Hg. The concentration of ATP in the plasma before forearm exercise was 10^{-8} g (0·02 n-mole)/ml. In order to obtain the third sample, the arterial occlusion cuff was reduced to a pressure of 140 mm Hg, just below systolic blood pressure. The cuff remained inflated to that pressure for the collection of the fourth and fifth samples. It can be seen that the concentration of ATP in the third sample has risen to nearly ten times that in the sample before exercise; the concentration in samples 4 and 5 has fallen rapidly as the blood flow through the sampling needle increased. A second experiment on the same subject (*b*) showed that similar values were obtained when the occlusion cuff was let down to 50 mm Hg during collection of the post-occlusion samples. An extra sample was obtained during the period of occlusion which gave a small light signal with no decay. The fourth sample in Fig. 5*c* was used to elute through the Sephadex column (Fig. 4). In Fig. 5*d* the sample indicated by the vertical arrow could only be obtained from the occluded forearm by momentarily letting the pressure down in the cuff. When the cuff was eventually deflated to 50 mm Hg the concentration, although reduced, still remained substantially higher than the resting level of ATP before exercise. In two experiments on the same subject (Figs. 5*e* and *f*) the cuff was let down to 100 and zero mm Hg. There was found to be a similar increase in the concentration of ATP over the resting level in both experiments. In the experiments shown in Figs. 5*d*, *e* and *f* the concentrations of ATP in the post-occlusion samples have risen by about 50 times. Table 1 gives the results of all the experiments on the nine volunteers. The maximum concentration of ATP found in the post-occlusion samples from each subject is given and ranged from $1{\cdot}8 \times 10^{-8}$ to 5×10^{-7} g (0·033–1·0 n-mole)/ml. plasma.

Assessment of ATP degradation. ATP is known to be rapidly destroyed in the bloodstream (Jørgensen, 1956; Ireland & Mills, 1964; Holmsen, Stormorken & Goote, 1965) and it was certain that the final concentration could represent only a small fraction of the ATP originally present in the blood samples. Experiments were done to assess the degree of degradation taking place from the time of exercise to the time of testing on the firefly extract. Degradation took place in two phases: an *in vivo* stage including the time of transfer of blood between the syringe and the centrifuge, and an *in vitro* stage, when the blood sample was centrifuged for 25 min at 5° C.

The loss occurring *in vitro* was assessed as follows. Samples of venous blood were obtained from resting subjects, transferred to centrifuge tubes and placed in a water-bath at 37° C. ATP was then added to the tubes to

TABLE 1. Assay results for all experiments

Expt	Subject	Age (yr)	Duration of exercise (min)	Cuff pressure during sample collection (mm Hg)	ATP equivalent (g/ml. plasma)	After correction for *in vitro* degradation (μg (n-mole)/ml. whole blood)
1	J.K.	43	5·0	140	$8{\cdot}3 \times 10^{-8}$	2·10 (4·20)
2	J.K.	—	4·3	50	$5{\cdot}9 \times 10^{-8}$	1·75 (3·50)
3	S.W.	31	5·5	100	$1{\cdot}8 \times 10^{-8}$	0·65 (1·30)
4	A.S.	41	5·5	0	$1{\cdot}2 \times 10^{-7}$	2·55 (5·10)
5	A.S.	—	3·5	100	$3{\cdot}7 \times 10^{-7}$	4·00 (8·00)
6	W.B.	35	5·0	50	$4{\cdot}6 \times 10^{-8}$	1·50 (3·00)
7	I.D.	22	5·0	50	$2{\cdot}3 \times 10^{-7}$	3·35 (6·70)
8	J.C.	54	3·0	*	$5{\cdot}0 \times 10^{-7}$	4·40 (8·80)
9	A. McL.	23	5·5	80–50†	$5{\cdot}5 \times 10^{-8}$	1·65 (3·30)

* Cuff let down momentarily in order to obtain sample, then immediately reinflated.
† Cuff pressure fell from 80 to 50 mm Hg whilst sampling.

give concentrations ranging from 1 to 5 μg (2–9 n-mole)/ml. whole blood. After addition of ATP the tubes were immediately centrifuged and the residual ATP in the supernatant plasma assayed. There was found to be a linear relationship between the amounts of ATP put into whole blood and the amounts recovered in the plasma after centrifugation. The concentrations of ATP found in plasma during exercise were then converted to amounts contained in the whole blood just before centrifugation using this relationship. The last column in Table 1 gives the corrected values for each

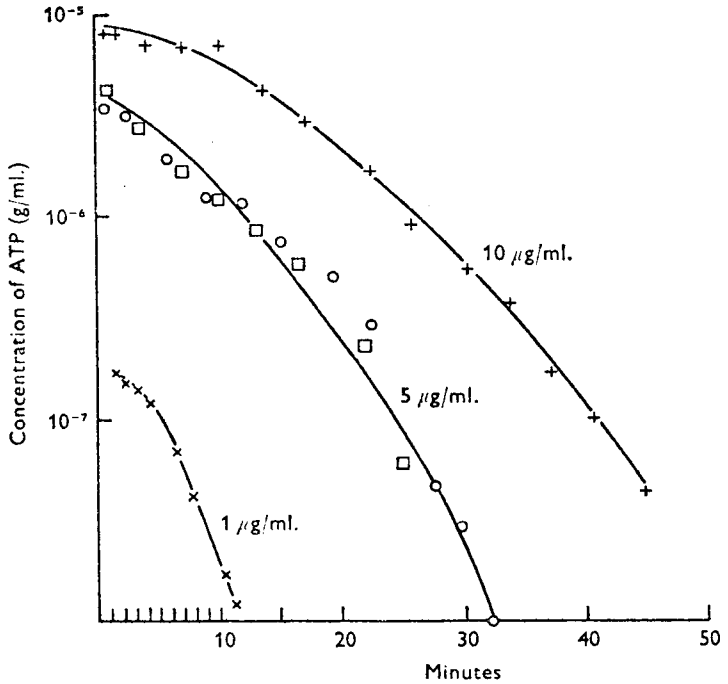

Fig. 6. Degradation of three concentrations of ATP in human plasma at 37° C. Each symbol represents plasma from a different subject. An initial concentration of 5 μg/ml. takes 32 min to degrade beyond the threshold of detection.

subject. The mean corrected value for the concentration of ATP just before centrifugation is 2·4 μg (4·4 n-mole)/ml. whole blood (range 0·65–4·40 μg/ml. or 1·2–8·0 n-mole/ml.).

Estimation of ATP degradation *in vivo* can only be approximate, since the accumulation of ATP in the blood is continuously offset by the ATPase activity of the plasma at 37° C. The effect of plasma at 37° C on low concentrations of ATP is shown in Fig. 6. ATP was added to plasma at 37° C in concentrations of 1, 5 and 10 μg/ml. Estimations of ATP were then made at frequent intervals using the firefly test. A consistent pattern of

degradation is seen with each concentration. If it is assumed that the ATP appears in the blood at a steady rate during the 4 min period of exercise, then a very large proportion of the amount appearing in the first minute will have been degraded. The ATP appearing in subsequent minutes will have had less time for degradation to take place. The average time taken for the post-occlusion samples to reach the centrifugation stage, taken together with an arbitrary time of 2 min allowed for degradation in the forearm, amounts to 6 min. It has been estimated that the concentration of ATP in the centrifuge tube just before centrifugation was 2·4 μg (4·4 n-mole)/ml. whole blood. After correction for the haematocrit this becomes 4·3 μg (7·8 n-mole)/ml. plasma. With the co-ordinates of 4·3 μg/ml. and 6 min a point is reached in Fig. 6 lying just above the curve of degradation of 5 μg/ml. The average concentration of ATP estimated to be present in the blood within the forearm at the time of exercise is therefore calculated to lie between 5 and 7 μg (9 and 13 n-mole)/ml. plasma.

Sjöstrand (1935) has estimated that the volume of small blood vessels in skeletal muscle is about 4% of the volume of the whole muscle. In a forearm containing 150 ml. muscle the volume of blood vessels into which ATP might be expected to diffuse is therefore 6 ml. The total amount of ATP calculated to be present at the time of exercise then ranges from 6×5 μg to 6×7 μg (54–76 n-mole). Over the 4 min period of exercise the rate of release is then calculated to be between 7·5 and 10·5 μg/min (14–20 n-mole/min). The average concentration of ATP in resting human quadriceps femoris muscle has been recently estimated as 4·3 μmole/g wet muscle (Karlsson, 1971). In a forearm containing 150 g wet muscle the total intracellular ATP is approximately 645 μmole. With an output of 20 n-mole/min released into the venous effluent the percentage loss of intracellular ATP over a 4-min period of exercise amounts to less than 0·02%.

DISCUSSION

The amount of ATP released into the venous effluent when human forearm muscles exercised at approximately 5% MVC in the presence of arterial occlusion has been calculated to lie between 7·5 and 10·5 μg/min (14–20 n-mole/min). No allowance has been made for any dilution that may have occurred in the passage between the vascular bed and the antecubital vein from where the samples were taken; nor has any allowance been made for the fact that ATP is degraded seven to eight times more rapidly in whole blood than in plasma (Jørgensen, 1956), since in this work the estimation of ATP degradation *in vivo* at 37° C was made in plasma.

The forearm exercise was performed in the presence of arterial occlusion and the obvious question arises, does ATP appear in the venous effluent

as a result of occlusion? Previous work has shown that when forearm muscles performed a non-fatiguing 10 % MVC for 4 min without arterial occlusion the ATP concentration in the venous effluent increased by 4 μg (7·3 n-mole)/ml. (Forrester & Lind, 1969, Fig. 7*B*). Although the forearms only performed a 5 % MVC in this study, the mean figure of 0·2 μg (0·36 n-mole)/ml. (ATP equivalent, Table 1) obtained would suggest that the arterial occlusion was not a factor in the production of ATP in the venous effluent.

Duff *et al.* (1954) have shown that an infusion of ATP at 16 μg (29 n-mole)/min into the brachial artery of human volunteers trebled the blood flow through the forearm. When forearm muscles sustain a contraction of 5 % MVC, such as the subjects performed in this study, the blood flow might be expected to increase by about three times (Lind & McNicol, 1967). Thus for approximately the same increase in blood flow the amounts of ATP infused intra-arterially are close to the amounts calculated to be released during forearm exercise. It is concluded that ATP could act as a satisfactory mediator of vasodilatation in exercising human forearm muscle.

Perhaps a significant result from these investigations is that after arterial occlusion for a minimum period of 4 min no ATP could be detected in the immediate post-occlusion samples of plasma. Even if it is assumed that the ATP concentrations in these samples were just *above* the threshold for detection, say 10^{-8} g (0·02 n-mole)/ml. then the original amounts present can be calculated as before. The amount just before centrifugation would be approximately 0·1 μg (0·19 n-mole)/ml. whole blood after correction for *in vitro* degradation (Fig. 6). When this amount is adjusted for haematocrit and the time of *in vivo* degradation taken as 6 min (see Results, p. 623, para. 1) then the original amounts present are calculated as 1 μg (0·02 n-mole)/ml. plasma, or 10 % of the amounts detected in the samples for exercising muscle. This would in itself suggest that muscular activity was responsible for the appearance of ATP in the venous effluent.

It has been generally supposed that cell membranes are impermeable to ATP under normal conditions; nevertheless, recent work has provided evidence that ATP is released from active skeletal muscle (Abood *et al.* 1962; Boyd & Forrester, 1968) and can enter skeletal muscle cells (Chaudry & Gould, 1970). In the present study the virtual elimination of background levels of ATP seems to exclude the blood platelet as a source of ATP. Erythrocytes have already been excluded as a direct source (Forrester & Lind, 1969), leaving skeletal muscle as the most likely source.

A comparison can be made with the amounts released from active frog sartorius muscle *in vitro* (Boyd & Forrester, 1968). When a sartorius muscle was maximally stimulated at 2/sec for a period of 30 min the output of

ATP was calculated as 8 μg (14·6 n-mole)/g wet weight of muscle. If there is an average of 150 g muscle in human forearm, then with a rate of release of 7·5 μg (14 n-mole)/min the equivalent release for a period of 30 min would be 1·5 μg (2·7 n-mole/)g wet weight of forearm muscle. Certainly the degree of forearm exercise was not as severe as that imposed upon the sartorius muscle and this would account for the lower figure for forearm muscle.

ATP as a contributor to ischaemic pain. In these experiments the subjects were unable to sustain, in the presence of circulatory occlusion, a grip of greater than 5% MVC for 4 min without experiencing considerable ischaemic pain. As it was, in the 30 sec period preceding deflation of the occlusion cuff there was a rapid development of pain, but as soon as the cuff was deflated to a pressure below that of systolic the pain was immediately relieved. The highest concentrations of ATP were always found in the post-occlusion period, the concentrations falling rapidly in successive samples, even when the cuff was kept at a point just below systolic pressure (Fig. 5*a*). Thus the relief of pain coincided with the washout of ATP from the forearm muscle. It is known that ATP can produce pain when applied to the base of a skin blister in a concentration of 100 μg (182 n-mole)/ml. (Keele & Armstrong, 1964) and it is possible that in more severe exercise than that performed in this work the concentration of ATP in the extravascular space might approach that level.

Other effects of circulating ATP. The question arises whether ATP can be released into the circulation during exercise in sufficient quantities to affect other systems. ATP is known to have profound effects on the cardiovascular and respiratory systems when injected into the circulation (Drury & Szent-Gyorgyi, 1929; Gaddum & Holtz, 1933; Gillespie, 1934; McDowall, 1944; Bielschowsky, Green & Stoner, 1946; Emmelin & Feldberg, 1948; Davies, Gropper & Schroeder, 1951). Emmelin & Feldberg gave 200 μg (364 n-mole) ATP I.V. to cats and found that a rapid and marked fall in blood pressure was produced. They concluded that this was due to pulmonary vasoconstriction, bradycardia and vasodilatation. They also noted an effect on respiration, namely an initial apnoea immediately followed by hyperpnoea. The effect on respiration was thought to be due to ATP affecting the respiratory centre directly and indirectly through the vagi from the lungs, although the possibility that the hypernoea was reflexly caused by the hypotension cannot be ruled out. Davies *et al.* have shown that I.V. infusion of ATP, 800 μg (1·5 μ-mole)/ml., into human subjects produced similar effects on the circulatory and respiratory systems. In this study the amounts of ATP estimated to be present in the blood would have little effect upon respiration, but perhaps in more severe exercise, when hyperpnoea does occur, the respiratory centre is stimulated by greater concentrations of ATP in the blood stream.

The action of ATP upon the coronary arteries has been studied by many workers (Gillespie, 1934; Folkow, 1949; Green & Stoner, 1950; Winbury, Papierski, Hemmer & Hambourger, 1953; Wolfe & Berne, 1956; Rowe, Afonso, Gurtner, Chelius, Lowe, Castillo & Crumpton, 1962). Rowe *et al.* recorded rises in coronary blood flow of up to 500 % in response to doses of ATP, 2 μmole/kg body weight and minute, introduced into the right atrium. Recent work in this laboratory has shown that the threshold of coronary dilatation in response to ATP lay between 0·03 and 0·2 μg (0·06 and 0·56 n-mole)/ml. entering rabbit coronary arteries (McCruden, 1970). ATP is known to be degraded in the lungs (Folkow, 1949) and a rapid degradation takes place in whole blood (Jørgensen, 1956); nevertheless, it is likely that in exercise when a large amount of ATP is being produced in working skeletal muscle, the threshold for coronary vasodilatation is exceeded. Further investigation is necessary to find whether ATP is completely destroyed in one passage through the lungs. Even if most of the ATP is dephosphorylated in the circulation, the concentrations of ADP, AMP and adenosine may reach significantly high levels during muscular exercise.

I wish to thank the volunteers for their co-operation during these studies. The excellent technical services of Mrs Anne Murray and Mrs Irene Thomson are gratefully acknowledged. This work is supported by an M.R.C. grant.

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Commentary - paper 7.

This abstract appeared in response to some earlier questions about whether the *in vitro* experiments with frog muscle were indeed dealing with damaged tissue. In paper 6 a quantitative comparison was made between the amounts of ATP released from human forearm muscle compared with those released from a frog sartorius muscle into a surrounding bathing solution. Approximately 5 - 6 times the amount was released from the frog muscle *in vitro*, probably indicating the reduced extracellular ATPase activity in the frog muscle experiment. However the problem of cellular damage was reasonably answered since this preparation excluded direct dissection of the musculature.

[*From the Proceedings of the Physiological Society*, 13–14 *April* 1973]
Journal of Physiology, **232**, 86–88*P*

Appearance of adenosine triphosphate in the perfusate from active frog skeletal muscle

By T. Forrester and M. O. Hassan.* *Institute of Physiology, University of Glasgow*

When an isolated frog sartorius muscle is made to twitch repetitively, it is possible to detect small quantities of adenosine triphosphate (ATP) in the surrounding bathing solution (Boyd & Forrester, 1968). In view of the recent identification of ATP in the venous effluent from exercising human forearm muscle (Forrester & Lind, 1969) and its estimated amount

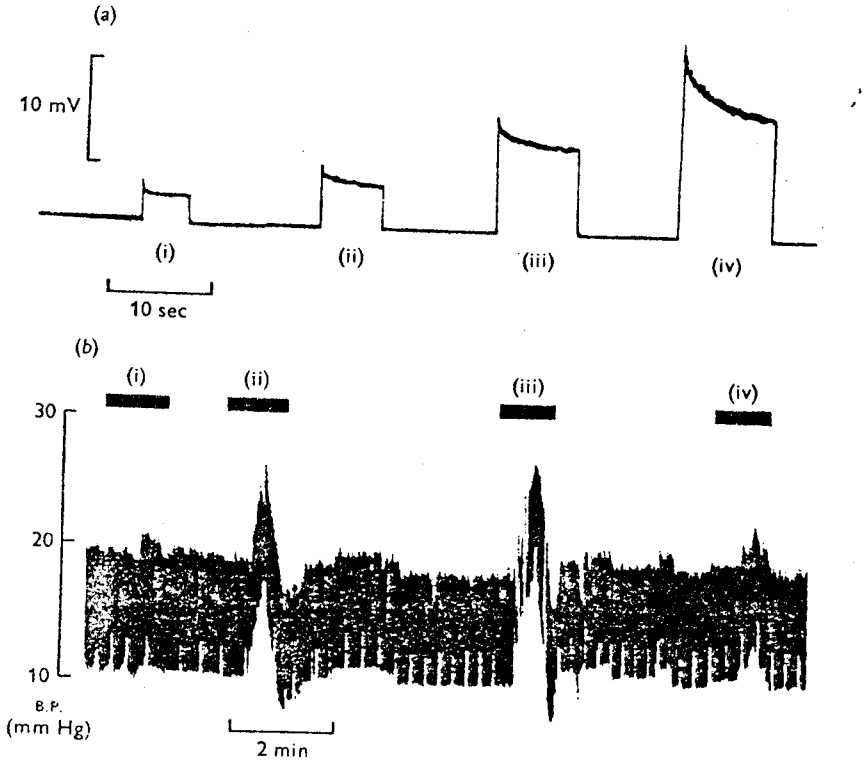

Fig. 1. Response of (*a*) firefly extract and (*b*) a perfused frog heart to solutions of perfusate and ATP. (*a*) (i), perfusate from unstimulated hind limbs; note no decay of light signal; (ii), ATP, 10^{-8} g/ml.; (iii), perfusate from hind limbs stimulated at 5 Hz; (iv), ATP, 5×10^{-8} g/ml. (*b*) (i), perfusate from unstimulated hind limbs; (ii), perfusate from hind limbs stimulated at 5 Hz; (iii), ATP, 10^{-8} g/ml; (iv), sample (ii) after incubation with apyrase. Horizontal bars, time of perfusion.

* Department of Physiology, University of Khartoum. W.H.O. Grant.

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(Forrester, 1972), it was of interest to analyse the perfusate from frog skeletal muscle for ATP.

The hind-limb musculature of decapitated, pithed frogs was perfused with frog Ringer through the abdominal aorta and the perfusate was collected from the anterior abdominal vein (Dale, Feldberg & Vogt, 1936). In each experiment the perfusion pressure was adjusted so that the flow rate became 0·5 ml./min. The muscles were stimulated via the sciatic nerve bundles at frequencies ranging from 1 to 10 Hz for periods of 2 and 5 min during perfusion. Perfusion took place for 3 min before stimulation.

The perfusate produced a light signal when mixed with firefly extract (Fig. 1*a*). When perfused through a frog heart the perfusate had a positive inotropic effect which was abolished after incubation of the perfusate with the enzyme apyrase (Fig. 1*b*). It is concluded that ATP appears in the perfusate from active muscles. No ATP was detected in the perfusate from unstimulated muscles (Fig. 1*a*).

The amounts of ATP produced in the perfusate at various frequencies of stimulation were measured, using the firefly assay. Stimulation at 1, 2, 5 and 10 Hz produced 4·0 (± S.D. 2·5), 1·5 (± 0·48), 1·4 (± 0·8) and 3·0 (± 1·5) p-moles per volley per 100 g muscle respectively, while the total amounts of ATP produced were calculated as 240, 180, 420 and 1800 p-moles per 100 g muscle per min. This compares with an output of 50 n-moles per 100 g muscle per min from an isolated sartorius muscle stimulated at 2 Hz (Boyd & Forrester, 1968).

This work is supported by an M.R.C. Grant.

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Commentary - paper 8.

This abstract could be subtitled 'the mysterious peak at (a).' Not only did this peak of elution grow more prominent with the time of soak, but it was markedly accentuated when the muscle was twitched or continuously depolarized with KCl. Although no positive identification was made of this elution peak, it did seem to indicate the efflux of a larger molecule than adenosine, possibly ATP. Comparative elution with ^{14}C-ATP was not performed and direct testing with firefly extract was precluded because of the effect of radioactive emission upon the photomultiplier tube used for light detection from the firefly luminescence procedure. Modern techniques would now make this a worthwhile project.

[*From the Proceedings of the Physiological Society*, 19–20 *April* 1974]
Journal of Physiology, **241**, 43–44*P*

Adenosine flux in frog skeletal muscle

By T. Forrester and R. Lindsay.* *Institute of Physiology, University of Glasgow.*

Adenine nucleotides have recently been considered as mediators of vasodilatation in skeletal muscle. The presence of ATP and its derivatives has been demonstrated in the bloodstream after muscular activity (Forrester, 1972; Parkinson, 1973) and the possibility exists that these substances are taken up by skeletal muscle.

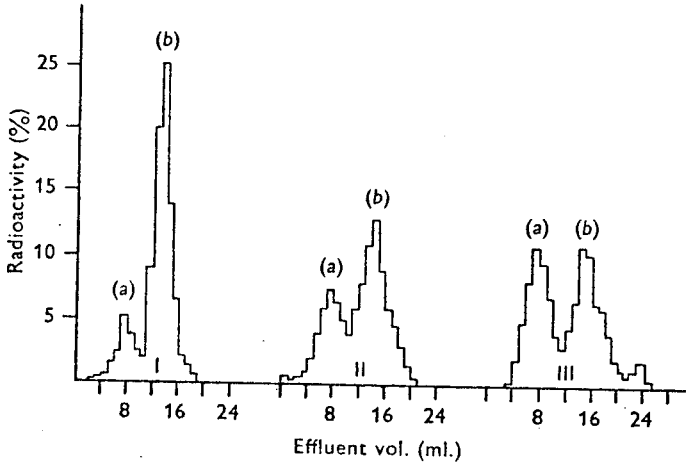

Fig. 1. Gel filtration of (Sephadex G-25, void volume 11·5 ml.) washout fluid (50–55 min sample) from sartorius muscles which had been previously soaked in 10 mM [^{14}C]adenosine for 1 hr. (I), 3 hr (II), and 16 hr (III). In each profile the peak at (*b*) corresponds to adenosine. Note that peak (*a*) increases with time of soak. The substance responsible for this peak has not yet been identified. Total radio-activity was the same in all three tests.

We have examined uptake and release of [^{14}C]adenosine by the sartorius and toe muscles of the frog *Rana temporaria*. Intracellular content of the label reached a steady value of 13 mM/kg dry weight after $1\frac{1}{2}-2$ hr incubation in 10 mM [^{14}C]adenosine. The washout curve obtained after short ($1\frac{1}{2}$–3 hr) periods of incubation showed two exponentials, the slow efflux phase having $t_{\frac{1}{2}} = 26$ min. The calculated flux was 7·3 p-mole/cm^2.sec. Prolonged incubation (> 12 hr) resulted in a change in the character of the efflux. The intracellular content of label remained steady

* Present address: University Department of Medicine, Western Infirmary, Glasgow.

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but now three exponential terms were required to fit the efflux curve, the two slower movements having $t_{\frac{1}{2}} = 26$ and 240 min. This suggests that the label is either held up in a separate, slowly exchanging compartment, or is incorporated into two separate compounds, each having a different rate of efflux. The latter alternative is supported by results from gel filtration of the washout fluid; two peaks are evident in the elution profile (Fig. 1, peaks *a* and *b*).

Efflux of label from the muscles was increased by indirect stimulation or by depolarization with high KCl ringer. Gel filtration of the washout fluid from these muscles showed a larger peak at (*a*) than that obtained from the fluid surrounding resting muscles. The substance eluted at this peak has not yet been identified, but the chromatography procedure indicates that it is probably a larger molecule than adenosine.

This work was supported by an M.R.C. grant.

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Commentary - paper 9.

Although not directly concerned with the problem of nucleotide efflux from skeletal muscle, this short project did emphasize that a much larger molecule, creatine kinase, could be released from sartorius muscle under identical conditions to those in which nucleotide efflux had been demonstrated. These conditions also seemed satisfactory to other workers in the field of muscle physiology.

Effect of temperature on creatine phosphokinase released from frog skeletal muscle

By T. Forrester, J. C. Sweetin and W. H. S. Thomson. *Institute of Physiology, University of Glasgow, G12 8QG, and Research Laboratory, Knightswood Hospital, Glasgow G13 2XG*

Zierler (1958) demonstrated increased enzyme efflux from stimulated rat skeletal muscle. We examined frog sartorius muscle (*Rana temporaria*).

Sartorii at resting length were bathed at room temperature (< 20° C) in changes of pH 7·2 phosphate-glucose-Ringer until efflux of muscle-specific creatine phosphokinase (CPK) ceased, then twitched isometrically to exhaustion at 2 Hz for 10 min, and changes resumed as before. CPK release after stimulation was far less than expected; and any dilution of effluent caused marked irregular loss of CPK activity, unlike the human enzyme (Thomson, 1969). This instability was investigated.

In both sartorius effluent and human sera CPK was assayed simultaneously by two different methods, each measuring the same reaction in the same direction using the same substrates:

$$\text{ADP} + \text{creatine phosphate} \xrightarrow{\text{CPK}} \text{ATP} + \text{creatine}.$$

The first measured ATP kinetically during formation at 25° C using a U.V. enzyme/coenzyme indicator (Thomson, 1968); the second measured creatine colorimetrically after formation at 37° C using diacetyl-α-naphthol (Hughes, 1962). Fig. 1 shows frog CPK activity measurable by the first but scarcely by the second method, while human CPK activity was adequately measured by both. Though optimal at 37° C, human CPK shows tenfold inactivation in 5 min at 56° C (Thomson, 1969); evidently frog CPK is even more labile.

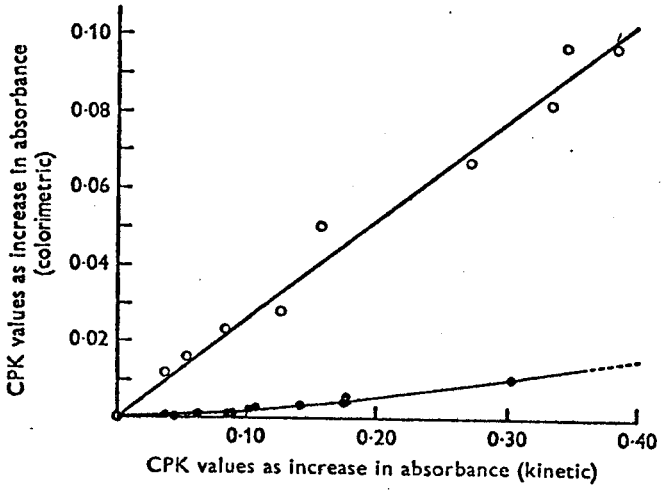

Fig. 1. Simultaneous measurement of CPK activity at 25° C (kinetic) and 37° C (colorimetric) in frog sartorius effluent (●) and human sera (○). Lines fitted by eye.

This thermal lability was examined in CPK released from frog sartorius into pH 7·2 Ringer at 0° C after ½ hr immersion in pH 6·1 Ringer at 0° C. Aliquots were assayed forthwith and at 45 min intervals after incubation at 0, 20, 25, and 30° C; all assays were by the first (kinetic) method at 20° C. Progressive inactivation with time, marked even at 20° C, became precipitous at higher temperatures, indicating a thermal lability so great as to render these methods quite unsuitable for the frog. Latent molecular differences may thus prevent comparisons of tissues in different species.

This study was supported by the Muscular Dystrophy Group of Great Britain.

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Commentary - paper 10.

The characteristic exercise hyperaemia of skeletal muscle is superimposed upon a resting blood flow that varies according to the type of muscle. Soleus muscle has a high resting flow in comparison to fast twitch muscles and thus the hyperaemia of exercise in this muscle does not have the dramatic percentage increase seen in fast muscle. The consistent release of ATP at rest and the augmentation of release during stimulation provided further evidence linking metabolic events and local blood flow control by ATP.

[*From the Proceedings of the Physiological Society*, 21–22 *March* 1975]
Journal of Physiology, **249**, 20–21*P*

Functional hyperaemia in soleus muscle of the cat

By T. Forrester and I. J. D. Hamilton, *Institute of Physiology, University of Glasgow, G12 8QQ*

Blood flow through the soleus muscle of the cat has a resting value 3–4 times greater than that through fast muscle, and functional hyperaemia is reported to be reduced (Folkow & Halicka, 1968; Hudlicka, 1968) and sometimes absent (Hilton, Jeffries & Vrbová, 1970). It is generally accepted that alteration in local chemical environment is responsible for functional hyperaemia, therefore it was of interest to examine further certain characteristics of the soleus vascular bed.

In heparinized cats anaesthetized with intra-peritoneal sodium pentobarbitone, the soleus blood supply was isolated. The femoral vein was cannulated and the venous outflow from soleus was passed through an integrating drop counter and then recycled proximally into the femoral vein. Blood pressure was monitored via a cannula in the common carotid artery. The muscle was held isometrically. Supramaximal pulses of 1 msec duration were applied to the soleus nerve at frequencies of 1–20 Hz for periods of 1 min. After each experiment Naphthol green dye was injected to confirm the selective perfusion of soleus.

The resting flow ($n = 6$) was 34 ml./100 g.min ($\pm$ S.E. 3·8) and during stimulation at 1, 5, 10 and 20 Hz was found to be 38 ($\pm$ 3·7), 39 ($\pm$ 2·0), 45 ($\pm$ 3·7) and 57 ($\pm$ 6·6) respectively. It is concluded that the soleus muscle has a high resting blood flow and a reduced functional hyperaemia in agreement with previous workers. Close arterial infusion at 1 ml./min of 10 μM-ATP, adenosine and NaH_2PO_4 into soleus muscles ($n = 6$) produced a 2·1 ($\pm$ S.E. 0·34), 1·6 ($\pm$ 0·17) and 1·4 ($\pm$ 0·26) increase in flow ($\times$ resting flow) respectively. Finally, samples were collected from the soleus muscle perfused with blood or an oxygenated Krebs solution. All blood samples were collected into melting ice and centrifuged at 25000 *g* for 1 min. The cell-free plasma was assayed for ATP using a modification of the firefly technique (Strehler & McElroy, 1957). ATP levels in plasma ($n = 8$) were: resting, 0·07 μM ($\pm$ S.E. 0·04) and during stimulation at 10 Hz, 0·3 μM ($\pm$ 0·05); this difference is probably significant ($0{\cdot}05 > P > 0{\cdot}01$). Corresponding values for muscles perfused with Krebs solution ($n = 12$) were 0·9 μM ($\pm$ 0·26) and 1·8 μM ($\pm$ 0·48); this difference is not significant.

The lower levels of ATP detected in plasma could be due to surface ATP-ase activity of R.B.C.s (Parker, 1970) and the higher levels in the Krebs perfusate might result from the release of ATP by hypoxic muscle.

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In conclusion, as the soleus blood vessels are sensitive to ATP, could a continuous release of ATP by soleus account for its high resting blood flow and the reduced functional hyperaemia result from only a modest increase in the release of ATP?

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Commentary - paper 11.

Much of the work published in papers 5 & 6 was verified in the whole body exercise experiments of Parkinson (1973). With the realization that ATP could exist in the plasma for a short time it was necessary to obtain further information about the rate of its neutralization as a vasodilator. Much information already existed in the context of platelet 'clumping' and the effects on the clotting process of ADP. The conclusions from this short study could have signficant bearing on the clotting procedure in pathological circumstances.

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Journal of Physiology, **248**, 25–26*P*

Fate of adenosine triphosphate in human plasma

By T. Forrester and L. M. Morrison. *Institute of Physiology, University of Glasgow, Glasgow G12 Q8Q*

Mills (1966) demonstrated that ATP was primarily depyrophosphorylated (ATP → AMP + pyrophosphate) when added to human plasma. He also showed the presence of a secondary pathway in which ATP was dephosphorylated to ADP. Although much work has focused upon the break-down of ADP in plasma (Ireland & Mills, 1964; Mills, 1966; Holmsen & Holmsen, 1971), little attention has been paid to the reaction ATP → ADP + phosphate. It was of interest to initiate a study of the kinetics of this pathway, since ATP has been detected in human plasma after exercise (Forrester & Lind, 1969; Parkinson, 1973).

The conversion of ATP to ADP was followed using a modified technique of Marsh (1959). Phosphate released during the reaction can be monitored without any interference from the presence of ATP, ADP, AMP or pyrophosphate. A 0·2 ml. volume of ATP in concentrations ranging from 0·16 to 8·5 μM was added to 1 ml. human plasma at 37° C. The reaction was terminated at specified times by the addition of 7·3 ml. ice-cold Krebs solution; the plasma proteins were then precipitated by addition of 1·5 ml. 12 % trichloracetic acid. The standard Marsh procedure was then adopted, except that centrifugation of the sample just prior to optical measurement was carried out to eliminate residual turbidity.

It was found that phosphate appearance reached a peak rate at 2 min after addition of ATP to the plasma. Subsequently the concentration fell until, in some cases, there was clear evidence of phosphate uptake; therefore the first 2 min were taken to assess the kinetics of the reaction. It exhibited a simple Michaelis–Menten relationship up to a concentration of 10 μM ATP. Beyond this the kinetics became complex. The apparent K_m for ATP ranged from 0·5 to 2 μM, while the V_{max} range was 6·4–14 n-moles/min.

Two main conclusions arise from this study. First, the reaction ATP → ADP + PO_4^{2-} is probably not responsible for the levels of ADP found in plasma after exercise (Parkinson, 1973). Secondly, it has been shown that ADP causes clumping of blood platelets (Gaarder, Jonsen, Laland, Hellem & Owren, 1961) when added to plasma in a concentration of 0·5 μM (Born & Cross, 1963); the depyrophosphorylation pathway clearly avoids any dangerous accumulation of ADP which would predispose to intravascular clotting.

L.M.M. is grateful to the Physiological Society for a grant from the Dale Fund.

[P.T.O.

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Commentary - paper 12.

This short abstract resulted from a brief discussion between the author and Dr. Murray Harper about five years prior to the commencement of the project. It seemed a very unlikely prospect that ATP injected into the carotid artery would have any effect upon the cerebral blood vessels. In order to investigate the possible involvement of ATP in the local control of cerebral blood flow the substance would have to be applied to the outside of the resistance vessels - quite a sophisticated procedure was necessary. However the intracarotid experiment was easy to perform and would quickly verify that ATP did not cross the blood-brain barrier. No effect on cerebral blood flow was anticipated. It was with some disbelief that the results reported in this abstract were received. The seeds of 'mens sana in corpore sano' had been sown and would not reach the published page until 1979.

[*From the Proceedings of the Physiological Society*, 25–26 *April* 1975
Journal of Physiology, **250**, 38–39*P*]

Effects of intracarotid adenosine triphosphate infusions on cerebral blood flow and metabolism in the anaesthetized baboon

By T. Forrester, A. M. Harper and E. T. MacKenzie. *Institute of Physiology, University of Glasgow, Glasgow G12 8QQ, and Wellcome Surgical Research Institute, University of Glasgow, Garscube Estate, Bearsden Road, Glasgow G61 1QH*

Roy & Sherrington (1890) proposed that 'the chemical products of cerebral metabolism contained in the lymph...can cause variations of the calibre of the cerebral vessels'. Adenosine triphosphate (ATP) has been implicated in the local chemical control of muscle blood vessels (Forrester, 1972), and its release from stimulated peripheral and central nervous tissue has been reported (Abood, Koketsu & Miyamoto, 1962; Kuroda & McIlwain, 1974). We have examined the response of the cerebral vasculature to intracarotid infusions of ATP.

The studies were carried out in anaesthetized baboons maintained at normocapnia. Full details of the anaesthesia and the measurement of cerebral blood flow (CBF) by the ^{133}Xe clearance technique have been reported elsewhere (Deshmukh & Harper, 1973). Cerebral oxygen consumption ($CMRO_2$) was calculated as the product of CBF and the arterial and cerebral venous (superior sagittal sinus) oxygen content difference. ATP was infused (1 ml./min) into one internal carotid artery at 37° C.

A dose-dependent relationship was established between the infused concentrations of ATP and CBF (Table 1). Arterial pressure was reduced slightly and no changes in P_{a,CO_2} were noted during ATP infusion.

It is interesting that systemic ATP can have effects beyond the blood–brain barrier. This is supported by the finding that ATP increased $CMRO_2$ in a dose-related manner. Evidence that extracellular ATP can stimulate the metabolism of ortical tissue *in vitro* has been provided by Sattin & Rall (1970).

Table 1. Cerebral blood flow and oxygen consumption during ATP infusions in five baboons. Figures presented are mean ± S.E.M.

		ATP (moles/min, intracarotid)			
	Base line	10^{-9}	10^{-8}	10^{-7}	10^{-6}
CBF (ml./100 g.min)	49 ± 11	66 ± 11	71 ± 13	94 ± 24	116 ± 10
		*$P < 0.05$	$P < 0.01$	$P < 0.05$	$P < 0.001$
$CMRO_2$ (ml.O_2/100 g.min)	2·9 ± 0·3	3·2 ± 0·5	3·4 ± 0·4	4·3 ± 0·7	4·5 ± 0·5
		P = N.S.	P = N.S.	$P < 0.05$	$P < 0.01$

* By Student's paired *t*-test (base line *vs.* ATP infusion). N.S. = not significant.

[P.T.O.

It would appear from this study that ATP is a more potent cerebral vasodilator than adenosine (Berne, Rubio & Curnish, 1974) when compared on a equimolar basis. Our findings would support the hypothesis that adenine nucleotides have some role to play in the control of local cerebral blood flow.

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Commentary - paper 13.

This paper has been included in the assembly to emphasize the importance of techniques in the measurement of small concentrations of ATP. We had noted that previous work always included the use of perchloric acid for the extraction of the nucleotide and nucleoside material. Some criticism was extended by these workers who doubted that ATP was released in such low concentrations (see paper for references). This paper demonstrated that they could not detect these small amounts for two reasons. First, the perchloric acid desensitised the firefly extract to ATP and secondly, ATP was adsorbed to the perchlorate precipitate upon neutralization.

Pflügers Arch. 366, 281–283 (1976)

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European Journal of Physiology

Loss of ATP in Micromolar Amounts after Perchloric Acid Treatment

CAROLE WILLIAMS and THOMAS FORRESTER

Department of Physiology, St. Louis University School of Medicine, 1402 South Grand Boulevard, St. Louis, Missouri 63104, U.S.A.

Summary

Treatment of fluid samples containing known amounts of ATP with 6.0 N perchloric acid (PCA) results in a total loss of 65–71% when the initial concentrations of ATP ranged between 0.5 to 50 μM. Half of this loss was attributed to desensitization of firefly extract (luciferin-luciferase reaction) while the remaining loss was presumably due to adsorption of ATP to perchlorate precipitate upon neutralization. Similar treatment of solutions with higher initial concentrations (100–1000 μM) resulted in apparent total losses averaging 22%. These losses were due solely to desensitization of firefly extract by neutralized PCA. Both the adsorption and desensitization phenomena must be taken into account when the ATP content is measured from tissue extracts and fluid samples subjected to this procedure.

Keywords

ATP, perchloric acid, luciferin-luciferase reaction.

The release of ATP from active skeletal and cardiac muscle and its level in plasma during normal and hypoxic conditions has been related to the degree of vasodilatation found in those tissues (5, 6, 12). However, others have disputed this relationship, ascribing the vasodilator function to adenosine, since they have consistently failed to find ATP in plasma (1) and tissue perfusates (1, 9). The experiments reported here show that the procedures used by those workers to identify and quantify ATP in plasma, tissue perfusates, and tissue extracts (2, 3, 4, 8, 11) in fact eliminate a substantial proportion of ATP.

A method commonly used to deproteinize biological tissue and fluid samples involves treatment with perchloric acid (PCA) following neutralization of the extract with KOH. Recently (10), it was reported that a significant loss (40–60%) of ATP occurred when the acidified sample was titrated with KOH to the phenolphthalein end-point; this loss was attributed to adsorption of ATP to the potassium perchlorate precipitate. It is interesting that this phenomenon had been previously noted by Berne (1) who presumed that the ATP was adsorbed to the precipitated protein. Since the perchloric acid technique has been used in the preparation of samples for adenine nucleotide estimation, and since the luciferin-luciferase reaction is widely used for the direct estimation of ATP following this procedure, it seemed important to verify and extend this finding (10).

All levels of ATP reported here were quantified using a modification (5) of the luciferin-luciferase technique devised by Strehler and McElroy (13). It was found that ATP estimations from samples treated with PCA are substantially underestimated due not only to adsorption of ATP to the precipitate but also to the alteration in the sensitivity of the assay procedure.

Figure 1A (open columns) indicates that the apparent loss of ATP was 65–70% (mean 68% ± 1.7) when ATP is mixed with PCA and this mixture neutralized. This represents the amount lost due to both desensitization of the firefly extract and adsorption of ATP to potassium perchlorate precipitate. The proportional amount lost remained constant irrespective of the initial concentration of ATP over the range 0.5–2.5 μM. In order to determine the proportional loss due to adsorption of ATP to potassium perchlorate precipitate, the effect of desensitization of firefly extract alone was measured by two methods. Solutions of ATP were mixed with neutralized PCA following the removal of potassium perchlorate precipitate and then assayed. This resulted in a 35% ± 6.3 loss of ATP and is indicated in Figure 1A as the difference between the heights of the open and black columns. Desensitization was independently verified by mixing the firefly extract with neutralized PCA before the addition of pure ATP samples. It was possible to determine the loss attributed to adsorption of ATP to the precipitate (Figure 1A black columns, mean 33.2% ± 6.1) by taking the difference between the % total loss and the % loss due to desensitization. This confirmed the losses previously reported (10). Note that while the percentage lost due to adsorption declined steadily as the concentration of ATP increased up to 2.5 μM,

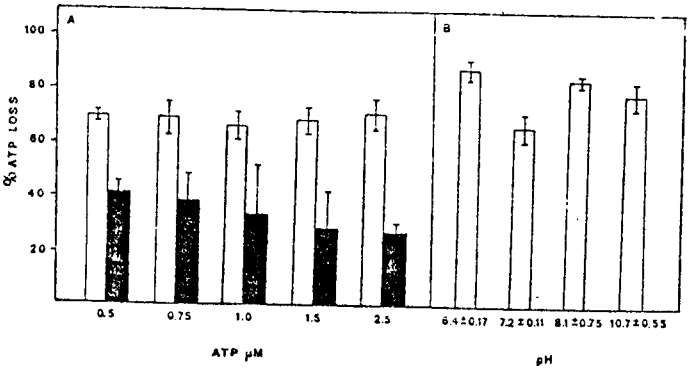

Fig. 1: Influence of concentration and pH on loss of ATP in solutions treated with perchloric acid. A. Open columns, total percentage loss of ATP treated with perchloric acid (PCA) and assayed on firefly extract. Black columns, percentage loss due to adsorption of ATP to potassium perchlorate precipitate. Difference between black and open columns is due to desensitization of firefly extract in presence of neutralized PCA. Total loss determined by treating 4 ml samples containing 0.5-2.5 µM ATP with 0.2 ml of 6.0 N PCA. Solutions were neutralized to pH 7.1 ± 0.2 using 7.5 N KOH containing 50 mM K_2HPO_4; usually 280-300 µl were sufficient for neutralization. Samples were allowed to stand on ice for 10 min to facilitate precipitation of potassium perchlorate and then were centrifuged for 5 min at 2000 x g; 0.2 ml supernatant was added to 0.2 ml firefly extract (Sigma type FLE-50) and assayed using a modification (4) of the technique devised by Strehler & McElroy (14). The standard solutions used for assay were not treated with PCA. Percentage loss due to desensitization was obtained by mixing equal volumes of ATP solutions with previously neutralized solutions of 6.0 N PCA; 0.2 ml of this mixture was assayed on firefly extract. Standard solutions used for assay were treated with PCA; n=4 for each concentration tested. Bars represent one standard deviation. B. Effect of end-point pH on percentage loss of ATP from solutions treated with PCA. Solutions of 1 µM ATP were treated with 6.0 N PCA as before; pH adjusted to the values indicated with 7.5 N KOH containing 50 mM K_2HPO_4. Assay conducted on firefly using four standard curves corresponding to each pH range; n=4 for each range. Bars represent one standard deviation.

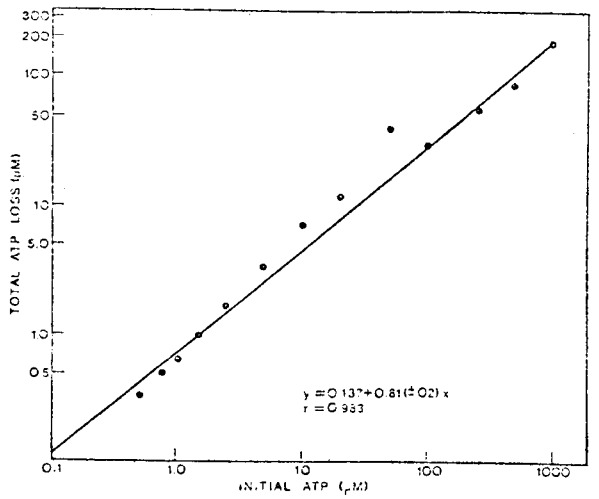

Fig. 2 Total amount of ATP lost as a function of initial ATP concentration in solutions treated as described in Figure 1. Each point indicates the quantity lost due to both desensitization and adsorption phenomena, and represents the average of 4 determinations at each of the initial concentrations indicated. Slope of the line computed by linear regression.

(Figure 1A black columns), these losses were not negligible at ATP concentrations greater than 1 µM as previously reported (10). Also, the addition of K_2HPO_4 to the reaction as recommended for the prevention of ATP adsorption (10) did not, in fact, do so at initial ATP concentrations in this range. Experiments performed using the same procedures outlined in Figure 1 with higher initial concentrations of ATP (5-50 µM) indicated that the average 'loss' (i.e. due to adsorption and firefly desensitization) amounted to 71.1% ± 7.9 so that the absolute loss was dependent on the initial concentration. However at initial concentrations of 100-1000 µM, the total loss was only 21.9% ± 5.9 and was due solely to desensitization of the assay reaction. This result probably indicates that adsorption has reached a saturation point.

In order to define whether neutralization played a critical role in the assessment of ATP, the end-point pH was varied. Figure 1B indicates the apparent losses over the pH range 6.4-10.7. The highest recovery was observed with solution at pH 7.2. Figure 2 indicates the average amounts of ATP 'lost' from treating solutions containing initial ATP concentration of 0.5-1000 µM with perchloric acid and assayed by the luciferin-luciferase reaction. The linear relationship demonstrates that the total loss

of ATP increases with the initial amount of ATP and when the anticipated amount of ATP in a sample is approximately 1 mM, an average of only 177 ± 82 μM will remain undetected. Therefore such treatment of samples with ATP content in this range will allow sufficient quantities of the nucleotide to remain for detection by the luciferin-luciferase reaction.

These findings are particularly relevant to the problem of ATP detection in tissue perfusates, and hence to the possible function of ATP as a local vasodilator. In cases where PCA has been used as a deproteinizing agent no ATP was detected either in plasma (1) or tissue perfusate samples (1, 9) while in samples which had not been treated with PCA, ATP was readily measured (5, 6, 7, 12).

Acknowledgement

We wish to thank Professor A. R. Lind for helpful criticism in the preparation of the manuscript.

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Commentary - paper 14.

The following full paper extended our knowledge of the behaviour of myocardial cells in response to hypoxia. The instant release of ATP in response to hypoxia was clear. Also, the reversibility of the response was one indication of cell viability. A discussion of other evidence for cell viability is presented in paper 21.

Paddle & Burnstock (1974) had previously detected ATP in the coronary sinus effluent from hypoxic hearts perfused in the Langendorff mode. They could not determine whether the ATP had as its source the myocyte or nervous tissue, but suggested that 'purinergic' nerves may be the source. This work showed that at least one source was the hypoxic myocyte.

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With 7 text-figures
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RELEASE OF ADENOSINE TRIPHOSPHATE FROM ISOLATED ADULT HEART CELLS IN RESPONSE TO HYPOXIA

BY T. FORRESTER AND CAROLE A. WILLIAMS

From the Department of Physiology, Saint Louis University School of Medicine, 1402 South Grand Boulevard, Saint Louis, Missouri 63104, U.S.A.

(*Received 20 September 1976*)

SUMMARY

1. Adult rat heart cells were isolated enzymically and ATP was identified in the cell suspension using the firefly luminescence technique. Adenosine 5′-triphosphate (ATP) was not detected from cell suspensions obtained from hearts which had been left asystolic for 10 min.

2. It was found that ATP $0{\cdot}34 \pm 0{\cdot}22$ μM/mg protein was released by cells kept in an oxygenated condition, while ATP $1{\cdot}28 \pm 0{\cdot}41$ μM/mg protein was initially released by cells made hypoxic.

3. Addition of Ca^{2+} in a concentration of 2 mM caused cells to initially extrude ATP $0{\cdot}40 \pm 0{\cdot}14$ μM/mg protein. This was attributed to an inotropic effect.

4. Extracellular ATPase activity in the fluid suspension was partially characterized, giving a K_m of 13 μM and a $V/2$ of hydrolysed ATP 18·3 μM/min at 37° C. Q_{10} was found to be 4 between 25 and 37° C. Enzyme activity remained unaffected by either hypoxic conditions or ouabain.

5. If these amounts of ATP are released from myocardial cells rendered hypoxic *in vivo*, then it must be concluded that ATP plays a principal role in the local control of myocardial blood flow.

6. It is proposed that release of ATP occurs through the sarcolemma from an intracellular pool, and that alteration of the configuration of structural membrane protein controls the amounts of ATP extruded.

INTRODUCTION

The search for underlying mechanisms of coronary vasodilatation in response to an increased myocardial work load was initiated many years ago (Barcroft & Dixon, 1907; Wiggers, 1909; Cow, 1911; Markwalder & Starling, 1913) and remains the subject of intensive investigation. It has become clear that the resistance of the coronary vasculature is regulated

by the metabolic demands of the tissue and it is most probable that the primary stimulus comes from the lowered oxygen tension in the fluid environment of the myocardial cells (Hilton & Eichholtz, 1925). It is postulated that the cells release substances which cause local blood vessels to dilate, allowing delivery of more oxygen to the tissues. Many substances have been implicated in the role of local vasodilator (see review by Berne, 1964) but none seem to fulfil the role completely, either on the basis of potency or of actual amounts detected in the coronary effluent blood. The widely accepted adenosine hypothesis proposed by Berne (1963) has recently had doubts cast upon it by the work of Afonso, Ansfield, Berndt & Rowe (1972). They have shown that aminophylline, a drug which inhibits the coronary vasodilator action of adenosine, had no influence on the response of the coronary circulation to hypoxia.

Adenosine 5′-triphosphate (ATP) has been long considered as a candidate for local vasodilatation in the coronary circulation (Drury & Szent-Gyorgyi, 1929; Folkow, 1949; Winbury, Papierski, Hemner & Hambourger, 1953). Many attempts have been made to detect this substance in the coronary effluent blood, but it is not surprising that these attempts have failed, since erythrocytes (Parker, 1970), plasma (Mills, 1966; Holmsen & Holmsen, 1971; Forrester & Morrison, 1975) and extracellular ATPases in the myocardium (Williamson & DiPietro, 1965) are all capable of rapidly degrading exogenous ATP. Another important, though less well defined, consideration might be the binding of ATP by nerve membranes (Abood & Matsubara, 1968) or other proteins, further reducing the amounts available for assay. Despite these latter difficulties Paddle & Burnstock (1974) have been able to demonstrate an increase in the appearance of ATP in the coronary sinus effluent of perfused guinea-pig hearts whenever the myocardium was rendered hypoxic. It was, however, uncertain as to whether the ATP came from nerves, vascular smooth muscle or myocardial cells.

In the present work the use of isolated heart cells confers some advantages over the perfused heart preparation. There is no danger of contamination from the blood elements, the ATPases present in the blood stream are eliminated, no nerves are present in the medium and no circulating hormones can influence the metabolism or performance of the cells. The extremely high ratio of surrounding fluid volume to cellular volume provides satisfactory conditions for oxygenation. Also, no alteration in the fluid volume occurs after rendering the cells hypoxic, thus avoiding the problem with intact hearts of having to estimate amounts of ATP coming from a vascular bed which is continuously dilating in response to hypoxia. Therefore the opportunity was provided to test the fluid environment for the presence of ATP, establishing the myocardial cells as the source, and

to assess whether any change took place in the ATP concentration in response to hypoxia without the attendant complications of a perfused whole heart preparation.

METHODS

Isolation of cells

Ventricular cells were enzymatically isolated from hearts of adult rats according to the method devised by Vahouny, Wei, Starkweather & Davis (1970) and modified by C. A. Williams and A. H. Gold (unpublished). Male Sprague–Dawley rats (175–200 g) were decapitated, their hearts removed and placed in ice-chilled bicarbonate-phosphate buffer within 1 min. The buffer contained (in mM): NaCl, 116; KCl, 5·3; $Na_2HPO_4.2H_2O$, 1·9; $NaH_2PO_4.H_2O$, 0·5; $NaHCO_3$, 4·2; and glucose, 5·5 (see DeHaan, 1967). The osmolarity was adjusted to 285–289 m-osmole/l. with sucrose. Before the isolation procedure, the buffer was equilibrated with 95% O_2:5% CO_2 (referred to as oxygenated buffer) by bubbling for 45 min at room temperature and the final pH adjusted to $7·4 \pm 0·05$ with 1 N-NaOH. The atria were cut away and the ventricles cut from base to apex with a scalpel, washed repeatedly with fresh buffer and then cut into pieces averaging 3–5 mm^3 in size. The minced tissue was transferred to a polyethylene Erlenmeyer flask containing 5 ml. buffer with 0·1% trypsin, 0·1% collagenase and 0·2 u insulin/ml. In addition, this digestion mixture was supplemented with bovine serum albumin (BSA) 5 mg/ml., since it was shown by Kono (1969) that enzymatically isolated fat cells regained insulin sensitivity upon incubation with bovine serum albumin. The contents of the flask were agitated with a vortex mixer for 10 sec and then digested at 32° C under a humidified atmosphere of 95% O_2:5% CO_2 in a shaker bath at 100 strokes/min. After 17 min the supernatant was discarded, the tissue mince washed with enzyme-free buffer and the digestion continued in successive 17 min periods for the next 1–2 hr in bovine serum albumin-supplemented buffer containing 0·1% trypsin and 0·05% collagenase. Freed cells were collected beginning with the third digestion period with this combination of enzymes and continued for the next 70 min (four consecutive digestion periods). Cells were obtained by centrifuging the supernatant from the digestion mixture combined with the enzyme-free buffer used for washing the tissue mince at 50 *g* for 3 min at 0–4° C. The supernatant from centrifugation was discarded and each aliquot of packed cells was re-suspended in enzyme-free buffer supplemented with bovine serum albumin and insulin and stored at 0–4° C. Each new aliquot of collected cells was re-suspended and then added to the previous ones for storage. It was previously determined that 20–30% of the isolated cells were beating (C. A. Williams and A. H. Gold, unpublished).

Cell-suspension measurements

The ATP present in the fluid bathing samples of re-suspended cells was measured by using the luciferin–luciferase reaction (see Strehler & McElroy, 1957) as modified by Forrester (1972). The total population of isolated cells kept in oxygenated buffer was divided into two after gently inverting the polyethylene collection tube two or three times and transferring half the volume to another tube. Both tubes were then centrifuged at 50 *g* for 3 min at 0–4° C. Supernatant fluid from this centrifugation, whether the total population of cells was divided or not, is termed 'suspension fluid'. One of the aliquots was re-suspended in a volume of fresh oxygenated buffer at 37° C and served as the control, while the other aliquot was re-suspended in an equal volume of buffer equilibrated with 100% N_2, pH 7·4, referred to as 'nitrogen buffer'. A 0·2 ml aliquot of re-suspended cells in either condition was removed at

various intervals and added to 0·2 ml. firefly tail crude extract (Sigma Chemical Co. FLE-50). Five to six seconds elapsed between the addition of cell samples to the firefly extract and the application of 1225 V to the photomultiplier tube. ATP concentration was determined by comparing the intensity of the initial light signal caused by the addition of cells to the firefly tail extract to that caused by known amounts of ATP (Sigma, crystalline, disodium 'Sigma Grade').

Calculation of percentage loss of ATP from each cell. Experimentally the amount released was found to be 10^{-15} mole/min from each cell exposed to hypoxia; therefore the number of molecules released per minute $= 6{\cdot}02 \times 10^{23} \times 10^{-15} = 6 \times 10^{8}$. Calculation of intracellular amount assumed a cell concentration of 5 mM ATP $= 5 \times 10^{-6} \times 10^{-12}$ mole/μm^3. Assuming a cell volume of $20 \times 20 \times 100 = 4 \times 10^{4}$ μm^3, the number of molecules of ATP per cell $= 1{\cdot}2 \times 10^{11}$.

ATP levels following rapid removal of cells. After a basal level of ATP extrusion had been established (see Fig. 2) a 0·4 ml aliquot of suspended cells was centrifuged in a Microfuge (10,000 *g*) for 10 sec. Within 6 sec following the end of centrifugation 0·2 ml. supernatant was removed and tested for ATP levels.

Correction for variation in cell population. Total protein was determined on aliquots of homogenized cells by the Lowry (1951) method using bovine serum albumin (Sigma, Fraction V) as the standard to correct for variations in the cell population between oxygenated and nitrogen buffer fractions from each digestion and for slight variations in the number of cells yielded from one digestion to another.

Calcium experiments. The same procedure as above was followed in a series of experiments in which the effect of the addition of 2 mM-$CaCl_2$ to cells kept in an oxygenated environment was studied. Cells were re-suspended in equal volumes of oxygenated buffer and oxygenated buffer + Ca^{2+}.

EDTA experiments. One millilitre aliquots of cells were incubated at 37° C in the presence of 0, 5, 25, 50, 100, 200 and 2000 μM ethylenediaminetetra-acetate (EDTA) and the amount of ATP released was measured.

Apyrase test. Aliquots of cells were subjected to the apyrase test as described by Forrester (1972) in which 4 mg apyrase (Sigma, Grade 1) was incubated with 1·0 ml. of re-suspended cells for 5 min at 30° C. ATP signals following this reaction were compared to those elicited by cells kept at 30° C for 5 min without apyrase.

Alteration of oxygenated and hypoxic environments. Another variation of this procedure was carried out such that the total population of cells was again divided into two fractions, *A* and *B*, one being resuspended in the nitrogen buffer at 37° C (*A* fraction) while the other was resuspended in fresh oxygenated buffer (*B* fraction). The amount of ATP released from cells in both samples after 0–2 min of resuspension was measured and then the nitrogen buffer fraction was centrifuged at 50 *g* while the oxygenated buffer fraction remained suspended at 37° C. The packed centrifuged cells were then suspended in oxygenated buffer which had the same volume before centrifugation, and the ATP released from both fractions was measured. Cells from fraction *A* were recentrifuged and then taken up in the nitrogen buffer again to the same volume before centrifugation. Resuspension of *A* cells in the oxygenated buffer was also repeated.

Extracellular ATPase activity

Cell-free suspension fluid was derived from the centrifugation at 50 *g* for 3 min of the total isolated cell population which had been stored for at least 30 min at 0–4° C. The ATPase activity was determined by measuring the decrease in the initial intensity of light signals caused by known amounts of ATP, ranging from 0·5 to 50 μM, which had been added to cell-free suspension fluid. Equal volumes (0·5 ml.) of undiluted suspension fluid and ATP standards were mixed and maintained at

0–4° C; 0·2 ml. aliquots of this mixture were removed at 30 sec, 2, 5, 10 and 15 min and the ATP signal measured as described above. The ATP signal in millivolts was converted to micromolar concentrations and then plotted as a function of time. The rate of hydrolysis of ATP was calculated as the change in ATP signal from the initial slopes of the degradation curves for a specific time interval and then expressed as μM ATP hydrolysed per hour. Michaelis-Menten curves describing the relationship between rates of hydrolysis and initial ATP concentrations were made. Similar reactions were carried out at 37° C with the suspension fluid diluted tenfold before the addition of ATP.

Ouabain experiments. The effects of adding various concentrations of ouabain (E. J. Lilly) to the suspension fluid before the addition of ATP standards was performed for both the oxygenated and anoxic conditions. Mixtures of ouabain and suspension fluid were allowed to stand at 37° C for 10 min prior to the addition of ATP. ATPase activity was then determined as described above.

RESULTS

Release of ATP from heart cells

When a suspension of isolated cells was added to firefly tail extract a light signal of considerable magnitude was always detected, while suspension fluid without cells did not elicit a signal. Fig. 1*A* illustrates a typical light signal produced by cells which had been suspended in oxy-

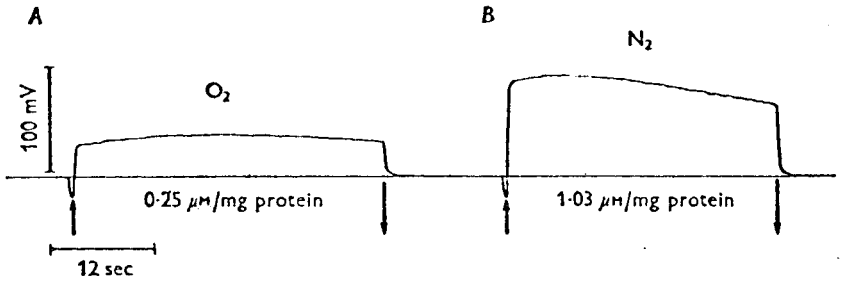

Fig. 1. Emission of light from firefly lantern extract upon addition of cells re-suspended in either: *A*, oxygenated; or *B*, nitrogen-equilibrated buffer solution. Both buffers at pH 7·4; ↑, power supply to photomultiplier tube switched on; ↓, power switched off. Each of the signals represent the ATP extruded after cells had been in contact with respective buffer solutions for 30 sec at 37° C.

genated buffer for 30 sec at 37° C and is equivalent to ATP 0·25 μM/mg protein. In every case the light signal recorded showed a peak 0–10 sec after mixing the sample with firefly extract; this was followed by a gradual decline in light intensity. The latter response resembles one caused by solutions containing known amounts of pure ATP. The implication that ATP was the cause of the light signal produced by addition of cells to firefly extract was further supported by the results of the apyrase test. When oxygenated cells had been incubated with the enzyme apyrase, the

light signal caused by the reaction with luciferin–luciferase was found to be decreased by 34% in contrast to the one caused by cells incubated without apyrase. This enzyme converts ATP to adenosine 5′-phosphate (AMP) which does not elicit a light signal from the firefly extract. It was concluded that the cells release ATP into the fluid medium. While it has recently been reported that no nucleotide triphosphate other than ATP can induce light emission by the luciferase reaction (Kimmich, Randles & Brand, 1975) transphosphorylase activity may exist in the crude firefly extract preparation (Balfour & Samson, 1959; Kimmich *et al.* 1975) so that other triphosphates which may be present in the suspended cell samples might cause a light reaction. However, the other triphosphates must be present in concentrations ten times greater than ATP for this light reaction to occur (Kimmich *et al.* 1975). The presence of bovine serum albumin in the suspension buffer did not in any way alter the extent of the signal, since the standard curve for ATP in the presence or absence of BSA was the same.

A feature which was almost always present in the recorded signal was the 'hump' in the time course of light production (Figs. 1 and 3). This phenomenon, also noted in the data reported for plasma samples by Forrester & Lind (1969) and Forrester (1972), may be due to the combination of transphosphorylases present in the firefly extract, and the small amounts of other triphosphates in the cell samples causing a slow production of ATP. Attempts at generating ATP from mixtures of adenosine 5′-pyrophosphate (ADP) and AMP by pyruvate kinase and myokinase (see Kimmich *et al.* 1975) failed to produce a hump in the resulting light signal.

ATP levels after exposure of cells to nitrogen. The signal in Fig. 1*B* was caused by cells introduced to hypoxic buffer for 30 sec at 37° C and represents ATP 1·03 μM/mg protein. The extracellular ATP concentrations for cells introduced to the hypoxic buffer were always much greater than those for cells re-suspended in fresh oxygenated buffer (also note this difference in Fig. 3).

A summary of the differences in the amounts of ATP released by cells incubated for various intervals in either oxygenated or hypoxic buffer is presented in Fig. 2. The media of cells made hypoxic initially contained nearly four times as much ATP (1·28 ± 0·41 μM/mg protein) as those of oxygenated cells (0·34 ± 0·22 μM/mg protein). The oxygenated cells continued to release a constant amount of ATP leading to a constant amount in the medium equivalent to 0·28 μM/mg protein for at least 60 min of incubation at 37° C. In contrast, the amounts of ATP released by the hypoxic cell population were reduced leading to a lower constant medium level which nevertheless remained higher (0·45 μM/mg protein) than that observed for the cells maintained in the oxygenated buffer. A similar

response has been noted by Paddle & Burnstock (1974) who reported a threefold increase in the amounts of ATP released after a 1·5 min hypoxic period of perfusion in the guinea-pig heart. Repeated periods of hypoxia produced successively decreasing levels of ATP. The isolated cell system

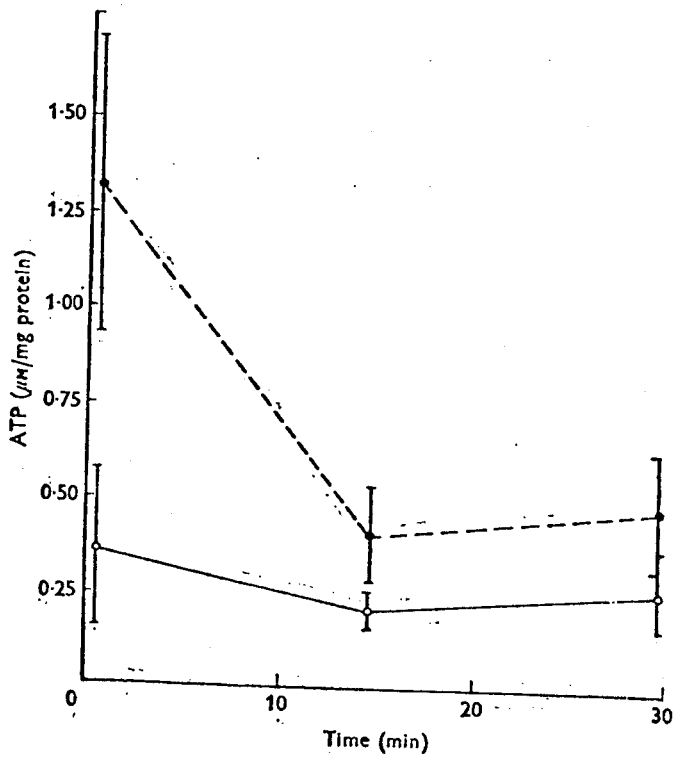

Fig. 2. Amounts of ATP present in the medium for cells in oxygenated (○) or nitrogen-equilibrated buffer solution (●) at 37° C. Difference between points at 1 min of incubation is highly significant ($0{\cdot}001 < P < 0{\cdot}01$). Cell population divided in half such that each preparation served as its own control. Each point represents the average from six determinations. Bars indicate ± 1 S.D. of observation.

permits this response to be expressed in terms of the output of ATP per individual cell. It has previously been determined that each cell contains 18·9 ng protein (C. A. Williams and A. H. Gold, unpublished); therefore over the first 15 min of incubation at 37° C ATP $2{\cdot}9 \times 10^{-16}$ mol/oxygenated cell.min were released in contrast to ATP $1{\cdot}1 \times 10^{-15}$ mol/hypoxic cell.min. It can be calculated that over a period of 1 min the release of ATP from the total cell population in the hypoxic state is 0·5 % of the total intracellular amount, while in the oxygenated state the proportion released is only 0·05 %.

In order to exclude the possibility that dead cells were the source of ATP, rats were decapitated in the usual way and left to bleed out. The hearts were removed 20 min later when all circulatory and heart move-

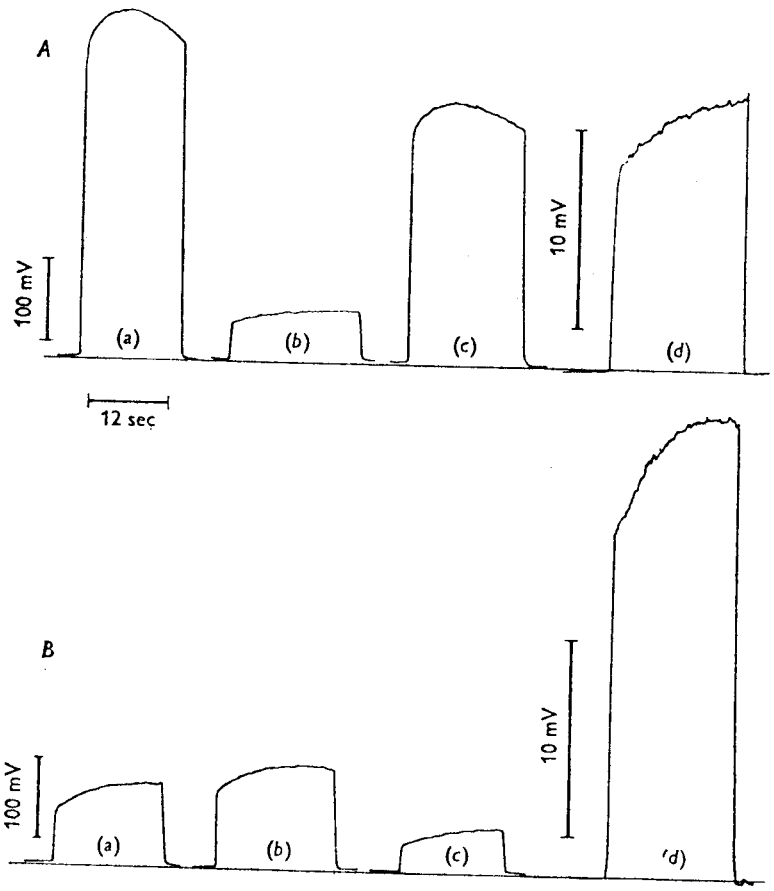

Fig. 3. Emission of light from firefly extract in response to a cell suspension exposed alternately to hypoxic and oxygenated buffer solution. *A*(*a*), signal after exposure to cells to hypoxic buffer; (*b*), response after cells had been returned to oxygenated medium; (*c*), response when returned to hypoxic buffer; (*d*), response when finally returned to oxygenated medium; note alteration in amplification. *B*, (*a*)–(*d*), paired oxygenated controls matching the solutions tested in *A*.

ments had ceased. Cells were isolated in the usual manner, and the cell suspension in hypoxic buffer was tested on the firefly extract. The resultant signal was consistently smaller than that given by the firefly extract plus buffer at 37° C.

ATP released by cells recovering from hypoxia. The amounts of ATP

released are related to the gaseous environment of the cells since the high levels associated with cells in the hypoxic state (see Fig. 2) could be reverted to those levels associated with cells initially in the oxygenated

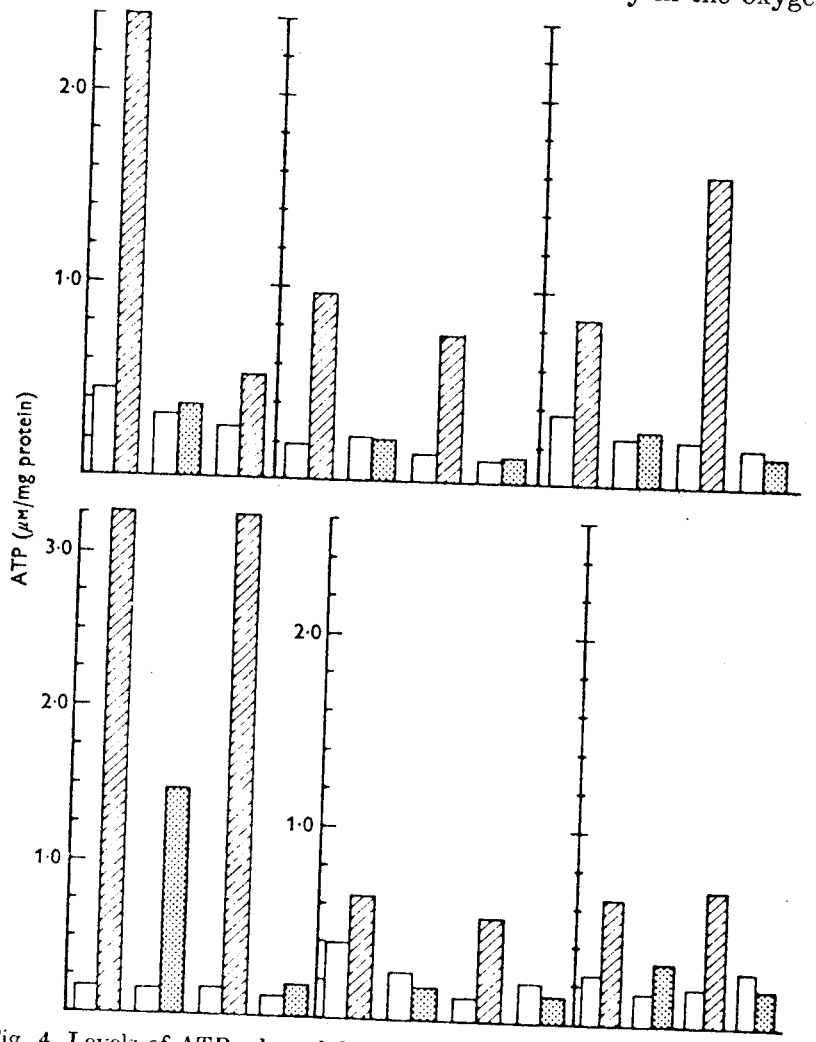

Fig. 4. Levels of ATP released from cells alternately exposed to hypoxic and oxygenated condition; results from six experiments. □, cells continuously oxygenated. ▨, cells first introduced to hypoxic buffer. ▩, cells previously suspended in hypoxic buffer restored to oxygenated medium.

state. A cell suspension was divided into two volumes, *A* and *B*. Volume *A* was immediately exposed to nitrogen buffer, while *B* remained continuously oxygenated. Cells were maintained in either condition for 1–2 min before an aliquot was removed for measurement of ATP. Volume *A* was

then immediately centrifuged and the cells re-suspended in oxygenated buffer for 1–2 min before the next ATP estimation. The same procedure was carried out in returning the cells to the hypoxic buffer for ATP measurement. Finally in the same manner the cells were returned to the oxygenated medium. They were never allowed to remain in the hypoxic suspension for more than 3 min, avoiding any possibility of the ATP levels diminishing to the levels recorded in Fig. 2 (●). The results of one such experiment are presented in Fig. 3*A*. There is a striking increase in the light signal obtained from the hypoxic cell suspension, while the paired oxygenated controls remained relatively constant throughout the same time course.

The results obtained from six experiments when cell suspensions were first made hypoxic and then returned to the oxygenated buffer are shown in Fig. 4. In each of the cases illustrated, it is clear that cells in the hypoxic condition always released greater amounts of ATP than the cells which were continuously kept in the oxygenated environment. Each time the hypoxic cells were introduced to the oxygenated condition, the levels of ATP released matched the amounts of ATP released by the cells that had always been kept in the oxygenated condition.

ATP release in the presence of calcium. No calcium was added to the buffer used for the isolation procedure or for the storage of dispersed cells. A better yield of cells was obtained upon dialysis of collagenase before its use for digestion (C. A. Williams and A. H. Gold, unpublished) in which the amount of calcium associated with the enzyme was decreased from 12·4 to 5·8 μg/ml. This observation supports and extends the findings reported by others (Vahouny *et al.* 1970; Glick, Burns & Reddy, 1974; Moustafa, Skomedal, Osnes & Oye, 1976) in which minimum cell dispersion occurred in the presence of calcium in addition to the lack of rhythmic contraction for any of the single cells. Furthermore, Fabiato & Fabiato (1972) have isolated cells using either trypsin or hyaluronidase and collagenase in the absence of additional calcium; they reported concentrations of calcium from 0·05 to 0·07 mM in the digestion buffer solution. The data presented in Fig. 5 indicate that alterations in the amounts of ATP released occurred as a result of addition of calcium to the oxygenated buffer. In the presence of 2 mM-$CaCl_2$, cells released ATP 0·40 ± 0·14 μM/mg protein. These levels declined over the next 5 min and became similar to the basal levels extruded by the control cells, which again remained constant over the time interval studied. Addition of calcium did not alter the response of the luciferin–luciferase reaction to ATP.

Influence of EDTA on release of ATP. Previous studies (Abood, Koketsu & Miyamoto, 1962; Kuperman, Volpert & Okamoto, 1964*b*;

Okamoto, Askari & Kuperman, 1964) have shown that ATP can be released from cells by applying a powerful calcium chelator, EDTA. It was postulated that the source of this ATP was the membrane itself (see

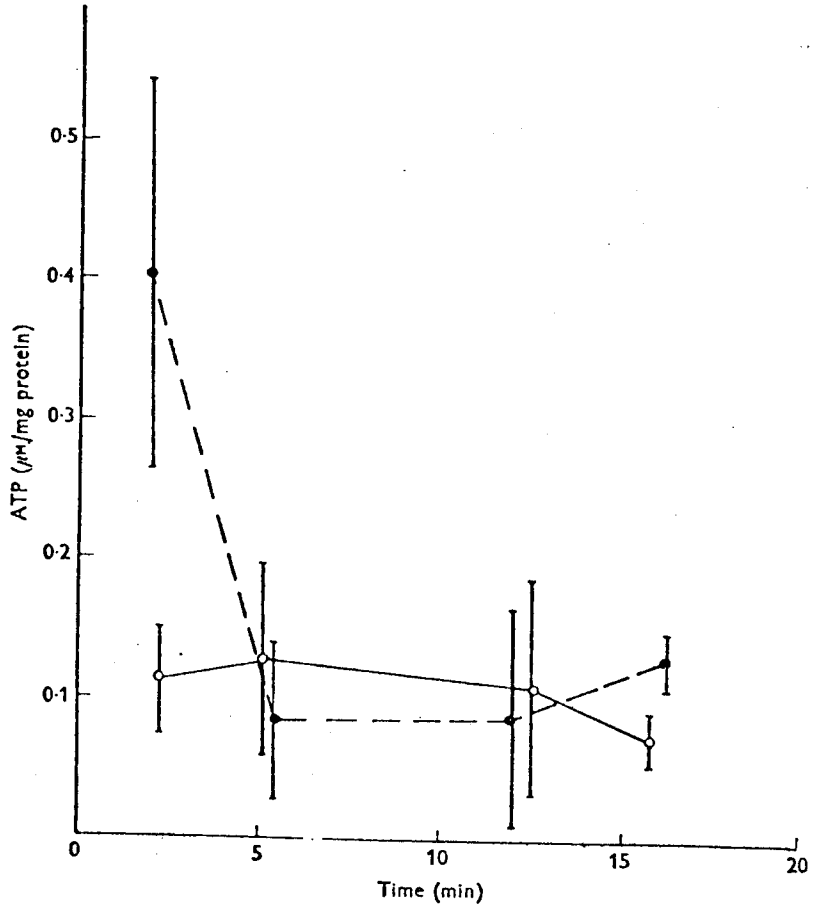

Fig. 5. Effect of adding Ca^{2+} upon ATP extrusion from cells in suspension. Cells were re-suspended in oxygenated buffer (○) or oxygenated buffer containing 2 mM-$CaCl_2$ (●). Each point represents the average of three determinations. Bars indicate ± 1 S.D. of observation. Initial levels of ATP released are significantly different ($0{\cdot}001 < P < 0{\cdot}01$).

Discussion, p. 387). EDTA was applied to the heart cells in order to see whether chelation of calcium from the membranes was associated with an increase in the levels of ATP detected. The amounts of ATP released in response to incubating cells with increasing concentrations of EDTA is given in Fig. 6. When EDTA was added in concentrations below 50 μM there was no significant effect on the ATP released, averaging 0·15 μM/mg

protein in contrast to cells incubated without EDTA which extruded 0·14 μM/mg protein. Concentrations of EDTA above 50 μM increased the levels of ATP by sevenfold during the first 5 min of incubation. This indicates that a certain level of EDTA must be present before ATP efflux is altered.

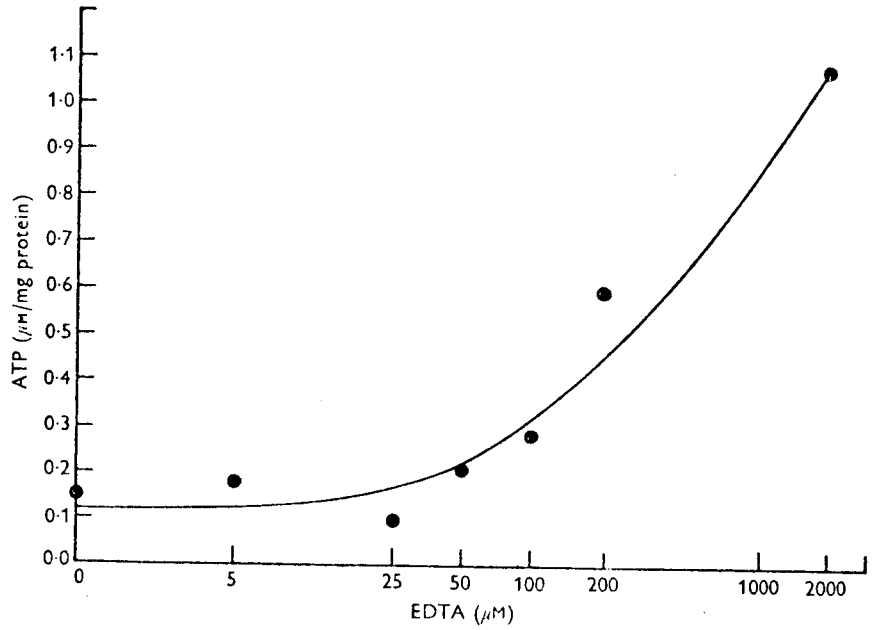

Fig. 6. Effect of ethylenediaminetetra-acetic acid (EDTA) on ATP release from isolated cells. Oxygenated cells were incubated at 37° C with increasing amounts of EDTA. Points represent amount of ATP released after incubation for 5 min with EDTA.

Extracellular ATPase activity

ATP was not detected in the fluid suspension after the cells had been removed, suggesting the presence of potent extracellular ATPase activity. A preliminary characterization of this activity was therefore undertaken in order to determine whether the total disappearance of the ATP in the absence of cells could be explained by this activity. The results of these experiments are reported in Fig. 7 and Table 1. Cells isolated and then incubated in oxygenated buffer were removed by centrifugation and known amounts of ATP ranging from 0·5 to 50 μM were then added to the fluid and incubated at 0° C for up to 15 min in order to establish the initial rates of degradation. Aliquots of this mixture were removed at 30 sec, 2, 5, 10 and 15 min and added to firefly extract. The amount of ATP remaining, plotted as the initial intensity of the light signal in relationship

to their time of removal of samples is indicated in Fig. 7. The rate of degradation was determined from the initial slopes of these curves. Similar decay curves were obtained for the reaction at 37° C. The initial rates of

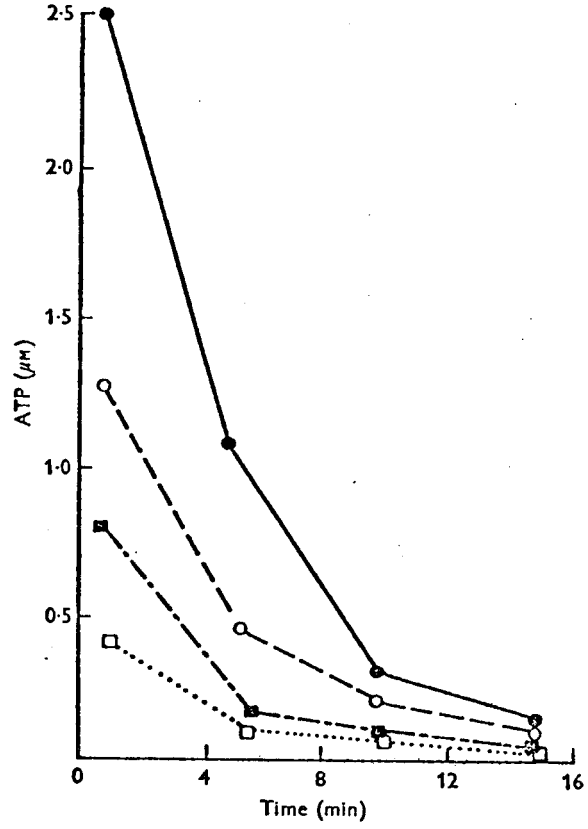

Fig. 7. Rate of disappearance of ATP added to cell-free suspension fluid. Reactions measured by mixing equal volumes of undiluted suspension fluid with ATP (in μM): 2·5, ●; 2·0, ○; 1·0, ▣; and 0·5, □. An aliquot (0·2 ml.) of the mixture was removed at intervals and added to 0·2 ml. firefly extract for assay. Temperature 0° C.

hydrolysis increased as the initial ATP concentration increased. A plot of velocity against initial substrate concentrations revealed that the hydrolysis reaction present in the crude enzyme preparation followed Michaelis–Menten kinetics. Initial ATP concentrations above 15 μM were saturating for the reaction at 0° C; V/2 hydrolysed ATP was approximately 1·5 μM/min and K_m was determined to be 10·5 μM. However, initial ATP concentrations above 25 μM were saturating for the reaction at 37° C, yielding a V/2 of ATP hydrolysed 18·3 μM/min and a K_m of approximately 13 μM. The saturation nature of the kinetics was emphasized by the close

agreement of the K_m values at the two divergent temperatures in view of the crude enzyme preparation which was used for the determination of the rates of hydrolysis. This agreement also indicated that the degradation of the ATP signal with time in the presence of the cell-free suspension fluid is truly an enzymic reaction. Increasing dilutions of the suspension fluid resulted in a loss of enzymic activity, rendering the ATPase as the

TABLE 1. Initial characterization of extracellular ATPase

Initial ATP concentration (μM)	Rate of hydrolysis (μM/hr) 0° C (in O_2:CO_2)	Rate of hydrolysis (μM/hr) 37° C (in O_2:CO_2)	Rate of hydrolysis (μM/hr) 37° C (in N_2)
0·5	4	33	77
1·0	10	147	88
2·0	18	598	185
5·0	—	—	540
10·0	111	690	1050
15·0	147	900	1230
25·0	132	2220	2520
50·0	180	2160	1257
	K_m 10·5–11 μM V/2 1·5 μM/min	K_m 12–13 μM V/2 18·3 μM/min	K_m 12·5 μM V/2 20·8 μM/min

limiting factor in the reaction, or inactivating it, possibly due to conformational change. The Q_{10} of the reaction was determined to be 4 when a comparison of the rates of hydrolysis was made between 25 and 37° C.

The dynamic nature of the ATP release process from single cells was partially revealed from the reduction in the light signal following rapid removal of cells. When cells were present, a signal equivalent to 0·39 μM ATP was elicited, whereas following the 10 sec centrifugation, the cell-free fluid sample generated a signal equivalent to only 0·11 μM ATP. This meant that ATP 0·027 μM/sec was lost after the removal of cells. According to the rate of hydrolysis determined at substrate concentrations between 0·5 and 1·0 μM (see Table 1), ATP 0·009 and 0·040 μM/sec can be hydrolysed, thus the disappearance of 0·274 μM ATP after the 10 sec rapid centrifugation seemed accountable by this process. Together, these results seemed to indicate that the normally functioning isolated cell system is composed not only of a source for ATP release, i.e. the cells themselves, but also a mechanism by which the ATP released is maintained within certain limits of concentration.

The possibility that the increased amounts of ATP detected from cells in the hypoxic condition were due to an inhibition of ATPase activity had to be ruled out, therefore ATPase activity was determined in suspension fluid samples derived from cells incubated in hypoxic buffer for 1 hr. The

results of these experiments are also presented in Table 1 and indicate that the enzyme is no less active in the hypoxic condition than in the well oxygenated condition. The K_m was determined to be 12·5 μM and V/2 20·8 μM/min. Thus, it appeared that the increased amounts of ATP measured from the re-suspended cell samples represented a direct cellular response to hypoxia. Attempts at further characterization of the enzyme by testing its activity in the presence of ouabain in both the oxygenated and hypoxic buffers were carried out. There was no inhibition of ATPase activity in the oxygenated buffer when 1·0 μM ATP was added. This was confirmed by increasing ouabain from 2·5 to 25 μg in the incubation mixture (1·2 ml.) while keeping the ATP concentration constant, so that the rate of hydrolysis at 1 μM ATP in the absence of ouabain was 147 μM/hr while in the presence of 25 μg ouabain there was a slight increase to 172 μM/hr. Similarly, at 2·0 μM ATP concentration, there was no consistent effect of either 25 or 50 μg ouabain on the rate of hydrolysis. Ouabain had no inhibitory effect on the rates of hydrolysis of ATP at initial concentrations of 1·0 and 2·0 μM in the hypoxic conditions, but some inhibition occurred at an initial concentration of 0·5 μM ATP. However, since the initial levels of ATP released (1·28 μM/mg protein) by cells incubated in 100% N_2 were much greater than 0·5 μM, there is no indication that rates of ATP hydrolysis from 1 to 2 μM are inhibited by ouabain, therefore alteration of enzyme activity as a possible explanation for higher ATP levels in hypoxia was not supported by the ouabain experiment.

DISCUSSION

ATP has been identified in the fluid medium surrounding adult heart cells isolated from rat ventricle. The most striking feature demonstrated was the consistent increase in the ATP concentration upon exposure of the cells to an hypoxic environment and the diminution of these levels upon restoring cells to an oxygenated medium.

There is little doubt that the source of the ATP detected is the myocardial cell, but obviously an assessment of viability was necessary. Previous studies suggest that enzymically isolated heart cells retain some metabolic and physiological characteristics which are comparable to the intact myocardium. C. A. Williams and A. H. Gold (unpublished) have concluded that the insulin receptor, which is known to be membrane bound, remains functionally intact since both glucose incorporation into glycogen and the activity of the I-form of glycogen synthetase (E.C. 2.4.1.11) were stimulated by the presence of insulin. Glick *et al.* (1974) have shown that octanoate is preferentially oxidized over glucose and its oxidation is stimulated by the presence of triiodothyronine (Burns &

Reddy, 1975). Berry, Friend & Scheuer (1970) have indicated that oxygen uptake will be dependent on the substrate provided and they noted that addition of calcium increased the percentage of contracting cells. Their studies on the fine structure of these cells revealed a normal morphology. Powell & Twist (1975) found that respiration increases in the presence of an uncoupler but is completely inhibited by a respiratory poison, indicating normal function of the mitochondrial oxidative apparatus. Moustafa *et al.* (1976) have reported that a transient increase in cyclic AMP occurred in response to isoprenaline when cells were pre-treated with theophylline, while calcium reduced the levels of cyclic AMP, presumably by inhibiting adenyl cyclase. In this study, reduction of the amounts of ATP released seen upon restoring cells to oxygen further demonstrates viability. Certainly the possibility that dead cells are responsible for the levels of ATP is excluded by the results of the experiments in which no ATP was detected when hearts were allowed to remain *in situ* for 20 min before cell isolation was performed. The argument that the cell membranes might be 'moderately' damaged, allowing ATP to leak steadily out is difficult to support in view of the results with calcium. Since there was no calcium added to the fluid medium it might be expected that increased levels of calcium would 'stabilize' the membrane and diminish the output of ATP. In fact, the addition of calcium increased the ATP initially released by cells, presumably through an inotropic effect (Berry *et al.* 1970; Fabiato & Fabiato, 1972). These results strongly suggest that release of ATP is a normal function of viable cardiac cells and emphasizes its role as one of the principle metabolites involved in the mechanism of local vasodilatation in the myocardium.

The question now arises whether enough ATP is released in order to satisfy vasodilatory requirements in the myocardium. Wolfe & Berne (1956) produced maximum coronary flow in dogs with ATP 0·2 μM/min and McCruden (1970) has shown that the threshold of coronary vasodilatation in response to ATP lay between 0·06 and 0·56 μM entering rabbit coronary arteries. The levels reported here in the oxygenated condition (ATP 0·28 μM/mg protein) fall well within this range. Continual release of ATP from isolated cells supports the possibility that coronary vessel tone is a function of this level. A continuous release of ATP from the soleus muscle of cat was noted by Forrester & Hamilton (1975) and was thought to be the probable reason for the high resting flow in this muscle. Furthermore, the significant increases noted in response to hypoxia resemble the pattern described by Paddle & Burnstock (1974). In the present study, ATP 1×10^{-15} mol/cell.min is calculated to be released under hypoxic conditions.

Efflux of ATP has already been demonstrated from nerve axons (Abood

et al. 1962; Kuperman, Okamoto, Beyer & Volpert, 1964*a*) nerve terminals (Holton, 1959; Silinsky & Hubbard, 1973) the post-synaptic membrane of the electroplaque (Israël, Lesbats, Muenier & Stinnakre, 1976) and skeletal muscle (Abood *et al.* 1962; Boyd & Forrester, 1968; Forrester & Lind, 1969; Forrester, 1972). The efflux of ATP demonstrated in this work may thus represent a general physiological phenomenon.

Since the rate of efflux is maintained for a period of 60 min, it is likely that a 'pool' of ATP is available for release. This pool is either large enough to maintain the efflux or is continuously replenished in some way. These findings are important to consider in relation to the extracellular ATPase activity reported. Williamson & DiPietro (1965) have observed surface ATPase activity from rat cardiac muscle quite similar to the one presented here. It is probable that this ATPase functions to maintain a constant basal level of ATP in the extracellular fluid and this level, in turn, determines the 'resting' tone of coronary vessels. It is possible to arrive at this conclusion because the same levels of ATP as reported here, during oxygenated conditions, were found to cause a certain degree of vasodilatation (Wolfe & Berne, 1956; McCruden, 1970), therefore, almost the same extracellular ATPase activity as measured here must be operating in these intact systems. Perhaps the most important finding of this study is the demonstration that the high levels of ATP seen in response to hypoxia are the result of increased amounts of ATP released and not the result of an inhibition of extracellular ATPase activity. These findings reveal the dynamic nature existing between the amounts of ATP released and the resulting degree of vasodilatation.

The site from which ATP is released remains to be determined. The experiments completed can only suggest that either ATP is released from the membrane or through it. A nucleotide–Ca–protein complex was first conceived by R. J. P. Williams (1959) and a similar model has been proposed for excitable membranes (Abood *et al.* 1962; Kuperman *et al.* 1964*b*; Okamoto *et al.* 1964). It has been noted by these workers that conditions which affect nucleotide release also increase the rate of calcium efflux. Since EDTA was used this phenomenon was presumably a membrane effect (Koketsu & Miyamoto, 1961). Experiments conducted in this work showed that incubation of isolated heart cells in the presence of increasing amounts of EDTA permitted larger amounts of ATP to be detected. It is possible that ATP was freed from these protein–Ca–nucleotide complexes along the sarcolemma in association with the removal of calcium by EDTA. However, it is not suggested here that the effects seen in the presence of EDTA account for the ATP released by cells maintained in an oxygenated or hypoxic condition, but it may indicate that myocardial cells enzymically isolated have retained this structural entity.

Possible mechanism of ATP release. The current most plausible model of the molecular organization of cell membranes concludes that a two dimensional solution of oriented globular proteins alternates with a phospholipid bilayer (Singer & Nicolson, 1972). This view can be co-ordinated with the association–induction hypothesis presented by Ling (1962), in which the physical state of the cell is maintained by protein–ATP complexes occurring along various cardinal sites on membrane-bound proteins. The adsorption of ATP most likely helps to establish the conformation of the integral proteins of the cell membrane. The binding of ATP to charged sites along the protein would certainly affect the affinity of various ions for these structures and might further explain the concept of selective ionic channels.

It is proposed here that alteration of protein configuration and protein–protein aggregate interactions, by whatever means, will release ATP which may not necessarily be that ATP involved with structural membrane proteins. In the present work, the continuous release of ATP into the fluid medium despite strong ATPase activity would suggest that it is replenished by intracellular ATP. This pool of nucleotide readily available for release may be situated close to the integral protein on the inner side of the membrane. The presence of oxygen may be necessary to allow membrane bound protein–protein interactions to occur. The absence of oxygen, independent of pH changes, would effect a sufficient alteration of the inherent membrane structure such that new charge–charge interactions would take place resulting in a different protein conformation (membrane structure) allowing ATP to pass through it.

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Commentary - paper 15.

This short abstract was a report upon experiments intended to throw some light upon the mode of release of ATP from the cell. Either the nucleotide is released through the cell membrane or from it. The well defined effects of pH and calcium could support either suggestion. Further work is in progress to clarify these points.

[*From the Proceedings of the Physiological Society*, 8–9 *July* 1977
Journal of Physiology, **272**, 44–45*P*]

Effect of pH and Ca^{2+} on ATP release from isolated adult heart cells

By T. Forrester and Carole A. Williams. *Department of Physiology, St Louis University School of Medicine, St Louis, Missouri 63104*

Adult rat heart cells isolated enzymically (Williams, 1977) release a constant amount of ATP which is increased upon exposure of the cells to hypoxia (Forrester & Williams, 1977) at pH 7·4. Addition of Ca^{2+}, 2 mM, to the oxygenated cell suspension caused an increase in the levels of ATP released; this was attributed to a positive inotropic effect (Fabiato & Fabiato, 1972). It was calculated that if these amounts of ATP are released *in vivo*, then ATP could satisfy the vasodilator requirements of hypoxic cardiac muscle. The present experiments set out to explore the effect of pH and applied Ca^{2+} on the output of ATP, since pH and Ca^{2+} levels are likely to change locally in increased cardiac muscle activity (Hasselbach, 1964).

Cardiac ventricular cells were enzymically isolated from adult male rats according to the method of Vahouny, Wei, Starkweather & Davis (1970) as modified by Williams (1977), and described in detail by Forrester & Williams (1977). The ATP present in the fluid surrounding the heart cells was measured by the luciferin-luciferase method modified by Forrester (1972).

Cells maintained in an oxygenated buffer at pH 7·4 released a constant amount of ATP, averaging 0·20 μM/mg protein during a 30 min period. When cells from the same digestion were equilibrated in the oxygenated buffer at pH 6·8, there was a significantly lower amount of ATP released, 0·15 μM/mg protein, during the first 15–20 min. Thereafter ATP levels were similar to those measured from the cells kept at pH 7·4. The affinity of extracellular ATPase activity increased with lowered pH, the K_m shifting from 11·3 to 5–7·5 μM. Levels of ATP released from hypoxic cells at both pH values were initially greater; reduction of pH caused less ATP to be released in relation to hypoxic cells maintained at pH 7·4. The lower amounts of ATP released by cells in an acidic environment indicates a relationship between H^+ and membrane permeability to anions such as ATP.

There was no modulation of the ATP released from oxygenated cells when various amounts of Ca^{2+} ranging from 0·1 to 2·0 mM were added. The higher levels of Ca^{2+} seemed equally to increase the amount of ATP released relative to the non-Ca^{2+} treated cells.

[P.T.O.

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Commentary - paper 16.

An opportunity arose to study the effects of ATP injected into the pulmonary artery in primates. Basically the rationale behind this experiment was the question: can intravenously administered ATP survive passageway through the lungs? If so, can vasodilator amounts significantly affect the calibre of the coronary blood vessels? If this is the case then perhaps ATP could be used therapeutically as a coronary vasodilator drug. As can be seen from the report, very high doses are necessary before ATP appears in the arterial blood.

[*From the Proceedings of the Physiological Society*, 16–17 *December* 1977
Journal of Physiology, 276, 70–71*P*]

Intravascular passage of adenosine triphosphate through lung of baboon

By R. G. Evans, T. Forrester and Hiltrud S. Mueller. *Division of Cardiology and Department of Physiology, Saint Louis University School of Medicine, 1402 South Grand Boulevard, Saint Louis, Missouri 63104, U.S.A.*

Adenosine triphosphate (ATP) has been detected in the circulation during isometric exercise (Forrester & Lind, 1969) and 5 min following whole body exercise (Parkinson, 1973). We have measured ATP in plasma within 2 min of sampling and have monitored the level during infusion of ATP into the pulmonary artery.

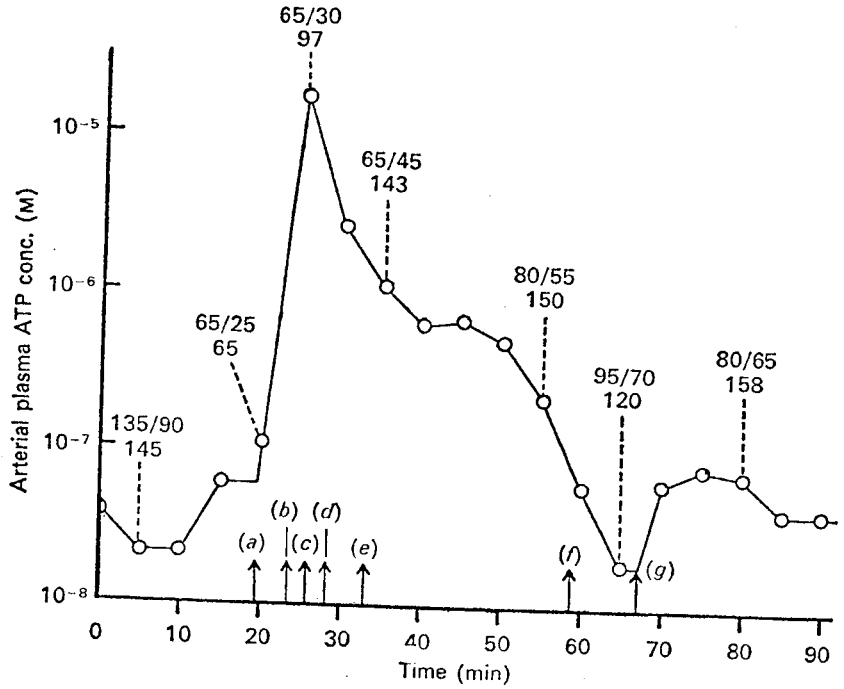

Fig. 1. Arterial plasma levels of ATP before, during and after infusions of ATP (0·167 M) into the pulmonary artery of a 17 kg baboon. Infusion (in ml./min. ↑) at (*a*) 1; (*b*) 0·5; (*c*) 0·25; (*d*) 0·125; (*e*) 0·0625; (*f*) stopped; (*g*) started at 0·0625. Interrupted lines indicate arterial B.P. (mmHg) and heart rate (beats/min).

Female baboons of 12–17 kg were sedated with ketamine HCl and anaesthesia was maintained with intravenous sodium pentobarbitone, 20 mg/hr. The tip of the infusion catheter lay approximately 1 cm beyond the pulmonary valve; the sampling catheter tip lay in the aortic arch. Both were inserted via the femoral vessels. ATP-Na, 0·167 M, was delivered by constant infusion at 0·06–1·0 ml./min.

Initial infusion of a high dose resulted in 10–20 % recovery in the arterial blood. This percentage was not substantially altered with subsequent infusions of lower doses (Fig. 1). The fall in arterial blood pressure was not accompanied by a corresponding increase in heart rate. Arterial levels were followed for 30 min without obviously harmful effects.

[P.T.O.

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Commentary - paper 17.

This manuscript represents the full account of experiments performed five years earlier in the laboratory of Dr. A.M. Harper. A few results were obtained at later intervals, but the essence of the work was described in the abstract of paper no. 12. The problems raised in this paper are of fundamental interest. How does ATP act beyond the blood-brain barrier? Do circulating nucleosides and nucleotides continuously modulate the metabolism of the brain tissue? In unpublished studies with Dr. Peter Corr, Department of Cardiology, Washington University Medical Centre, Saint Louis, the effect of intracarotid ATP in conscious dogs was observed. The uniform response was that the animal promptly became drowsy and in some cases sleep was induced. Levels of arterial blood pressure and heart rate were not significantly altered. No E.E.G. patterns were recorded but the sleep-provoking effect is a powerful one, since arterially cannulated conscious dogs in a noisy laboratory are usually difficult to control.

The question of central nervous synaptic transmission by nucleotides or substances affected by nucleotides must also be taken into consideration.

In any event, it became clear from this work and the studies by Pull & McIlwain (1972) that ATP and its derivatives must play an important role in the local control of the cerebral blood vessels.

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EFFECT OF ADENOSINE TRIPHOSPHATE AND SOME DERIVATIVES ON CEREBRAL BLOOD FLOW AND METABOLISM

By T. FORRESTER*, A. M. HARPER, E. T. MacKENZIE AND E. M. THOMSON†

From the Wellcome Surgical Research Institute, University of Glasgow, Garscube Estate, Bearsden Road, Glasgow G61 1QH

(*Received 21 November 1978*)

SUMMARY

1. Responses of cerebral blood vessels to peri- and intravascular doses of ATP (adenosine triphosphate) and some derivatives were studied in cat and baboon.

2. Perivascular application of ATP to cat pial arterioles gave a threshold dilatory effect at a concentration of 10^{-11} M. This figure is comparable to the amount of ATP calculated to be released from electrically stimulated brain slices.

3. It is concluded that adenine nucleotides have a major role to play in the local control of cerebral blood flow.

4. Intracarotid injection of ATP showed a calculated threshold effect at 4×10^{-8} M in the cat and 4×10^{-9} M in the baboon.

5. The threshold response of the vasculature to intracarotid adenosine lay between 4×10^{-7} M and 4×10^{-6} M in the baboon. Little effect was produced with AMP, pyrophosphate and inorganic phosphate.

6. Intracarotid ATP increased the oxygen consumption of the baboon brain parenchyma. This effect was attributed in part to an elevation of the cellular cyclic AMP levels.

7. Osmotic disruption of the blood–brain barrier in baboon did not affect the vasodilatory or metabolic effect of intracarotid ATP.

8. It is postulated that circulating purine compounds mediate a form of metabolic communication in the body. Also, release of purine compounds from active local nerves might influence cerebral blood flow.

INTRODUCTION

In 1890 Roy & Sherrington outlined two distinct mechanisms for the control of the cerebral circulation, one of them acting through the vasomotor nervous system, the other 'an intrinsic one by which the blood supply of various parts of the brain can be varied locally in accordance with local requirements'. Since that time evidence supporting the latter statement has steadily accumulated. For example, Cobb & Talbott (1927) found an increase in the vascularity of the olfactory bulb of rabbits

* Present address: Department of Physiology, St Louis University School of Medicine, 1402 South Grand Boulevard, St Louis, Missouri 63104, U.S.A.

† Present address: Hannah Research Institute, Ayr, Scotland KA6 5HL.

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when the nasal mucosa was stimulated with ammonia fumes. Many other examples of an increased blood supply occurring in an active region of the brain can be cited (Alexander & Révécz, 1912; Schmidt & Pierson, 1934; Gerard & Serota, 1936; Schmidt & Hendrix, 1937; Serota & Gerard, 1938; Penfield, von Santha & Cipriani, 1939). More recently, Ingvar & Risberg (1967) traced regional vasodilator responses in conscious man during mental activity. Roy & Sherrington concluded 'that the chemical products of cerebral metabolism contained in the lymph which bathes the walls of the arterioles of the brain can cause variation of the calibre of the cerebral blood vessels' and many such 'metabolites' have been implicated in the local control of cerebral vessel calibre, notably the hydrogen ion, carbon dioxide and oxygen (see reviews by Lassen, 1959; Sokoloff, 1959; Sokoloff & Kety, 1960).

Adenosine triphosphate (ATP) is well known as a powerful vasodilator in skeletal and myocardial muscle blood vessels (Folkow, 1949; Wolfe & Berne, 1956) and has recently been implicated in the local control of blood flow through these tissues (Boyd & Forrester, 1968; Forrester & Lind, 1969; Forrester, 1972; Forrester & Williams, 1977). Release of ATP and related compounds takes place from stimulated peripheral and central nervous tissue (Abood, Koketsu & Miyamoto, 1962; Pull & McIlwain, 1972) and it has been clearly demonstrated that this release is associated with the phase of membrane depolarization (Abood *et al.* 1962; Israel, Lesbats, Meunier & Stinnakre, 1976). Furthermore, there is the possibility that ATP and related nucleotides are released from nerves that supply the cerebral blood vessels themselves (Schubert & Kreutzberg, 1976; Burnstock, 1977). It therefore seemed pertinent to investigate the sensitivity of the cerebral vasculature to ATP and to ascertain the extent of its role as a local vasodilator of cerebral blood vessels.

Some of these results have been reported to the Physiological Society (Forrester, Harper & MacKenzie, 1975) and have been recently verified by Kozniewska, Trzebski & Zielinski (1976).

METHODS

Cat experiments

Nineteen cats of either sex weighing between 1·0 and 3·5 kg were used. Anaesthesia was induced with I.V. administration of alphaxalone and alphadalone mixture ('Saffan') given in a dose of 9 mg/kg. Anaesthesia was maintained with approximately 4 % fluothane in oxygen, and subsequently a 1 % chloralose solution infused IV in a dose of 6 ml/kg at 40 °C as necessary. The animals were intubated with an endotracheal tube and artificially ventilated with oxygen (Harvard Apparatus respiration pump). Stroke volume and pump rate were adjusted to obtain an end-inspired pCO_2 of 29 ($\pm$ 3·7) mmHg using a capnograph (Goddart). The pCO_2, pH and pO_2 of the arterial blood were monitored at intervals with a pH/blood gas analyser (Corning). Arterial blood pressure was continuously monitored (via a femoral artery catheter with a Statham gauge transducer. Body temperature was maintained between 35·5 and 37·5 °C with a heated blanket unit. An I.V. infusion of Hartmann's solution of composition (mM) Na^+ 131; Cl^- 111; K^+ 5; Ca^{2+}; HCO_3^- 29 at 0·1 ml./min was used to counteract fluid losses. The lingua artery was cannulated centripetally to provide the route for intra-arterial drug infusion. Drugs were infused at 0·2 ml./min.

Pial vessel calibre was measured using a modification of a technique developed by Baez (1966) and has been previously described in detail (Harper & MacKenzie, 1977*b*). The technique of perivascular application is one based on that developed by Wahl, Kuschinsky, Bosse & Thurau (1973) and has been previously described (Harper & MacKenzie, 1977*b*).

Baboon experiments

Twelve young, healthy baboons (*Papio anubis* and *P. cynocephalus*) were used. The mode of anaesthesia, measurement of cerebral blood flow, and oxygen consumption, and osmotic opening of the blood–brain barrier were carried out as described by Harper & MacKenzie (1977*a*). ATP and derivatives were infused at 0·2 ml./min into the internal carotid artery via a linguo-facial catheter. Mean arterial pressure was monitored through a catheter inserted into the abdominal aorta via the femoral artery. Another catheter lay in the inferior vana cava for administration of physiological saline if necessary. Artificial ventilation, adjusted if necessary, ensured that a normocapnic state was maintained. Body temperature was kept at approximately 37 °C using infra-red lamps. All test substances were made up in Krebs–Henseleit solution of composition (mM) NaCl 113; KCl 4·7; KH_2PO_4 1·2; $NaHCO_3$ 24·8; $CaCl_2$ 2·5; $MgCl_2$ 1·0 and glucose 1·0. The pH was 7·48. Drugs were obtained from Sigma Chemical Company.

RESULTS

Effect of ATP on pial vessels of cat

Infusion of a solution of ATP into the carotid artery of cats was followed within 10 sec by a profound increase in cerebral blood flow. Fig. 1 shows the relationship

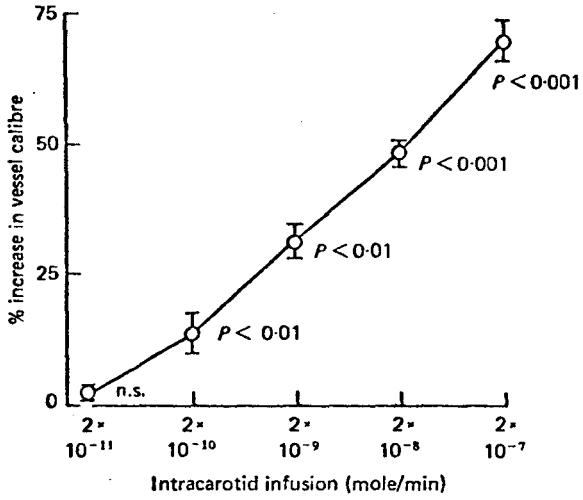

Fig. 1. Effect of intracarotid infusions of ATP on pial arteriolar calibre in eight cats (mean ± S.E.). *P* values obtained by comparing test effect with vessel calibre just before infusion. n.s., not significant.

obtained between the amounts of ATP infused and the maximum outside diameter produced in pial vessels ranging in calibre from 90–212 μm. Control infusions of Krebs solution did not affect the vascular diameter. The results were expressed as a percentage change since the reaction was found to be independent of the diameter over the range investigated. There was no change in end-tidal pCO_2 during the infusions, while the mean arterial blood pressure fell by no more than 10 mmHg. A significant increase in calibre ($0{\cdot}001 < P < 0{\cdot}01$) was produced with infusion of 2×10^{-10} mole/min. The concentration of ATP producing this vasodilatation is calculated as $4{\cdot}0 \times 10^{-8}$ M, assuming a common carotid blood flow of 5 ml./min. A response was seen in the vessels 3–4 sec after commencement of infusion. All responses

reached a maximum level within 30 sec and then the vessel constricted to a value above the control diameter. Fig. 2 shows the effect of three doses infused over a period of 3 min. In general the higher doses produced a more rapid time-to-peak effect and a longer time constant for the decay phase.

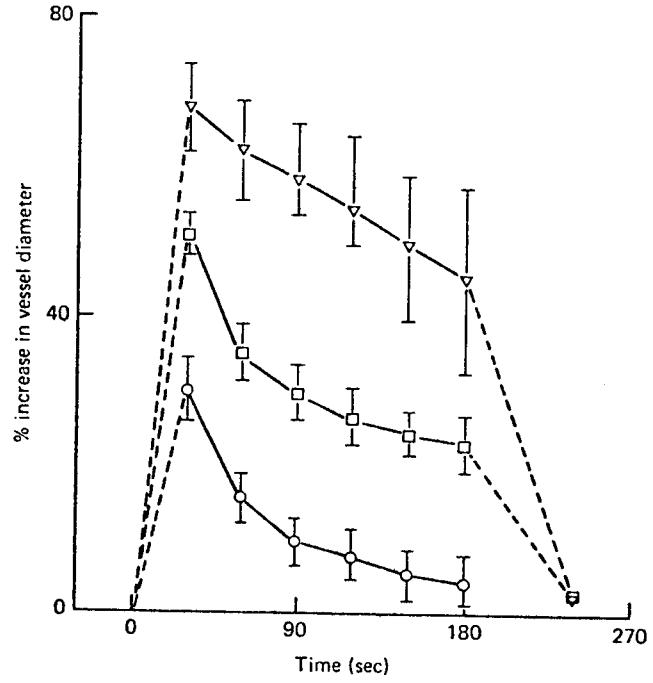

Fig. 2. Response of cat pial vessels to intracarotid infusions of ATP (mole/min), 2×10^{-7} (∇, $n = 9$, diameters 90–212 μm), 2×10^{-8} ($\square$, $n = 6$, diameters 95–212 μm) and 2×10^{-9} ($\bigcirc$, $n = 6$, diameters 112–212 μm) over a period of 3 min (0–180 sec on abscissa). Means ± S.E.

If ATP plays a role in linking the local control of blood flow to cellular metabolic demand the blood vessels should respond to the chemical when administered from the adventitial side. Thus the sensitivity of the pial vessels to perivascular application was tested. Fig. 3 gives a compilation of results obtained from eleven cats (seventy-three vessels tested) when doses of ATP were pipetted perivascularly. A peak effect is seen with 10^{-10} M solution. The threshold concentration lies between 10^{-12} and 10^{-11} M. It was interesting to note that with the higher concentrations of 10^{-4} and 10^{-3} M, there was an initial vasoconstriction; the cause of this effect has not yet been determined. The relative potency of peri- and intravascular ATP can be readily calculated, assuming a common carotid blood flow of 5 ml./min. The intra- and perivascular concentrations required to produce a 13% increase in pial diameter were 4×10^{-8} and 3×10^{-12} M respectively. Those amounts required for a 28% increase in diameter, the maximum achieved with perivascular application, were $2{\cdot}6 \times 10^{-7}$ M intravascularly and 10^{-10} M perivascularly (Figs. 1 and 3). It is clear that perivascular application is the more efficacious, and many factors obviously contribute to this difference (see Discussion, p. 350).

It was not possible to assess the actual cerebral blood flow using the diameter of pial vessels without information about changes also taking place at sites proximal and distal to the point of measurement (see Stromberg & Fox, 1972, for discussion). Application of Poiseuille's equation here is not valid, since the flow in these distensible vessels is pulsatile. Also, as vessel diameter decreases below 200 μm, the regional variation in blood viscosity becomes an important factor.

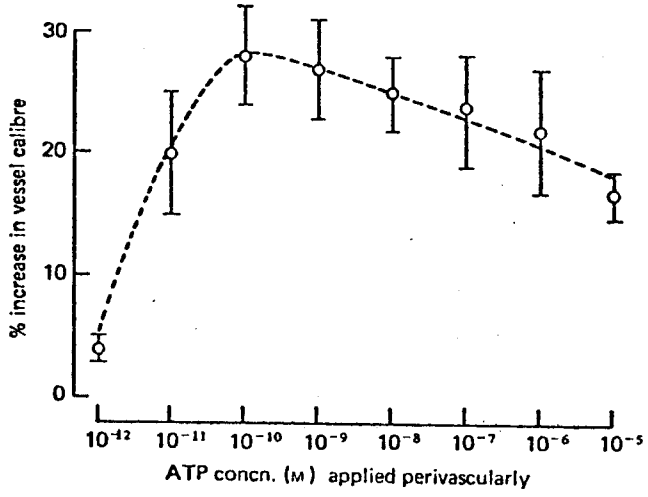

Fig. 3. Maximum response of cat pial vessels to doses of ATP applied perivascularly (mean ± S.E.). A 28 % increase in outside diameter was obtained with a concentration of 10^{-10} M. Line fitted by eye.

Effect of ATP on cerebral blood flow of baboon

The effect of intravascular ATP on the cerebral blood flow was assessed using the xenon clearance technique in baboon. The relationship between intracarotid doses of ATP and the increase in cerebral blood flows is shown in Fig. 4. A threshold response was obtained with 10^{-10} mole/min being infused. The threshold concentration is calculated as 4×10^{-9} M, assuming an average internal carotid blood flow of 25 ml./min. As with the cat, no allowance was made for degradation in the blood during the time of circulation between the tip of the infusion catheter and the cerebral vessels being scanned.

Comparison with other derivatives

The actions of pyrophosphate, adenosine and adenosine monophosphate were compared with that of ATP using the baboon preparation. Fig. 5 shows that the threshold response to adenosine was produced by infusion of 10^{-7} and 10^{-8} mole/min, i.e. between concentrations of 4×10^{-7} and 4×10^{-6} M. Little effect was seen with equimolar amounts of pyrophosphate. The basal values (twelve baboons) for cerebral blood flow, mean arterial pressure, Pa,CO_2 and $CMRO_2$ were $52{\cdot}5 \pm 5{\cdot}5$ ml./100 g min, $98{\cdot}3 \pm 5{\cdot}2$, $39{\cdot}7 \pm 0{\cdot}3$ and $2{\cdot}78 \pm 0{\cdot}25$ ml./100 g min respectively. In two animals inorganic phosphate and AMP were infused over the same range of concentrations; no significant effects were noted.

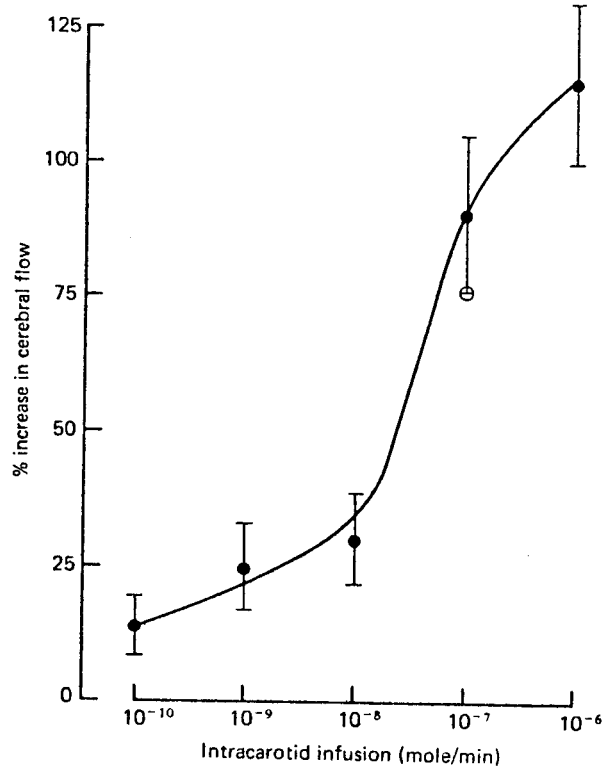

Fig. 4. Increase of baboon blood flow (●) in response to intracarotid ATP infused at 10^{-10} to 10^{-6} mole/min (mean ± S.E.). ○, response to 10^{-7} mole/min after disruption of the blood–brain barrier (see Table 1).

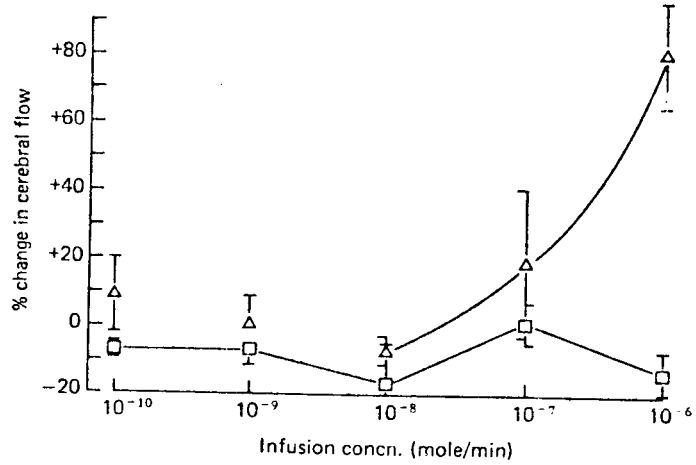

Fig. 5. Effects of intracarotid adenosine (△) and pyrophosphate (□) on baboon cerebral blood flow (mean ± S.E.).

Effect of ATP on cerebral metabolism

Arterial and cerebral venous oxygen saturations were measured during each estimation of cerebral blood flow. Table 1 shows that a relationship can be made between the actual blood flow produced by various concentrations of ATP infused and the cerebral oxygen consumption in the baboon. It was concluded that intracarotid ATP induced an increase in the oxygen consumption of the brain parenchyma.

TABLE 1. Relationship between oxygen consumption ($CMRO_2$) and cerebral blood flow (CBF) during ATP infusions into the carotid artery of baboons

ATP infusion (mole/min)	n	$CMRO_2$ (ml. ± S.E./ 100 g.min)	CBF (ml. ± S.E./ 100 g.min)
0	12	2·78 ± 0·25	52·5 ± 5·5
10^{-10}	3	3·01 ± 0·36	64·8 ± 17·4
10^{-9}	7	3·72 ± 0·34	72·3 ± 8·1
10^{-8}	7	3·26 ± 0·28	66·9 ± 10·1
10^{-7}	7	5·45 ± 0·72	104·9 ± 15·6
10^{-7}*	2	4·53 ± 1·03	86·1 ± 9·9
10^{-6}	1	4·11 ± 0·52	101·3 ± 8·4

* After osmotic disruption of the blood–brain barrier with urea.

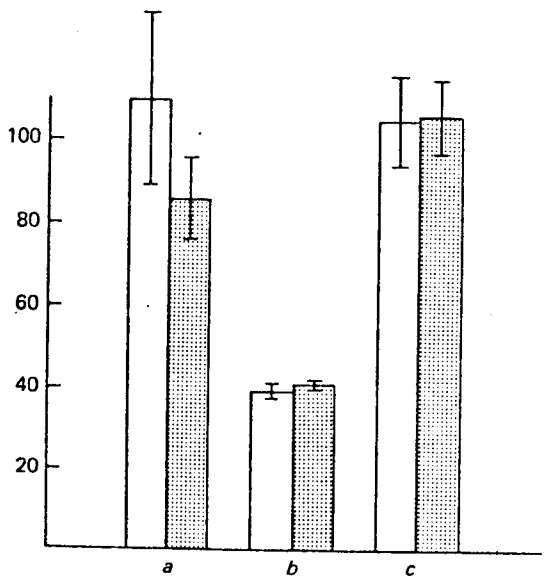

Fig. 6. The effect of intracarotid ATP (10^{-7} mole/min) on the cerebral blood flow (*a*, ml./100 g.min), *Pa* CO_2 (*b*, mmHg) and mean arterial pressure (*c*, mmHg) before (□) and after (▨) osmotic disruption of the blood–brain barrier. Mean ± S.E. plotted. No significant differences seen by paired Student *t* test. Ordinate units apply to the three parameters.

Effect of ATP after opening the blood–brain barrier

It was of interest to find whether osmotic disruption of the blood–brain barrier has any effect on the potency of intracarotid ATP. Previous studies have shown that osmotic disruption of the blood–brain barrier with intracarotid infusion of a 2 M solution of buffered urea opened the barrier to substances of widely differing molecular size, such as Evans Blue albumin, penicillin G, noradrenaline and 5-hydroxytryptamine. Cerebral blood flow, cerebral oxygen consumption and glucose uptake are unaffected 5 min after the administration of urea (Pickard, Durity, Welsh, Langfitt, Harper & MacKenzie, 1977; MacKenzie, McCulloch, O'Keane, Pickard & Harper, 1976). ATP, 10^{-7} mole/min, was infused via the intracarotid route and control measurements on cerebral blood flow, $PaCO_2$ and mean arterial pressure were made. 7–15 ml. urea, 2 M, was delivered through the intracarotid cannula and 5 min later ATP, 10^{-7} mole/min, was again infused. Fig. 6 shows the results obtained in seven baboons. There were no significant differences between the values obtained before and after osmotic disruption of the blood–brain barrier. Respiration and arterial blood pressure remained unaffected. Clearly the barrier, as represented by the vascular endothelial cells, does not modify the vasodilatory effects of intracarotid ATP. The value of 86 ± 10 ml./100 g. min is included in Fig. 6 to show that the effect of ATP on cerebral metabolism is probably little altered by opening the barrier.

Comparison with amounts released from brain tissue

Pull & McIlwain (1972) estimated the amounts of adenine nucleotide released from electrically stimulated brain slices *in vitro*. They calculated that the total adenine derivative released was 3–7 p-mole/g for a single electrical stimulus and suggested that much of the source could have been from released adenine nucleotide, probably ATP. They estimated that the extracellular volume associated with 1 g fresh weight of the neocortical tissue during incubation was 0·5 ml. Assuming that only a tenth of the adenine nucleotide was in the form of ATP, this would give a concentration of ~ 0·3 p-mole/0·5 ml. for each stimulus in a 1 g mass of cortical tissue. This value (6×10^{-10} M) can be compared to that concentration (10^{-10} M) which produced a 28 % increase in diameter when applied perivascularly to the cat pial vessel.

DISCUSSION

The results show that perivascular application of ATP to the cerebral resistance vessels in amounts equivalent to those released from activated nervous tissue causes marked vasodilatation. A major role for the adenine nucleotides in the local control of cerebral blood flow seems evident.

The great difference in concentration between peri- and intravascular routes to produce the same increase in vessel diameter is not surprising. It reflects a comparison between the adventitial and the vascular endothelial barrier; moreover, no allowance was made for any degradation or binding that might have occurred in the blood-stream in the 2–3 sec circulation time between the tip of the catheter and the site of

the pial vessel. The relative importance of other factors, such as the power of the ATP-ases, and the distribution of nucleotide receptors, remains to be elucidated.

The poor effects obtained with pyrophosphate and inorganic phosphate, previously noted by Kozniewska *et al.* (1976), seem to rule out the simple notion that the vasodilatation might be produced by these substances after cleavage from the adenosine moiety.

The results obtained with adenosine compare to those obtained earlier by Berne, Rubio & Curnish (1974). One interesting point is that although adenosine does not cross the blood–brain barrier in effective concentrations (Berne *et al.* 1974), nevertheless an effect for intracarotid adenosine was demonstrated in this work. The fact that ATP has a greater effect than adenosine when given intra-arterially may indicate the presence of a barrier-mediated nucleotide receptor or transport system.

The actions of intracarotid ATP on the cerebral vessels and brain parenchyma are of special interest and pose some fundamental questions. First, how can ATP traverse the endothelial lining of the cerebral blood vessels in order to reach the vascular smooth muscle? Recently Simionescu, Simionescu & Palade (1975) proposed a third type of transport through vascular endothelium in addition to filtration and diffusion. Their electron micrographs show that intra-endothelial vesicles exist, either singly or fused together. In some cases a complete channel was formed from the vesicles, traversing the whole breadth of the endothelial wall (Simionescu *et al.* 1975, fig. 7). If 'vesicular transport' also exists in the capillary endothelium of cerebral vessels then it is not hard to imagine that ATP might be transported through the endothelial cell as an intact molecule.

Other questions concern the action of ATP on the vascular smooth muscle to produce dilatation and also its effect on the brain tissue to raise the oxygen consumption. The fact that these actions are produced by concentrations of exogenous ATP so much lower than those found on the other side of the cell membrane would indicate that the membrane itself is intimately involved. A mechanism of action common to both vasodilatory and metabolic effects can be cited involving the interaction of exogenous ATP with membrane adenyl cyclase. Relaxation of vascular smooth muscle, produced in various ways, has been closely associated with an increase of cyclic 3′5′-adenosine monophosphate (Kukovetz, Poch & Holzmann, 1971; Kukovetz & Poch, 1972; Triner, Vulliemoz, Verosky, Habif & Nahas, 1972; Andersson, 1973). Relaxation of smooth muscle has also been associated with inhibition of phosphodiesterase, allowing cyclic AMP levels to accumulate (Lugnier, Bertrand & Stoclet, 1972). Sattin & Rall (1970, fig. 1) found that incubation of a brain slice for 5 min with exogenous ATP, 0·02 mM, increased the amount of cyclic AMP by over ten times. If exogenous ATP has the same effect on smooth muscle cyclic AMP, then it may exert its vasodilatory action via the adenyl cyclase system within the cell membrane. The action of exogenous ATP on brain cell metabolism could also be explained in the same way, since it is probable that accumulation of cyclic AMP in the brain cell will result in an increased oxygen consumption, both by the increased action of adenyl cyclase and the effect of cyclic AMP on other systems within the cell (Strubelt, 1968; Robison, Butcher & Sutherland, 1971). It must be remembered, however, that the exact role of cyclic AMP in this response has not yet been defined (Robison *et al.* 1971).

Exogenous ATP could interact with the adenyl cyclase system in several ways. One likely possibility would presume the existence of a specific receptor for ATP which might directly influence the activity of membrane adenyl cyclase. Evidence for brain receptors specific for exogenous adenine ribose monomers has already been provided (Sattin & Rall, 1970). The effect of these compounds was blocked competitively by methylxanthines despite the fact that these substances would be expected to cause a rise in cyclic AMP levels through the inhibition of phosphodiesterase (Hynie, Krishna & Brodie, 1966). Evidence for nucleotide receptors in other tissues is readily available. Cusack & Born (1977) have shown two types of receptor on the membrane of blood platelets, one for adenosine and one for ADP. Burnstock (1978), reviewing data from many sources, has proposed two types of purine receptor, one for adenosine and one for ATP (P_1 and P_2). An ATP receptor in the labellar sensilla of the tsetse fly has been characterized (Galun & Margalit, 1970; Mitchell, 1976) and is thought to be the sensor for feeding stimulation by adenine nucleotides (Galun & Margalit, 1969). Sattin & Rall (1970) suggested that ATP might be converted to adenosine close to the membrane, the adenosine then entering the cell and becoming phosphorylated to ADP with subsequent effects on the mitochochondrion. This view was supported by their finding that adenosine applied to brain slices has the same effect on the cyclic AMP levels as ATP. It is interesting to note that theophylline had no effect on the entry of adenosine into the cells, but did severely reduce the effect of adenosine on the accumulation of cyclic AMP, perhaps supporting the receptor theory. One other possibility must be considered. ATP could pass directly through the cell membrane and become part of the intracellular ATP 'pool' used for the adenyl cyclase reaction (Kakiuchi, Rall & McIlwain, 1969). Although this seems unlikely, nevertheless there is evidence to show that ATP can be taken up intact by skeletal muscle cells (Chaudry & Gould, 1970) and leucocytes (Wilkinson & Robinson, 1974). If intracellular dephosphorylation to ADP then took place, this in turn would stimulate mitochondrial activity and hence increase oxygen consumption.

Release of ATP from nerves supplying cerebral blood vessels. It has recently been demonstrated that substances synthesized in the nerve body can be transported into the dendrites and to the dendritic membranes, from where they can be released into the extracellular space (Schubert & Kreutzberg, 1976). Many substances could modulate the vascular function via this pathway and adenosine has been cited in this respect (Schubert & Kreutzberg, 1975, 1976). The extreme sensitivity of the pial vessels to perivascular application of ATP gives support to the concept of local release of nucleotides from neurons supplying the cerebral vessels (Schubert & Kreutzberg, 1976; Burnstock, 1977).

Possible effect of muscular exercise on cerebral blood flow and metabolism

ATP has been demonstrated in the venous effluent from exercising skeletal muscle (Forrester & Lind, 1969) and nucleotides have been detected in the circulation as long as 5 min after whole body exercise (Parkinson, 1973). The present work has demonstrated that circulating ATP can increase the metabolism and blood supply of cortical cells in the brain, probably through the effect of increasing intracellular

levels of cyclic AMP. The question then arises: can circulating nucleotides and derivatives released from active skeletal muscle achieve levels in the arterial blood significant enough to affect cerebral metabolism? Is there some substance to the aphorism *mens sana in corpore sano*? It remains to be determined what proportion of derivatives, or the nucleotides themselves, can survive passageway through the lungs. Other effects of circulating nucleotides have been well documented (see Discussion, Forrester, 1972, 1978) and recently the uptake and supply of purine compounds by the liver via the portal circulation has been outlined (Pritchard, O'Connor, Oliver & Berlin, 1975). These findings, together with the widely demonstrated presence of extracellular ATPase activity prompt the suggestion that there exists a system of 'metabolic communication' in the body mediated by circulating purine compounds.

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Commentary - paper 18.

This paper is one of a series of invited lectures given under the auspices of the Gif Lectures in Neurobiology. The series was published in the Journal of Physiology (Paris) under the collective title 'Nucleotides and Neurotransmission.' For the first time it drew together the development of ideas on extracellular nucleotides and the many various roles that may be played. The elegant work of Maurice Israel and his colleagues was the stimulus for such a meeting. Their clear demonstration of the post-synaptic release of ATP as a result of membrane depolarization, supported the previous observations that ATP was released from electrically excitable tissue.

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Gif lectures in Neurobiology
Nucleotides and Neurotransmission

Extracellular nucleotides in exercise : possible effect on brain metabolism*

T. FORRESTER

Department of Physiology, St. Louis University Medical School, St. Louis, Missouri, 63104, U.S.A.

SUMMARY:

ATP and other nucleotide derivatives have potent extracellular effects. Sensitive and rapid techniques of ATP detection have shown that ATP is (1) released from active skeletal muscle in vasodilator quantities and (2) released from isolated heart cells in response to hypoxia. Release of nucleotides from active brain tissue has also been demonstrated (Pull and McIlwain, 1972). It is calculated that active cerebral tissue releases sufficient ATP to satisfy local vasodilator requirements in the brain. Intracarotid infusions of ATP have the effect of stimulating oxygen uptake in brain tissue and profoundly increasing the cerebral blood flow. It is not understood how the ATP can exert these effects beyond the blood : brain barrier. Since exercising skeletal muscle releases ATP and other nucleotide derivatives into the circulation, it is postulated that the metabolism of the brain can be significantly affected in exercise. These data, together with the studies by Pritchard *et al.* (1975) on uptake and supply of purine compounds by the liver, prompt the suggestion that there exists a system of « metabolic communication » in the body mediated by circulating purine compounds.

Key-words : Adenosine triphosphate. Circulating nucleotides. Skeletal muscle blood flow. Cerebral blood flow. Myocardial blood flow. Brain metabolism.

INTRODUCTION

The significance of intracellular adenosine triphosphate (ATP) as an intracellular energy transfer substance is well recognized. However it is only recently that ATP and other nucleotide derivatives have come to be regarded as having a significant physiological role to play extracellularly. As long ago as 1929 DRURY and SZENT-GYORGYI demonstrated the great potency of ATP when applied exogenously to the heart and blood vessels. The vasodilator effect on skeletal and cardiac vessels was particularly impressive. These effects were verified (GADDUM and HOLTZ, 1933 ; GILLESPIE, 1934 ; MCDOWALL, 1944 ; BIELSCHOWSKY *et al.*, 1946 ; EMMELIN and FELDBERG, 1948 ; DAVIES *et al.*, 1951). In 1950 GREEN and STONER gave a clear account of the similarities between the shock produced by intravenous ATP infusions and ischaemic shock. They assembled much experimental evidence to show that the adenine nucleotides, principally ATP, might be closely involved with the production of ischaemic shock. They commented « that the problem was hindered by the lack of definition in the chemical methods available for estimating small changes in the body nucleotides ».

Little was published about the extracellular actions of adenine nucleotides from then until Pamela HOLTON (1959) demonstrated the presence of ATP in the effluent from rabbit ear associated with sensory nerve stimulation. Prior to this any physiological role for extracellular ATP had been regarded as highly unlikely, the general assumption being that an anion of such charge could not possibly pass across the cell membrane. With the development of more sensitive and rapid methods of ATP detection these longstanding and fundamental questions could once again be examined.

The following is a condensed account of the various pathways taken by this investigator in an effort to follow up a serendipitous ** finding and to gauge its full significance.

RELEASE OF ATP FROM ACTIVE SKELETAL MUSCLE.

Release of adenine nucleotides from active frog nerve and muscle *in vitro* was first noted by ABOOD *et al.* (1962) using indirect labelling methods. A direct bioassay system for ATP arose serendipitously * during a search for acetylcholine released from motor nerve terminals (BOYD and FORRESTER, 1968). When a solution bathing an indirectly stimulated frog sartorius muscle was perfused through a frog heart, a positive inotropic effect was produced. Fig. 1 shows the effect of such a solution on an isolated perfused frog heart beating against a constant peripheral resistance. There

* Reçu en première lecture le 24 mars 1978, en deuxième lecture le 2 mai 1978.

** Reviewer's note : Serendipitous finding = unexpected finding with interesting implications.

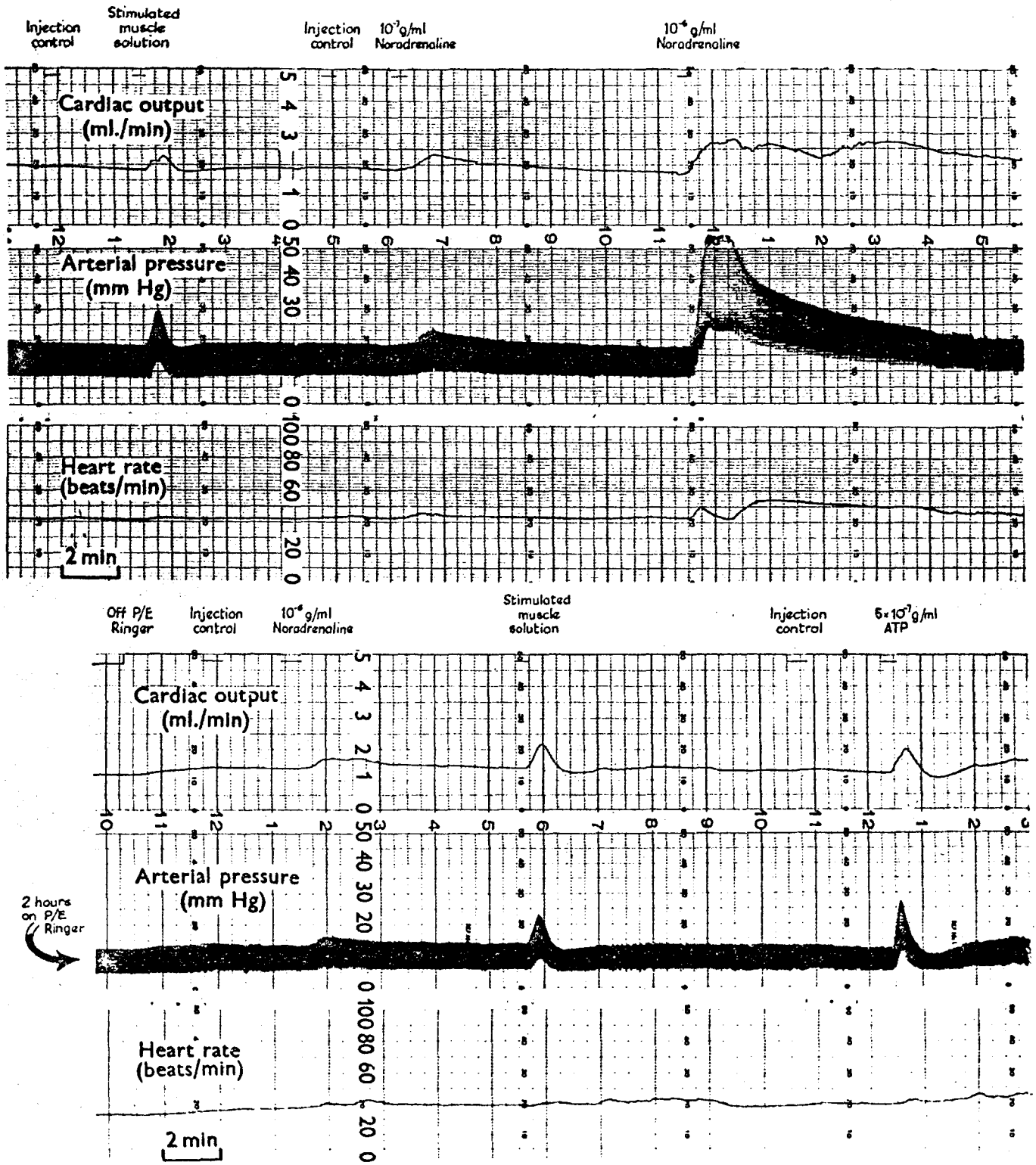

FIG. 1. — *Response of a perfused frog heart to a stimulated muscle solution and solutions of noradrenaline and ATP before and after adrenergic blockade.*

Lower trace continues directly from upper trace (arrow). Heart was perfused for two hours with a Ringer's solution containing pronethalol hydrochloride, 10^{-6}g/ml, and ergotamine tartrate, 10^{-6}g/ml. Adrenergic blockade was sufficient to block the effect of noradrenaline almost completely. The action of the stimulated muscle solution (and ATP) remained unaffected. It may be postulated that the ATP contained in this muscle solution was acting "beyond" the adrenergic receptor.

is an obvious similarity between the effect of the test solution and pure ATP. Adrenergic blockade of the heart with pronethalol and ergotamine did not affect the stimulatory effect of the muscle solution, thus ruling out the possibility that the stimulatory substance was a catecholamine. Further tests of identification were employed. It was found that the stimulatory substance could be eluted into one fraction using molecular sieve chromatography (Sephadex G-25). An Rf value similar to that of ATP was obtained. This procedure involved testing the eluted fractions from the column on the very sensitive frog heart perfusion system. A concentration of as low as 10^{-9} M could be detected. The molecular sieving also removed the protein fraction, thus making the solution available for spectrophotometric analysis. Maximum absorption of light occurred at 265 nm (pH 7.1), probably indicating the presence of substances containing a purine ring. Incubation of the test solution with the enzyme apyrase markedly reduced the stimulatory effect on the frog heart. Since apyrase catalyses the breakdown of nucleotide triphosphates to monophosphates, and monophosphates do not stimulate the frog heart, this provided strong evidence that the stimulatory substance contained a « high energy » phosphate chain. Finally, the test solution caused light to be emitted from firefly lantern extract, the pattern of light emission being similar to that produced by pure ATP. It was concluded that ATP, probably along with other nucleotides, was released from contracting skeletal muscle *in vitro*. At the time it seemed significant that no ATP could be detected when the muscles were stimulated indirectly, but curarized. In retrospect this did not indicate that no ATP came from active nerve terminals, but simply that the amounts released from that particular source did not achieve detectable levels in the bathing solution.

A justifiable criticism of this work was the possibility that the ATP had as its source muscle fibres damaged during dissection. It had been noted, however, that the potassium levels in the solutions bathing the inactive muscle remained at control levels. Nevertheless it was decided to use a frog muscle preparation in which the hindlimb musculature is left intact and perfused through the abdominal aorta. This preparation was first developed by DALE and his colleagues during their search for acetylcholine released from motor nerve terminals. We have clearly demonstrated the presence of ATP in the perfusate from indirectly stimulated hindlimb musculature, applying the tests for identification previously described (FORRESTER and HASSAN, 1973).

At this juncture it was realized that ATP could have a role to play in the hyperaemic response seen in skeletal muscle during exercise, since ATP is well known as a powerful vasodilator. Thus it was decided to apply these techniques of detection and identification to human peripheral blood despite the fact that human plasma contains enzymes capable of rapidly degrading ATP. When suitably diluted human plasma was perfused through a frog heart the same stimulatory response was seen, typical of ATP. The amount of ATP in plasma from the venous blood of resting subjects (antecubital vein sample) ranged from 0.19-0.95 μg/ml (0.37-1.9 nmoles/ml). Approximately half of this amount was attributed to platelet damage. Simultaneous arterial and venous samples from four subjects at rest had mean concentrations of 0.19 μg/ml (0.37 nmoles/ml) and 0.70 μg/ml (1.3 nmoles/ml) respectively, indicating that the ATP was added to the blood on its way through the skeletal muscle bed. The concentration of

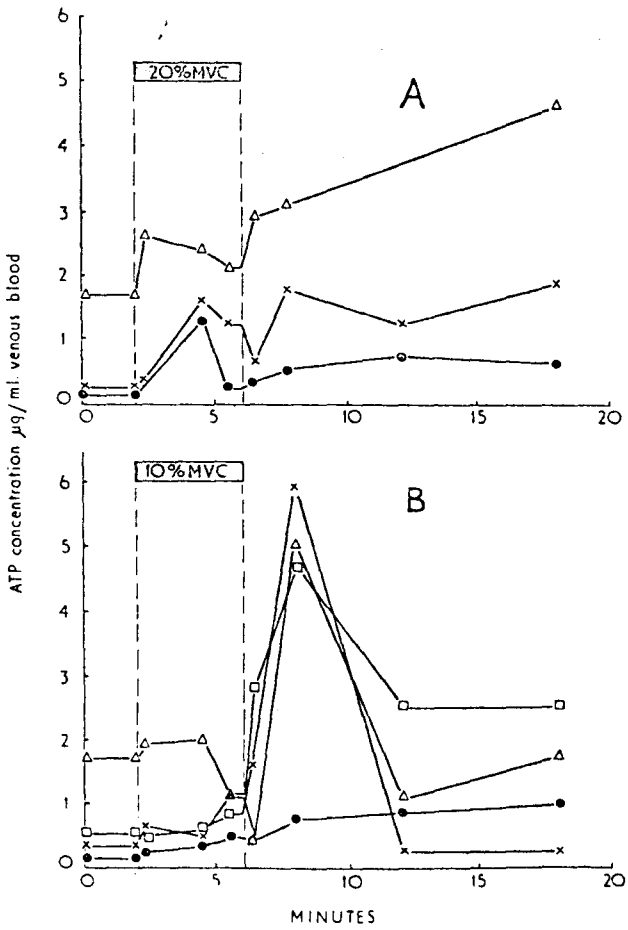

FIG. 2. — *The ATP concentration in venous effluent before, during and after 20 % (A) and 10 % (B) maximum voluntary contractions (MVC).*

Different symbols represent results obtained from different subjects. Rectangles indicate duration of isometric exercise. Note the slow rise in concentration of ATP during the post-contraction phase in the 20 % MVC (tension resulting eventually in fatigue) in contrast to the sharp rise and fall obtained in the non-fatiguing 10 % MVC. (FORRESTER and LIND, 1969).

ATP in the venous effluent from human forearm muscles performing a controlled amount of isometric exercise was measured (FORRESTER and LIND, 1969). Fig. 2 shows the results obtained from subjects performing 10 % and 20 % of their maximum voluntary contraction (MVC). Note that with the 20 % MVC the concentrations rise steadily after the end of the contraction period, presumably indicating that as the post-exercise hyperaemia diminished, the concentration will steadily rise if the musculature adds ATP to the venous effluent after contraction has ceased. With the 10 % MVC a peak of concentration is evident, when post-exercise hyperaemia was naturally not so great. It was established that the arterial levels remained low during and after the isometric exercise (FORRESTER and LIND, 1969, Fig. 8). These results were soon substantiated by PARKINSON (1973) who noted rises in blood levels of ATP, ADP and AMP occurring as long as five minutes after whole body exercise.

These results naturally led to the enquiry : is ATP released in sufficient quantities to satisfy the vasodilator requirements of active skeletal muscle ? Previous studies on the effect of infused ATP on the circulation through human forearm musculature (DUFF *et al.*, 1954) provided an opportunity to quantify the amounts of ATP released *in vivo*. The main difficulty with a quantitative study of this sort is the fact that an increase in blood flow through exercising skeletal muscle has the effect of washing out and diluting the ATP to an unknown extent. One way round this difficulty is simply to occlude the arterial supply to the muscles during the period of exercise, and then gradually restore the flow which will then pass through the line of least resistance, *i.e.* the dilated vessels in the skeletal muscle bed. Human subjects gripped a bar isometrically at 5 % MVC in the presence of arterial (and venous) occlusion. This exercise is a mild one, and normally gives rise to an exercise hyperaemia of only three times the resting flow (LIND and MCNICOL, 1967). DUFF *et al.* (1954) showed that a threefold increase in forearm flow could be produced by infusing ATP, 16 µg/min (30 nmoles/min), into the brachial artery. Fig. 3 shows the results obtained in six experiments on four subjects. It is important to note the logarithmic ordinate scale, indicating a 10-100 fold increase in the concentration at the end of the four minute exercise period. These concentrations represent only a small fraction of the ATP originally present because the rapid degradation in the bloodstream by plasma and blood elements could not be avoided. This factor would certainly diminish the concentrations of ATP infused by DUFF *et al.* (1954). A computation of the loss occurring was made and it was calculated that 7.5-10.5 µg/min (14-20 nmole/min) were released (FORRESTER, 1972). These amounts compare well with the 16 µg/min infused by DUFF *et al.* to produce the same threefold increase in flow.

RELEASE OF ATP FROM MYOCARDIAL CELLS IN RESPONSE TO HYPOXIA.

The findings with skeletal muscle *in vivo* encouraged further investigation using myocardial cells. The search for mechanisms of coronary vasodilatation has been going on for many years and is of obvious importance in view of the great morbidity associated with myocardial ischaemia. Many substances have been implicated in the search for « the vasodilator » (see review by BERNE, 1964) but because of potency and/or lack of detection, none so far have been found satisfactory. The adenosine hypothesis put forward by BERNE (1963) seems to be unsatisfactory on the grounds

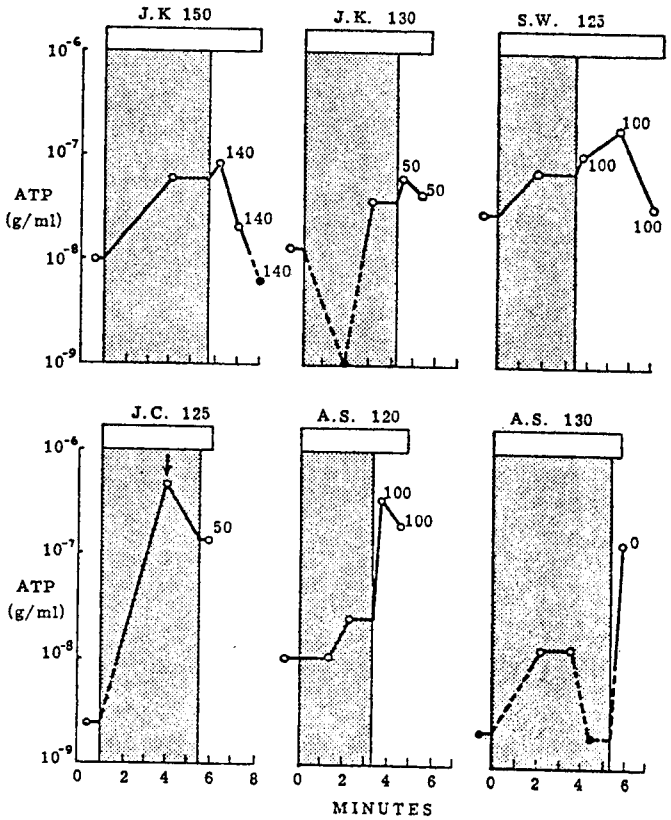

FIG. 3. — *Concentrations of ATP in human venous plasma before, during and after arterial occlusion + exercise (5 % MVC).*

Clear rectangles, duration of exercise ; shaded areas, time of arterial occlusion. Resting systolic blood pressure given above each figure. Filled symbols, samples without decay in light signal and therefore below assay threshold (dashed lines). Vertical arrow in *d* indicates time of brief reduction in cuff pressure in order to obtain a blood sample. Figures above post-occlusion samples show cuff pressure when sample was taken. (FORRESTER, 1972.)

that aminophylline blocks the dilatory action of adenosine but not that of hypoxia (AFONSO *et al.*, 1972). An increase in the levels of ATP in the coronary sinus effluent of perfused guinea-pig hearts has been observed whenever the myocardium was made hypoxic (PADDLE and BURNSTOCK, 1974) but it was not certain whether the ATP came from nerves, vascular smooth muscle or myocardium. The use of adult rat heart cells isolated according to a method devised by VAHOUNY *et al.* (1970), modified by C.A. WILLIAMS (1977), avoided many problems associated with whole heart preparations. The high ratio of surrounding fluid volume to cellular volume provides excellent conditions for oxygenation. No alteration in surrounding fluid volume occurs, thus avoiding the difficulty of having to estimate ATP concentrations in fluid coming from a vascular bed which is continuously dilating in response to hypoxia. The actual estimation of ATP remains free from both contaminating elements such as the blood platelets, and degrading elements, the blood ATPases. It was found that ATP, 0.34 μM/mg protein, was released from oxygenated cells, while 1.28 μM/mg protein was released within 30 seconds of rendering the cells hypoxic (FORRESTER and WILLIAMS, 1977). Fig. 4 shows the compiled results of the data. When hypoxic cells were restored to the oxygenated state the levels of ATP reverted to the previous low levels. The amounts of ATP released were calculated to fall well within the range necessary for production of maximum vasodilatation of the coronary vascular bed. Continuous release of ATP from these cells supports the possibility that the tone of coronary vessels is a function of this release. It is of interest at this point to note that GILES and WILCKEN (1977), working with dog heart, have shown that the response of the coronary blood flow to ATP was not changed by aminophylline.

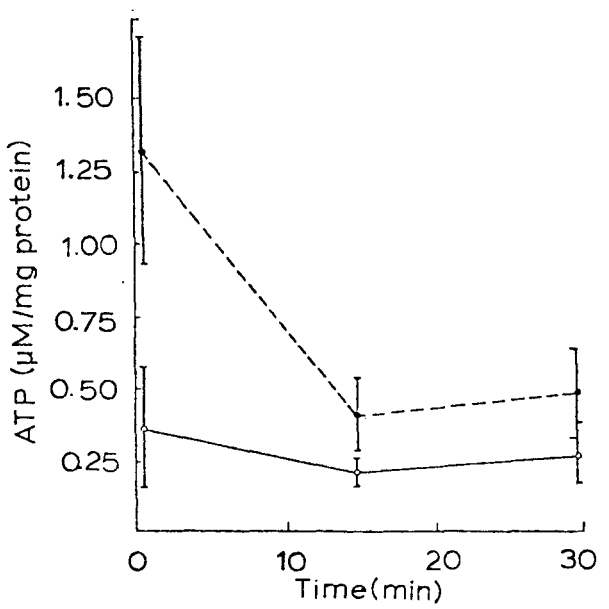

FIG. 4. — *Amounts of ATP present in the medium for isolated heart cells in oxygenated (○) or nitrogen-equilibrated (●) buffer solution at 37 °C.*

Difference between points at 1 min of incubation is highly significant ($0.001 < P < 0.01$). Cell population was divided in half such that each preparation was its own control. Each point represents the average from six determinations. Bars indicate ± 1 S.D. of the observation. (FORRESTER and WILLIAMS, 1977.)

EFFECT OF ATP ON CEREBRAL BLOOD FLOW AND METABOLISM.

The nature of local control of blood flow in the brain has also been a longstanding problem. In 1890 ROY and SHERRINGTON described an intrinsic control mechanism « by which the blood supply of various parts of the brain can be varied locally in accordance with local requirements ». In view of the probable major role that ATP plays in the control of local blood flow in skeletal and cardiac muscle, it seemed pertinent to explore its effects on the cerebral vasculature. It had already been shown by PULL and MC ILWAIN (1972) that adenine nucleotides were released from electrically stimulated brain slices. They calculated the amounts released per single stimulus and suggested that a large proportion of the nucleotide material may have been in the form of ATP.

The effects of ATP, AMP and adenosine on the cerebral blood vessels of cat and baboon were studied. Perivascular application of ATP to cat pial arterioles caused dilatation at a threshold concentration of 10^{-11}M. PULL and MCILWAIN calculated that the total adenine nucleotides released from stimulated brain slices lay between 3-7 pmole/g for a single stimulus. Assuming that only one tenth of this was ATP and taking the extracellular volume of the brain slice as 0.5 ml/g, a concentration of around 0.3 pmole/0.5 ml, or 6×10^{-10}M, can be computed. This value compares favourably with the threshold value of 10^{-11}M causing dilatation when applied perivascularly. It is thus concluded that ATP is released in amounts that are more than adequate to produce maximum local vasodilatation.

Intracarotid administration of ATP produced a surprising result in both cat and baboon. In both cases the cerebral blood flow was approximately doubled when ATP was infused into the carotid artery at a rate of 10^{-6}mole/min. Taking into account the carotid blood flow in each case, the calculated threshold concentration in cats was 4×10^{-8}M and in baboons 4×10^{-9}M. The threshold response to adenosine in the baboon was 4×10^{-7}M. No significant effects were seen with AMP, pyrophosphate or inorganic phosphate. An interesting finding was the relationship seen between

TABLE I. — *Cerebral blood flow and oxygen consumption during intracarotid ATP infusions in five baboons (means ± S.E.).*
(From FORRESTER *et al.*, 1975.)

	Base line	ATP (moles/min)			
		10^{-9}	10^{-8}	10^{-7}	10^{-6}
CBF (ml/100. min)	49 ± 11	66 ± 11	71 ± 13	94 ± 24	116 ± 10
$CMRO_2$ (ml. O_2/100 g. min)	2.9 ± 0.3	3.2 ± 0.5	3.4 ± 0.4	4.3 ± 0.7	4.5 ± 0.5

intravascular ATP and the oxygen consumption in the baboon experiments. Table I shows that the oxygen consumption was raised by about one and a half times when 10^{-6} mole/min ATP was infused into the carotid artery of baboon.

CONCLUSIONS

It now seems that active skeletal muscle, cardiac muscle and brain tissue release significantly high concentrations of ATP into the extracellular space to affect profoundly the local blood flow. A residual amount of ATP and other nucleotides is most likely to reach the general circulation and indeed nucleotides have been detected in the human circulation as long 5 min after whole body exercise (PARKINSON, 1973). The question then arises : can circulating nucleotides and derivatives released from active skeletal muscle achieve levels in the arterial blood sufficient to affect cerebral metabolism ? Is there some substance to the adage « *mens sana in corpore sano* » ? It remains to be determined what proportion of derivatives, or the nucleotides themselves, can survive passage through the lungs. Some preliminary data indicates that if ATP is infused in high enough quantities into the pulmonary artery of baboon micromolar levels can be sustained in the arterial circulation without obviously compromising the general circulatory welfare. Other effects of circulating nucleotides are well documented and recently the uptake and supply of purine compounds by the liver has been emphasized (PRITCHARD *et al.*, 1975). These findings prompt the suggestion that there exists a system of « metabolic communication » in the body mediated by circulating purine compounds.

ACKNOWLEDGMENTS :

It is a pleasure to thank Professor I.A. BOYD, Professor A.R. LIND, Dr. M.O. HASSAN, Dr. C.A. WILLIAMS, Dr. R.G. EVANS and Dr. Hiltrud MUELLER for their collaboration and support without which much of this work would not have been possible.
The cerebral blood flow studies were performed at the Wellcome Surgical Research Institute, University of Glasgow in collaboration with Dr. Eric MACKENZIE, Dr. Murray HARPER and Mr. Euan THOMSON. I am grateful to them and the technical assistance provised there for the progress in these blood flow studies in brain.

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Commentary - paper 19.

The American Physiological Society sponsor 'Tutorial Lectures' which are delivered to the Society at each Fall Meeting in order to make available the most up-to-date material in the various fields of physiology. This paper is the result of such a lecture and is more or less the same account as given in paper 18. Additional comments appear in the last section - 'Implications for the Future.'

EXTRACELLULAR NUCLEOTIDES IN EXERCISE: POSSIBLE EFFECT ON BRAIN METABOLISM*

TOM FORRESTER
Department of Physiology
St. Louis University Medical Center
St. Louis, MO 63104

Introduction

The roles that intracellular nucleotides play in cellular metabolism are well understood and formidable metabolic pathways controlled by nucleotides have been worked out in great detail. However the actions of nucleotides *extracellularly* have only recently come into prominence with the realization that these substances can exist, and have highly potent actions, outside the cell.

As long ago as 1929 Drury & Szent-Gyorgyi published a paper in *The Journal of Physiology* describing the action of adenosine triphosphate and derivatives when applied externally to the heart and blood vessels. They emphasized the great sensitivity of these tissues to the adenyl compounds. This might have prompted the immediate conclusion that there must exist nucleotide *receptors* to respond to the agonist nucleotide, a concept that has only now been put forward by Sattin & Rall (1970), Burnstock (1978) and others nearly fifty years later. Since then widespread actions of the nucleotides on many tissues have been described. The urgencies of World War II brought forward a significant report which largely summarized the extracellular actions of the nucleotides, particularly ATP, in the body organs. Much of this work is described by Green & Stoner (1950) who showed that circulating ATP could be responsible for the *wound shock* seen in battle casualties. The peripheral blood vessels seemed to dilate greatly and in some cases irreversibly when a large mass of tissue was destroyed. At this time Green & Stoner commented "that the problem was hindered by the lack of definition in the chemical methods available for estimating small changes in the body nucleotides." Little was published on extracellular activity of these substances until Pamela Holton (1959) demonstrated the presence of ATP in the effluent from rabbit ear artery associated with sensory nerve stimulation. Prior to this any physiological role for extracellular ATP had been regarded as highly unlikely, the general assumption being that an anion of such charge could not possibly pass across the cell membrane. However, with the development of more sensitive and rapid methods of ATP detection many studies have now been performed since then and many species have been used. A typical result is shown in Fig. 1.

This review will describe experiments demonstrating the release of ATP from skeletal muscle, cardiac muscle and active brain tissue. The effects of exogenously applied ATP to brain tissue will be discussed in relation to whole body exercise.

Release of ATP from active skeletal muscle.

Release of adenine nucleotides from active frog nerve and muscle *in vitro* was first noted by Abood, Koketsu & Miyamoto (1962) using indirect labelling methods. A direct bioassay system for ATP arose unexpectedly during a search for acetylcholine released from motor nerve terminals (Boyd & Forrester, 1968). When a solution bathing an indirectly stimulated frog sartorius muscle was perfused through a frog heart, a positive inotropic effect was produced. Fig. 2 shows the effect of such a solution on an isolated perfused frog heart beating against a constant peripheral resistance. There is an obvious similarity between the effect of the test solution and pure ATP. Adrenergic blockade of the heart with pronethalol and ergotamine did not affect the stimulatory effect of the muscle solution, thus ruling out the possibility that the stimulatory effect was produced by a catecholamine. Further tests of identification were employed. It was found that the stimulatory substance could be eluted into one fraction using molecular sieve chromatography (Sephadex G-25). An R_f value similar to that of ATP was obtained. This procedure involved testing the eluted fractions from the column on the very sensitive frog heart perfusion system. A concentration of as low as 10^{-9}M could be detected. The molecular sieving also removed the protein fraction, thus making the solution available for spectrphotometric analysis. Maximum absorption of light occurred at 265 nm (ph 7.1), probably indicating the presence of substances containing a purine ring. Incubation of the test solution with enzyme *apyrase* markedly reduced the stimulatory effect on the frog heart. Since apyrase catalyses the breakdown of nucleotide triphosphate to monophosphate, and monophosphate does not stimulate the frog heart, this provided strong evidence that the stimulatory substance contained a 'high energy' phosphate chain.

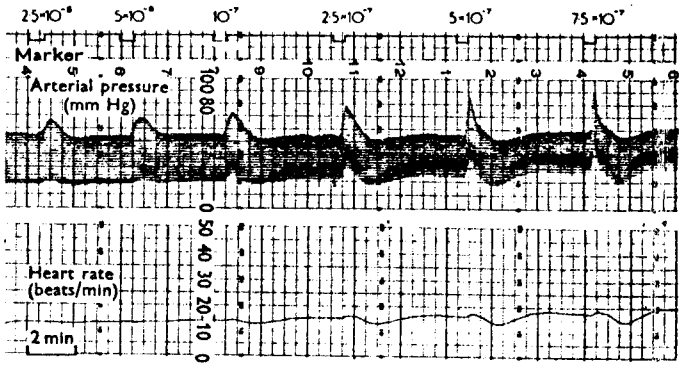


Fig. 1. The effect of graded concentrations of ATP solutions (g/ml) when perfused through a frog heart beating against a fixed peripheral resistance. There is a positive inotropic effect and a late increase in heart rate with the higher concentrations. (Boyd and Forrester, 1968, *J. Physiol, London).*

Finally, the test solution caused light to be emitted from firefly lantern extract, the pattern of light emission being similar to that produced by pure ATP. It was concluded that ATP, probably along with other nucleotides, was released from contracting skeletal muscle *in vitro*. At the time it seemed significant that no ATP could be detected when the muscles were stimulated indirectly, but curarized. In retrospect this did not indicate that *no* ATP came from active nerve terminals, but simply that the amounts released from that particular source did not achieve detectable levels in the bathing solution.

A justifiable criticism of this work was the possiblity that the ATP had as its source muscle fibers damaged during dissection. It had been noted, however, that the potassium levels in the solutions bathing the inactive muscle remained at control levels. Nevertheless it was decided to use a frog muscle preparation in

* Presented as a tutorial paper at the APS Fall Meeting in St. Louis.

which the hindlimb musculature is left intact and perfused through the abdominal aorta. This preparation was first developed by Dale and his colleagues during their search for acetylcholine released from motor nerve terminals. ATP was clearly demonstrated (Figs. 3,4) in the perfusate from indirectly stimulated hindlimb musculature, applying the tests for identification previously described (Forrester & Hassan, 1973).

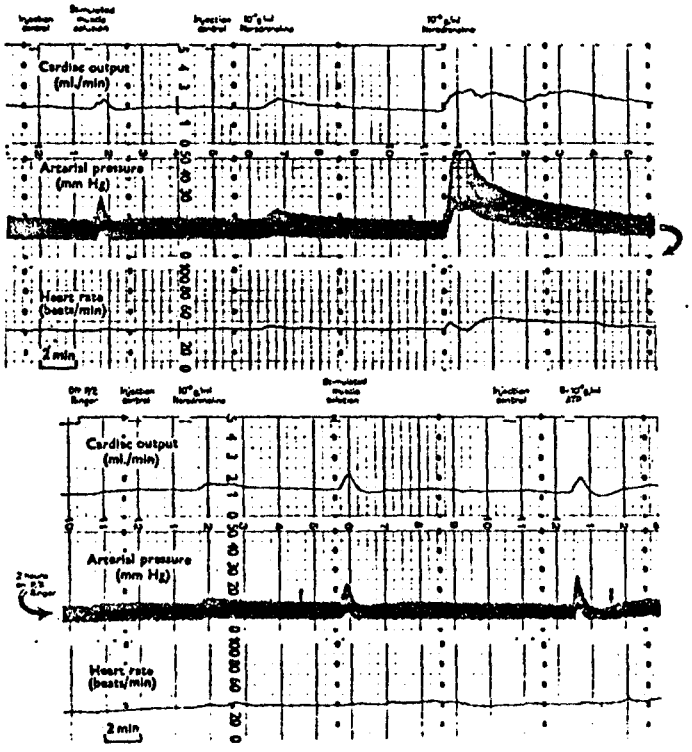


Fig. 2. Responses of a perfused frog heart to a solution in which a frog sartorius muscle had contracted and to solutions of norepinephrine and ATP before and after adrenergic blockade. Lower trace continues directly from upper trace. Heart was perfused for 2 hours with a Ringer's solution containing pronethalol hydrochloride, 10^{-6}g/ml, and ergotamine tartrate, 10^{-6}g/ml. Adrenergic blockade was sufficient to block the effect of norepinephrine almost completely. The action of the stimulated muscle solution (and ATP) remained unaffected. It indicates that the ATP contained in the muscle solution was acting "beyond" the adrenergic receptor. (From Forrester, 1967, Ph.D. Thesis, Glasgow).

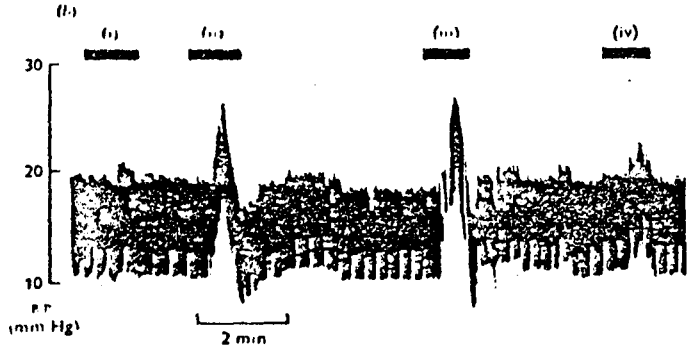


Fig. 3. Response of perfused frog heart to perfusate from frog hind limb muscles. Perfusate from (i): unstimulated hind limbs; (ii): limbs stimulated at 5 Hz.; (iii): response to ATP, 10^{-8}g/ml; (iv): sample (ii) after incubation with the dephosphorylating enzyme apyrase. Horizontal bar, time of perfusion (Forrester and Hassan, 1973, *J. Physiol., London*).

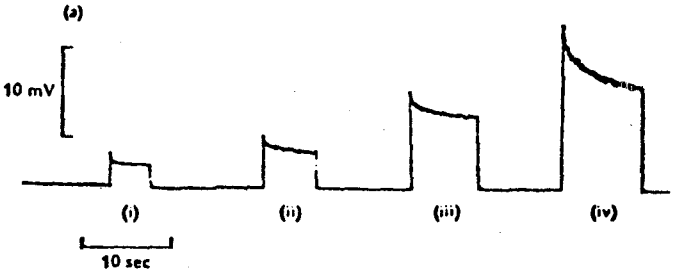


Fig. 4. Response of firefly extract to perfusate from frog hind limb muscles (i): perfusate from unstimulated hind limbs; note no decay of light signal; (ii), ATP, 10^{-8}g/ml; (iii) perfusate from limbs stimulated at 5 Hz.; note decay similar to that of pure ATP; (iv), ATP, 5×10^{-8}g/ml (Forrester and Hassan, 1973, *J. Physiol., London*).

At this juncture it was realized that ATP could have a role to play in the hyperemic response seen in skeletal muscle during exercise, since ATP is well known as a powerful vasodilator. Thus it was decided to apply these techniques of detection and identification to human peripheral blood despite the fact that human plasma contains enzymes capable of rapidly degrading ATP. When suitably diluted human plasma was perfused through a frog heart the same stimulatory response was seen, typical of ATP (Figs. 5,6). The amount of ATP in plasma from the venous blood of resting subjects (antecubital vein sample) ranged from 0.19 - 0.95 μg/ml (0.37 - 1.9 nmoles/ml). Approximately half of this amount was attributed to platelet damage. Simultaneous arterial and venous samples from four subjects at rest had mean concentrations of 0.19 μg/ml (0.37 nmoles/ml) and 0.70 μg/ml (1.3 nmoles/ml) respectively, indicating that the ATP was added to the blood on its way through the skeletal muscle bed. The concentration of ATP in the venous effluent from human forearm muscles performing a controlled amount of isometric exercise was measured (Forrester & Lind, 1969). Fig. 7 shows the results obtained from subjects performing 10% and 20% of their maximum voluntary contractions (MVC). Note that with the 20% MVC the concentrations rise steadily after the end of the contraction period, presumably indicating that as the post-exercise hyperemia diminished, the concentration will steadily rise if the musculature adds ATP to the venous effluent after contraction has ceased. With the 10% MVC a peak of concentration is evident, when post-exercise hyperemia was naturally not so great. It was established that the arterial levels remained low during and after the isometric exercies (Fig. 8). These results were soon substantiated by Parkinson (1973) who noted rises in blood levels of ATP, ADP and AMP occurring as long as five minutes after whole body exercise.

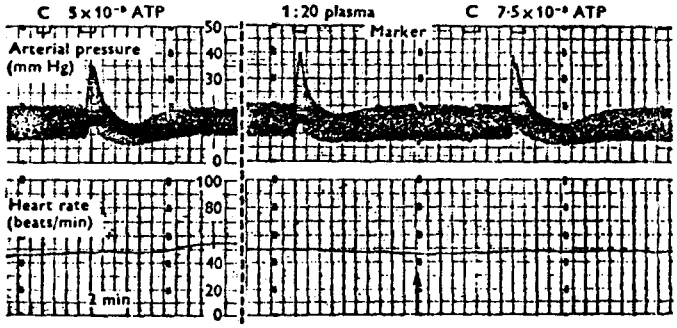


Fig. 5. Comparison of the action on a perfused frog heart of solutions of ATP (g/ml) and a diluted solution of fresh human plasma. Heart continuously perfused with Locke solution. C, control injection; vertical interrupted line 25 min period; ↑, fall in perfusion pressure during flushing of residual plasma solution from perfusion system. The actions of plasma and ATP are almost identical. (Forrester and Lind, 1969, *J. Physiol. London*).

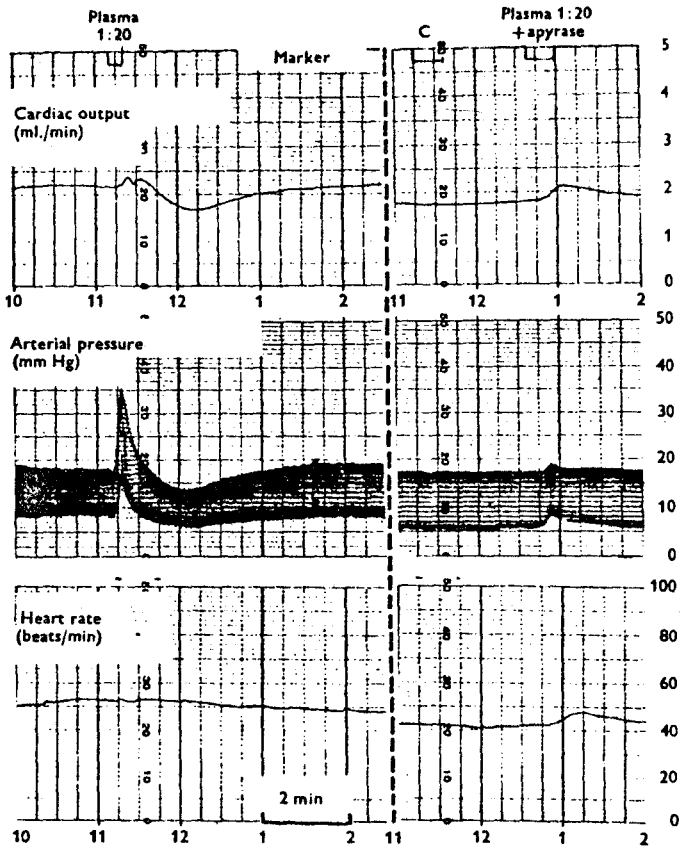


Fig. 6. The effect of a diluted plasma solution before and after incubation with apyrase on a perfused frog heart. Vertical interrupted line, 20 min period. After incubation with apyrase the same solution has a much reduced inotropic effect. The increase in cardiac output was caused by an increase in the heart rate. (Forrester and Lind, 1969, *J. Physiol., London*).

These results naturally led to the enquiry: is ATP released in sufficient quantities to satisfy the vasodilator requirements of active skeletal muscle? Previous studies on the effect of infused ATP on the circulation through human forearm musculature (Duff, Patterson & Shepherd, 1954) provided an opportunity to quantify the amounts of ATP released *in vivo* (Fig. 9). The main difficulty with a quantitative study of this sort is the fact that an increase in blood flow through exercising skeletal muscle has the effect of washing out and diluting the ATP to an unknown extent. One way round this difficulty is simply to occlude the arterial supply to the muscles during the period of exercise, and then gradually restore the flow which will then pass through the line of least resistance, that is, the dilated vessels in the skeletal muscle bed. Human subjects gripped a bar isometrically at 5% MVC in the presence of arterial (and venous) occlusion. This exercise is a mild one, and normally gives rise to an exercise hyperemia of only three times the resting flow (Lind & McNicol, 1967). Duff *et al* (1954) showed that a threefold increase in forearm flow could be produced by infusing ATP, 16 μg/min (30 nmoles/min), into the brachial artery. Figs. 10 & 11 show the results obtained in six experiments on four subjects. It is important to note the logarithmic ordinate scale, indicating a 10 - 100 fold increase in the concentration at the end of the four minute exercise period. These concentrations represent only a small fraction of the ATP orginally present because the rapid degradation in the bloodstream by plasma and blood elements could not be avoided. This factor would certainly diminish the concentrations of ATP infused by Duff *et al* (1954). A computation of the loss occurring was made and it was calculated that 7.5 - 10.5 μg/min (14-20 nmole/min) were released (Forrester, 1972). These amounts compare well with the 16 μg/min infused by Duff et al to produce the same threefold increase in flow (Fig. 9).

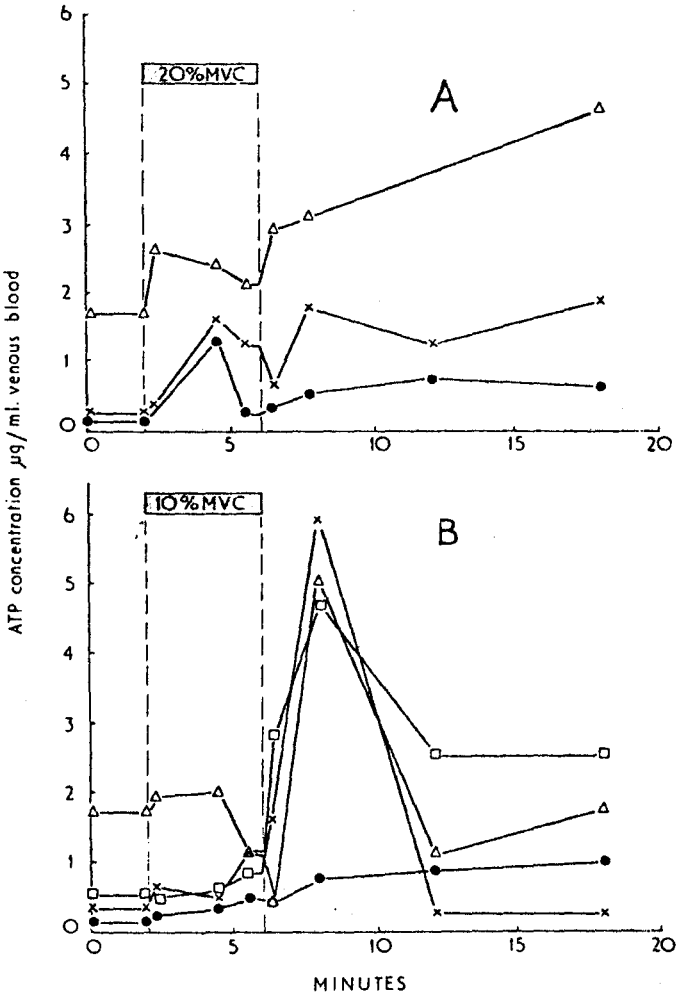


Fig. 7. The ATP concentration (μg /ml) in the venous effluent from human forearm muscle before, during and after 20% (A) and 10% (B) maximum voluntary contraction. Different symbols represent different subjects. Shaded bar indicates duration of contraction. Note the slow rise in concentration during the post-contraction phase in the 20% MVC (a tension eventually resulting in fatigue) in contrast to the sharp rise and fall obtained in the non-fatiguing 10% MVC. (Forrester and Lind, 1969, *J. Physiol., London*).

Release of ATP from myocardial cells in response to hypoxia

The findings with skeletal muscle *in vivo* encouraged further investigation using myocardial cells. The search for mechanisms of coronary vasodilatation has been going on for many years and is of obvious importance in view of the great morbidity associated with myocardial ischemia. Many substances have been implicated in the search for 'the vasodilator' (see review by Berne, 1964) but because of potency and/or lack of detection, none so far have been found satisfactory. The adenosine hypothesis put forward by Berne (1963) seems to be unsatisfactory on the grounds that aminophylline blocks the dilatory action of adenosine but not that of hypoxia (Afonso, Ansfield, Berndt & Rowe, 1972). An increase in the levels of ATP in the coronary sinus effluent of perfused guineapig hearts has been observed whenever the myocardium was made hypoxic (Paddle & Burnstock, 1974) but it was not certain whether the ATP came from nerves, vascular smooth muscle or myocardium (Fig. 12). The

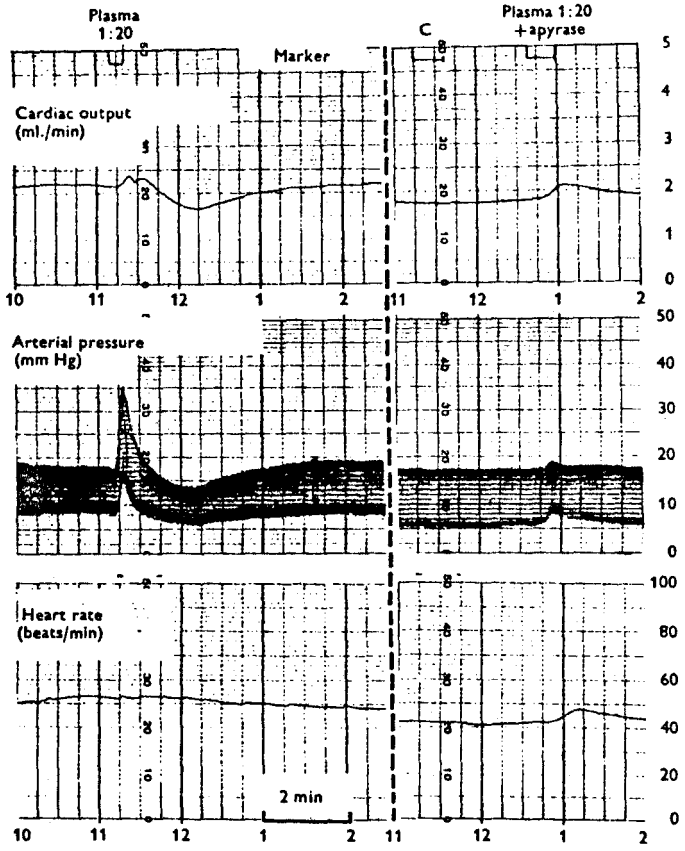
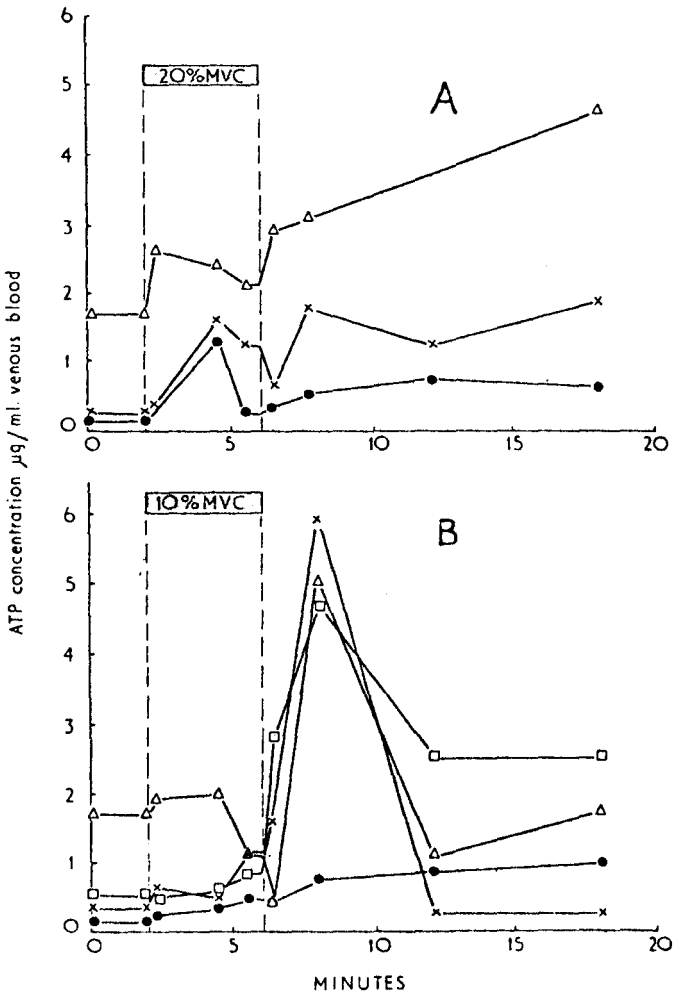


Fig. 6. The effect of a diluted plasma solution before and after incubation with apyrase on a perfused frog heart. Vertical interrupted line, 20 min period. After incubation with apyrase the same solution has a much reduced inotropic effect. The increase in cardiac output was caused by an increase in the heart rate. (Forrester and Lind, 1969, *J. Physiol., London*).

These results naturally led to the enquiry: is ATP released in sufficient quantities to satisfy the vasodilator requirements of active skeletal muscle? Previous studies on the effect of infused ATP on the circulation through human forearm musculature (Duff, Patterson & Shepherd, 1954) provided an opportunity to quantify the amounts of ATP released *in vivo* (Fig. 9). The main difficulty with a quantitative study of this sort is the 'act that an increase in blood flow through exercising skeletal muscle has the effect of washing out and diluting the ATP to an unknown extent. One way round this difficulty is simply to occlude the arterial supply to the muscles during the period of exercise, and then gradually restore the flow which will then pass through the line of least resistance, that is, the dilated vessels in the skeletal muscle bed. Human subjects gripped a bar isometrically at 5% MVC in the presence of arterial (and venous) occlusion. This exercise is a mild one, and normally gives rise to an exercise hyperemia of only three times the resting flow (Lind & McNicol, 1967). Duff *et al* (1954) showed that a threefold increase in forearm flow could be produced by infusing ATP, 16 μg/min (30 nmoles/min), into the brachial artery. Figs. 10 & 11 show the results obtained in six experiments on four subjects. It is important to note the logarithmic ordinate scale, indicating a 10 - 100 fold increase in the concentration at the end of the four minute exercise period. These concentrations represent only a small fraction of the ATP orginally present because the rapid degradation in the bloodstream by plasma and blood elements could not be avoided. This factor would certainly diminish the concentrations of ATP infused by Duff *et al* (1954). A computation of the loss occurring was made and it was calculated that 7.5 - 10.5 μg/min (14-20 nmole/min) were released (Forrester, 1972). These amounts compare well with the 16 μg/min infused by Duff et al to produce the same threefold increase in flow (Fig. 9).



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use of adult rat heart cells isolated according to a method devised by Vahouny, Wei, Starkweather & Davis (1970), modified by C. A. Williams (1977) avoided many problems associated with whole heart preparations. The high ratio of surrounding fluid volume to cellular volume provides excellent conditions for oxygenation. No alteration in surrounding fluid volume occurs, thus avoiding the difficulty of having to estimate ATP concentrations in fluid coming from a vascular bed which is continuously dilating in response to hypoxia. The actual estimation of ATP remains free from both contaminating elements such as the blood platelets, and degrading elements, the blood ATPases. It was found that ATP, 0.34 μM/mg protein, was released from oxygenated cells, while 1.28 μM/mg protein was released within 30 seconds of rendering the cells hypoxic (Fig. 13). Fig. 14 shows the compiled results of the data. When hypoxic cells were restored to the oxygenated state the levels of ATP reverted to the previous low levels (Fig. 15). The amounts of ATP released were calculated to fall well within the range necessary for production of maximum vasodilatation of the coronary vascular bed. Continuous release of ATP from these cells supports the possibility that the tone of coronary vessels is a function of this release. It is of interest at this point to note that Giles & Wilcken (1977), working with dog heart, have shown that the response of the coronary blood flow to ATP was *not* changed by aminophylline.

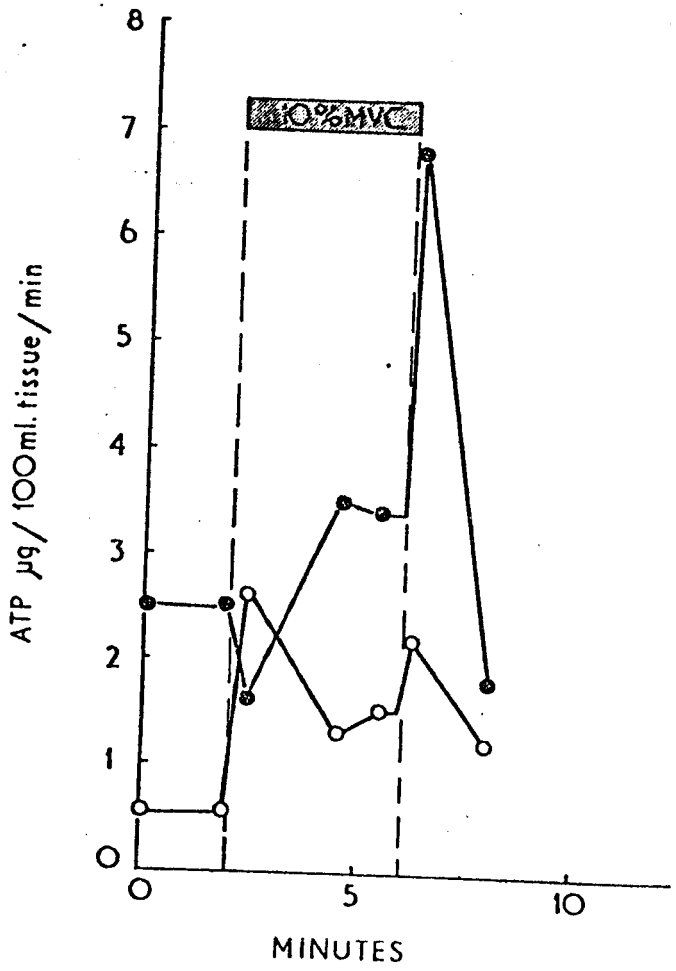


Fig. 8. Amounts of ATP measured in arterial (o) and venous (•) blood of one subject before, during and after a 10% MVC. Note the cross-over in first samples after onset of exercise. Arterial levels (from brachial artery) remain constant and below venous levels, indicating that the ATP was added to the blood in its passage through the exercising muscle. (Forrester and Lind, 1969, *J. Physiol., London*).

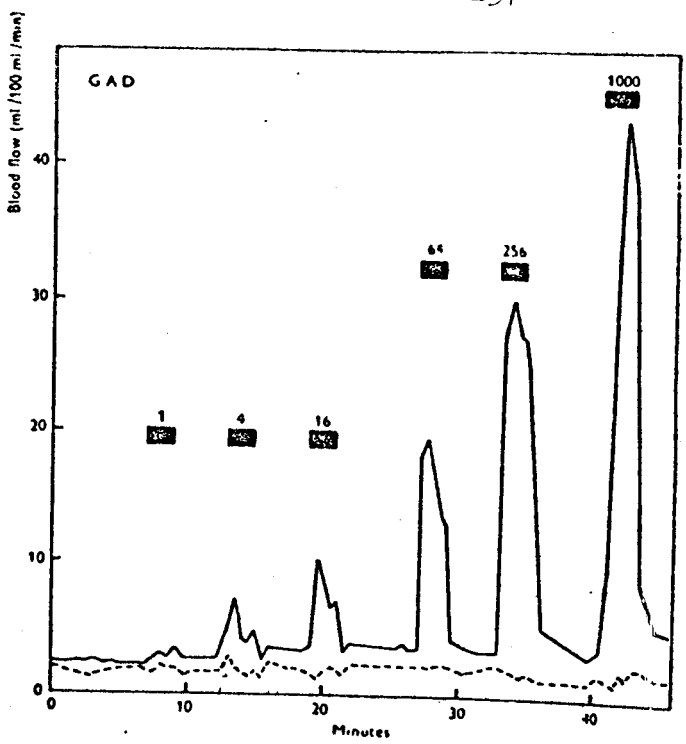


Fig. 9. The effect on forearm blood flow of various doses of magnesium ATP injected over 2-min periods into the brachial artery of a normal subject. Dotted line is control obtained from contralateral arm. Thick bars, times of perfusion with dose in μg/min. Note that 16 μg/min increases blood flow by about three times. (From Duff, Patterson and Shepherd, 1954, *J. Physiol., London*).

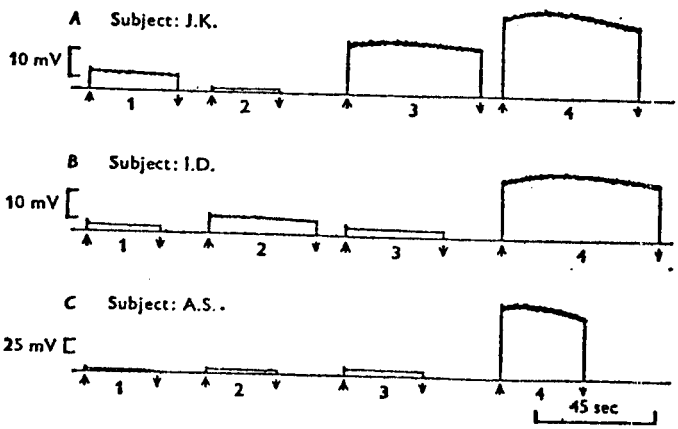


Fig. 10. Response of firefly extract to plasma samples from the forearms of three subjects, A, B and C. Sample 1 taken before onset of exercise (5% MVC); samples 2 and 3 taken during occlusion and exercise; sample 4 taken just after occlusions, exercise continuing. ↑, voltage switched on; ↓ voltage switched off. (From Forrester, 1972, *J. Physiol., London*).

Release of Nucleotides from active brain tissue

Pull & McIlwain (1972) showed that adenine nucleotides were released from electrically stimulated brain slices (Fig. 16). They calulated the amounts released per single stimulus (3 - 7 pmole/g) and suggested that a large proportion of the nucleotide material may have been in the form of ATP. Further discussion of these findings is continued below.

Effect of ATP on cerebral blood flow & metabolism

The nature of local control of blood flow in the brain has been a longstanding problem, similar to that in skeletal muscle. In 1890 Roy & Sherrington described an intrinsic control mechanism 'by

which the blood supply of various parts of the brain can be varied locally in accordance with local requirements'. In view of the probable major role that ATP plays in the control of local blood flow in skeletal and cardiac muscle, the effects of ATP, AMP and adenosine on the cerebral vasculature were explored (Forrester, Harper, MacKenzie & Thomson, 1979). Perivascular application of ATP to cat pial arterioles caused dilatation at a threshold concentration of 10^{-11}M. Pull & McIlwain calculated that the total adenine nucleotides released from stimulated brain slices lay between 3 -7 pmole/g for a single stimulus (see above). Assuming that only one tenth of this was ATP and taking the extracellular volume of the brain slice as 0.5 ml/g, a concentration of around 0.3 pmole/0.5 ml, or 6 x 10^{-10}M, can be computed. This value compares favourably with the threshold value of 10^{-11}M causing dilatation when applied perivascularly. It is thus concluded that ATP is released in amounts that are more than adequate to produce maximum local vasodilatation.

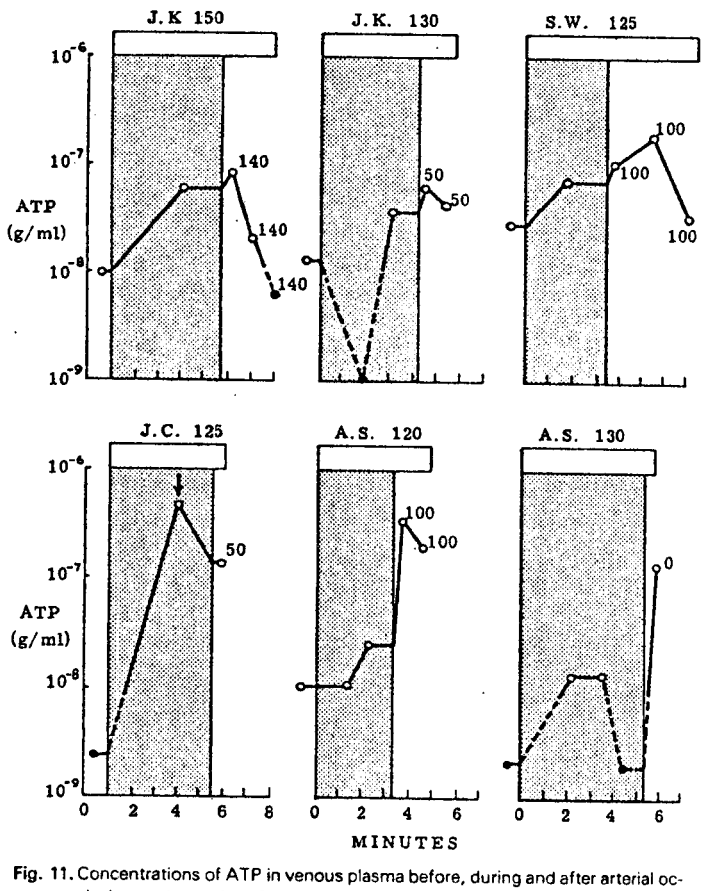


Fig. 11. Concentrations of ATP in venous plasma before, during and after arterial occlusion and exercise. Clear rectangles, duration of exercise; shaded areas, time of arterial occlusion. Resting systolic blood pressure given above each figure. Filled symbols, samples below assay threshold (dashed lines). Vertical arrow in *d*, cuff pressure lowered to obtain sample. Figures above post-occlusion samples indicate cuff pressure when sample was taken. (From Forrester, 1972, *J. Physiol., London*).

Intracarotid administration of ATP produced a surprising result in both cat and baboon (Figs. 17 & 18). In both cases the cerebral blood flow was approximately doubled when ATP was infused into the carotid artery at a rate of 10^{-6} mole/min. Taking into account the carotid blood flow in each case, the calculated threshold concentration in cats was 4 x 10^{-9}M and in baboons 4 x 10^{-8}M. the threshold response to adenosine in the baboon was 4 x 10^{-7}M. No significant effects were seen with AMP, pyrophosphate or inorganic phosphate. An interesting finding was the relationship seen between intravasuclar ATP and the oxygen consumption in the baboon experiments. Table I shows that the oxygen consumption was raised by about one and a half times when 10^{-6}mole/min ATP was infused into the carotid artery of baboon.

Table 1. Cerebral blood flow and oxygen consumption during ATP infusions in five baboons. Figures presented are mean ± S.E.M.

		ATP (moles/min, intracarotid)			
	Base line	10^{-9}	10^{-8}	10^{-7}	10^{-6}
CBF (ml./100 g.min)	49 ± 11	66 ± 11	71 ± 13	94 ± 24	116 ± 10
		*$P < 0.05$	$P < 0.01$	$P < 0.05$	$P < 0.001$
$CMRO_2$ (ml.O_2/100 g.min)	2·9 ± 0·3	3·2 ± 0·5	3·4 ± 0·4	4·3 ± 0·7	4·5 ± 0·5
		P = N.S.	P = N.S.	$P < 0.05$	$P < 0.01$

* By Student's paired *t*-test (base line *vs.* ATP infusion). N.S. = not significant.

(Forrester, Harper & MacKenzie, 1975, J. Physiol. London.)

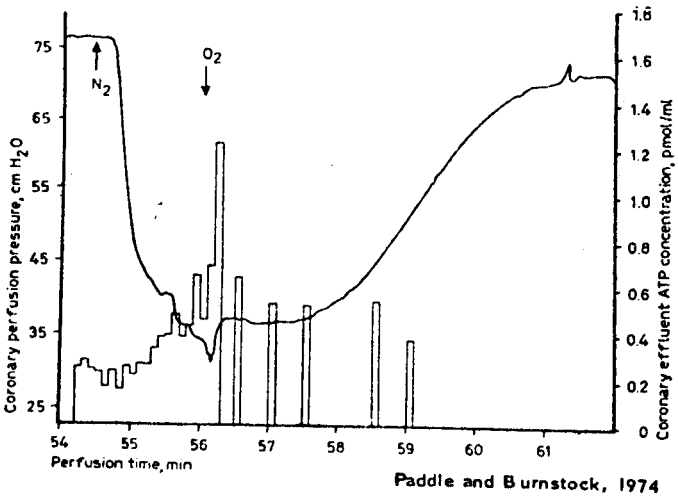


Fig. 12. Effects of a single period of hypoxia on the coronary perfusion pressure and effluent ATP concentration. Flow rate was constant at 4.8 ml/min. Perfusion pressure recorded continuously. Effluent ATP concentrations are average values during 6-sec collection periods. (From Paddle and Burnstock, 1974, *Blood Vessels, S. Karger AG. Basel*).

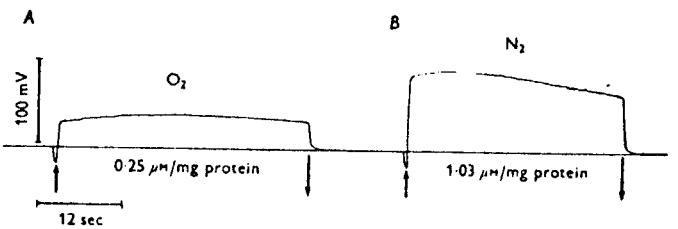


Fig. 13. Emission of light from firefly extract on addition of cells resuspended in either: A, oxygenated or B, nitrogen-equilibrated buffer solution. pH 7.4. Each signal represents amount of ATP extruded after cells had been in contact with respective buffer solutions for 30 sec. at 37°C. (From Forrester and Williams, 1977, *J. Physiol. London*).

Conclusions

It now seems evident that active skeletal muscle, cardiac muscle and brain tissue release significantly high concentrations of ATP into the extracellular space to affect profoundly the local blood flow. A residual amount of ATP and other nucleotides is most likely to reach the general circulation and indeed nucleotides have been detected in the human circulation as long as 5 min after whole body exercise (Parkinson, 1973). The question then arises: can circulating nucleotides and derivatives released from active skeletal muscle achieve levels in the arterial blood sufficient to affect cerebral metabolism? Is there some substance to the adage *'mens sana in corpore sano'?* It remains to be determined what proportion of derivatives, or the nucleotides themselves, can survive passage through the lungs. Some preliminary data in-

dicates that if ATP is infused in high enough quantities into the pulmonary artery of baboon, micromolar levels can be sustained in the arterial circulation without obviously compromising the general circulatory welfare (Evans, Forrester & Mueller, 1978). Other effects of circulating nucleotides are well documented and recently the uptake and supply of purine compounds by the liver has been emphasized (Pritchard, *et al*, 1975). These findings prompt the suggestion that there exists a system of 'metabolic communication' in the body mediated by circulating purine compounds.

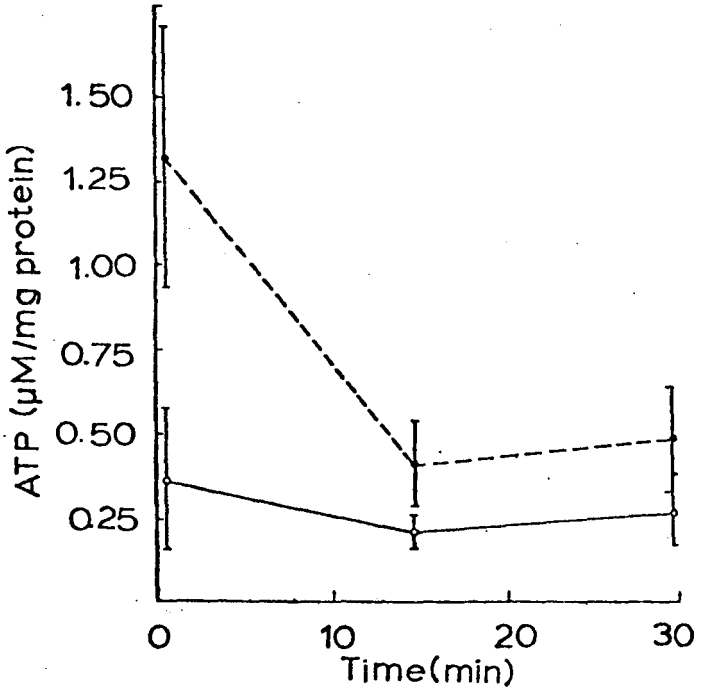


Fig. 14. Summated results from experiment shown in previous figure. Difference between points at 1 min of incubation is highly significant ($0.001 < P < .01$). Each point is average ± S.D. (n = 6). (From Forrester and Williams, 1977. *J. Physiol., London*).

Implications for the future

The evidenc· presented leaves us with an outstading problem that is difficult to interpret within current concepts. How does ATP traverse, or act beyond, the cell membrane?

The clearest evidence of release of ATP from (or through) an excited membrane has been provided by Israel, Lesbats, Meunier & Stinnakre (1976). They have demonstrated very elegantly the release of ATP in response to single nerve impulses applied to the electric organ of *torpedo*. A diagram of the experimental set-up is shown in Fig. 19. The electric organ can be regarded as a gigantic motor end-plate without the underlying skeletal muscle element. It is supplied by a cholinergic motor nerve. Israel *et al* stimulated the motor nerve and recorded the post-synaptic response from the electric organ. At the same time they arranged for the surface of the organ to be perfused with extract of firefly lanterns. Figure 20 shows the response of the superfusing firefly extract to single stimuli applied to the motor nerve. A discrete light signal is recorded for each nerve impulse, even in the case of three nerve stimuli delivered in quick succession. The post-synaptic source of this ATP release was demonstrated by the fact that the light signal became diminished when curare was applied to the synaptic juction *via* the superfusate (Fig. 21). The drug eserine, an anticholinesterase, had the effect of enlarging the light signal, (Fig. 22) indicating that the release of ATP was associated with activation of the acetylcholine receptor and subsequent depolarization of the post-synaptic membrane. This point was resolved neatly by blocking the receptor with curare and then superfusing a high potassium solution to produce depolarization of the post-synaptic membrane. A light signal was produced by this maneuvre, indicating that the ATP release was associated with membrane depolarization rather than with activation of the cholinergic receptor.

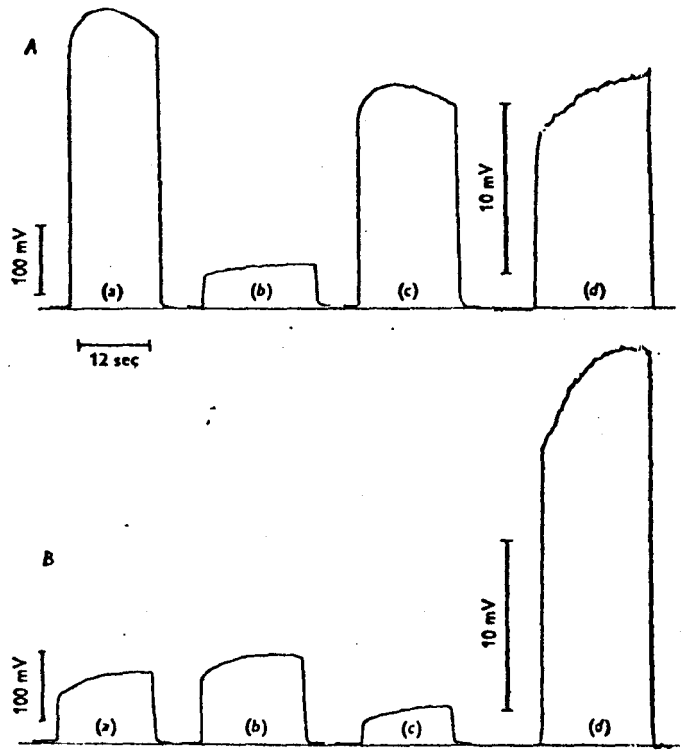


Fig. 15. Emission of light from extract in response to a cell suspension exposed alternately to hypoxic and oxygenated buffer solution. A (a), signal after exposure of cells to hypoxic buffer; (b), response after cells had been returned to oxygenated medium; (c), response when returned to hypoxic buffer; (d), response when finally returned to oxygenated medium; note increase in amplification scale. B, (a) - (d), paired oxygenated controls matching the solutions tested in A. (From Forrester and Williams, 1977, *J. Physio., London*).

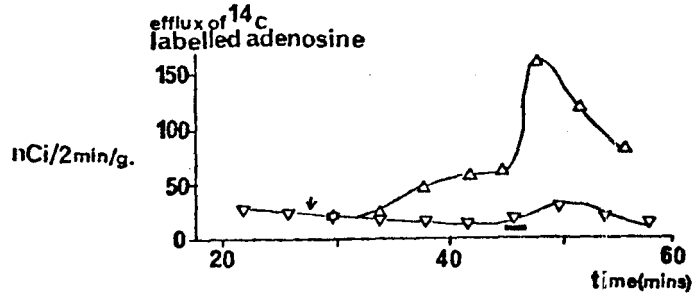


Fig. 15. Effect of stimulating isolated guinea-pig neocortex on output of ^{14}C-labelled derivatives of adenine into media lacking oxygen. The tissue was pre-incubated in oxygenated saline solution containing ^{14}C-adenine. Arrow indicates when hypoxic buffer was applied. Horizontal bar indicates period of stimulation at 32 Hz. for 2 min (▽), oxygenated solution; (Δ), hypoxic solution. Note that stimulation + hypoxia produce great outflux of label. (From Pull and McIlwain, 1972, *Biochemical Journal*).

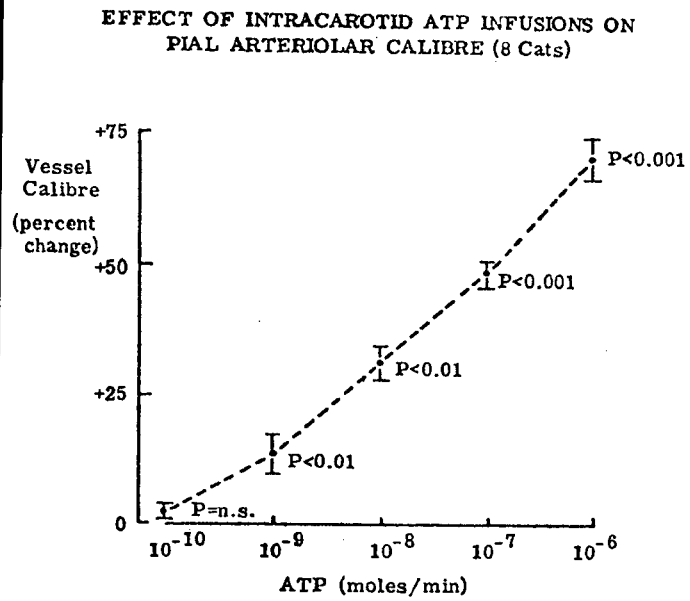


Fig. 17. Effect of intracarotid infusions of ATP on pial arteriolar calibre in 8 cats (Mean ± S.E.). P values obtained by comparing test effect with vessel calibre just prior to infusion. N.S., not significant. (Forrester, Harper, MacKenzie and Thomson, 1979, *J. Physiol. London*, in Press).

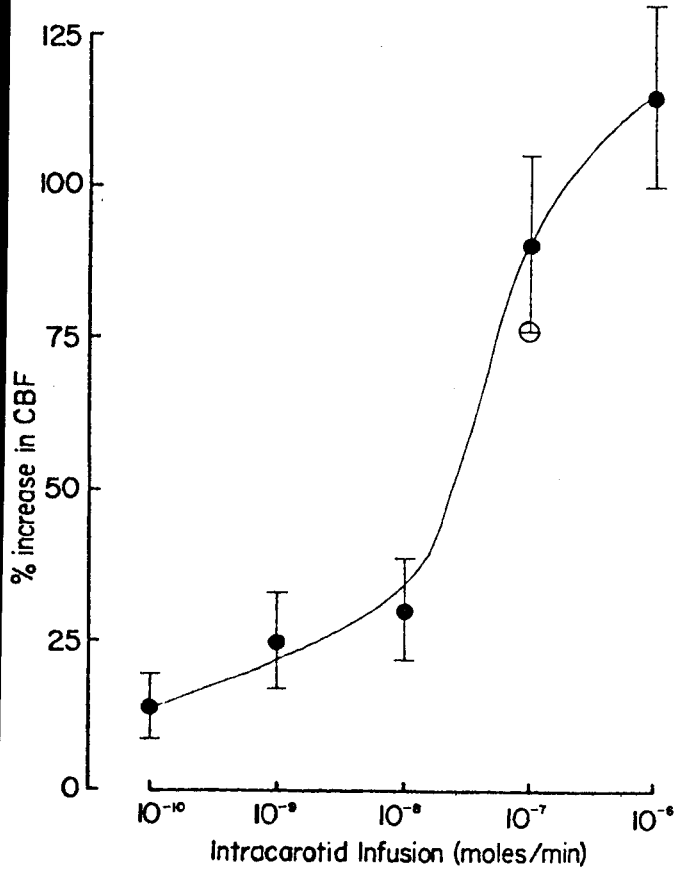


Fig. 18. Increase of baboon blood flow (•) in response to intracarotid ATP infused at 10^{10}-10^{-6} moles/min (Mean ± S.E.). o, response to 10^{-7} moles/min after disruption of the blood-brain barrier. (Forrester, Harper, MacKenzie and Thomson, 1979, *J. Physiol., London*, in Press).

We are left, then, to speculate about the mode of release of ATP from depolarizing membranes. Two possibilities suggest themselves at the present time.

The first can be considered within current concepts of membrane structure and takes into account the protein moieties 'floating' within the lipid bilayer (Singer & Nicolson, 1972). Alteration of protein configuration during depolarization of the membrane may release ATP from certain ATP-Ca-protein complexes in the membrane. The existence of these complexes was proposed by R.J.P. Williams in 1959 and there is now much evidence to support his view. Indeed the action of calcium chelating agents such as EDTA and EGTA on exitable membranes is to increase the levels of ATP in the bathing medium (Koketsu & Miyamoto, 1961; Kuperman, Volpert & Okamoto, 1964; Forrester & Williams, 1977).

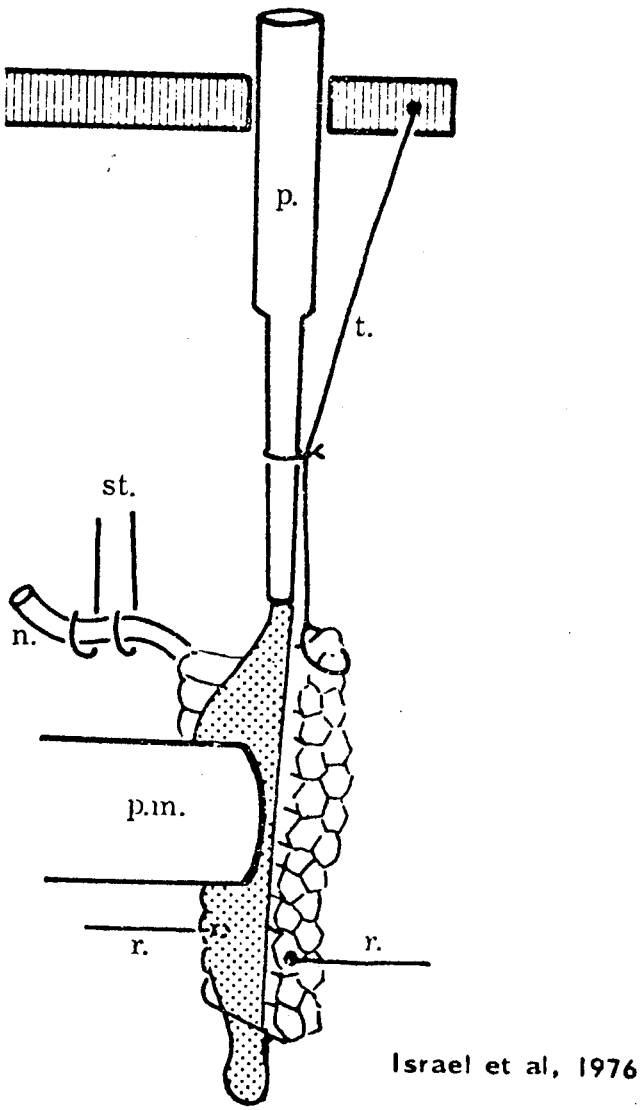
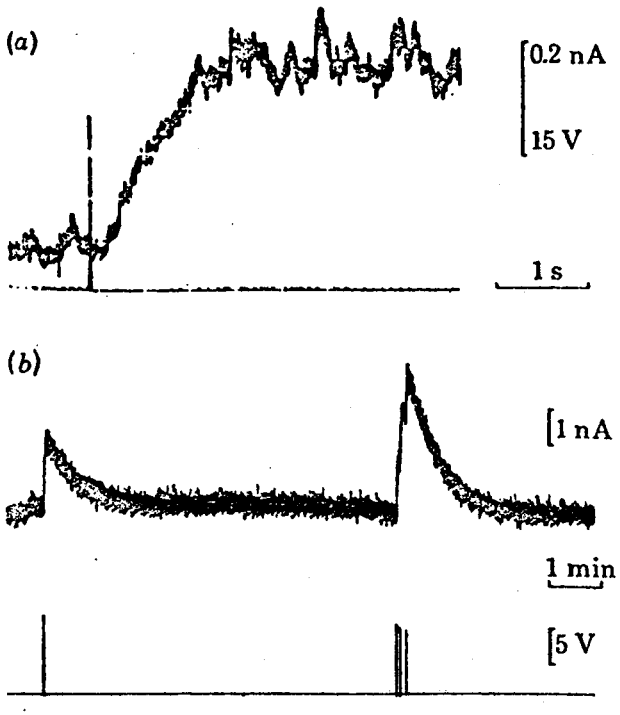
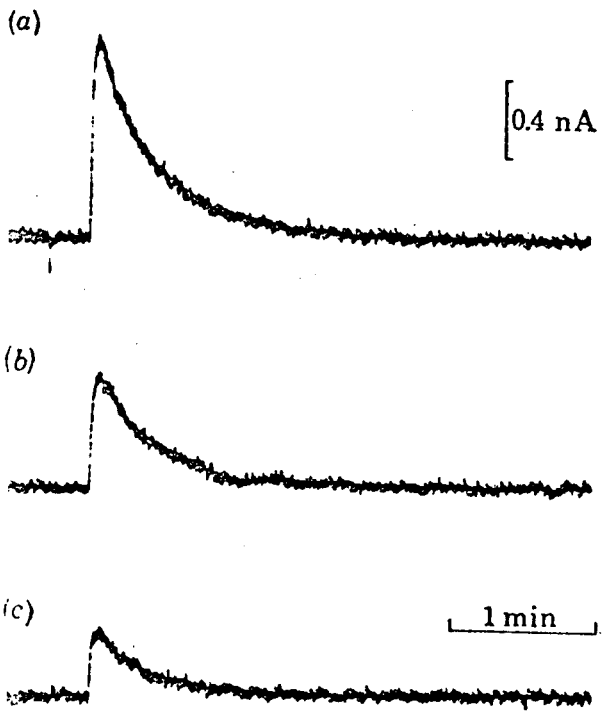


Fig. 19. Experimental set-up to measure post-synaptic release of ATP from electric organ of torpedo. Firefly extract is perfused over the surface of the organ from a pipette (p). Thread (t) stabilizes pipette and tissue. Nerve (n) stimulated through silver-silver chloride electrodes (st). Light signal from firefly recorded by photomultiplier tube (p.m.). Post-synaptic electrical response recorded with platinum electrodes. (From Israel, Lesbats, Meunier and Stinnakre, 1976, *Proc. Roy. Soc. B., London*).



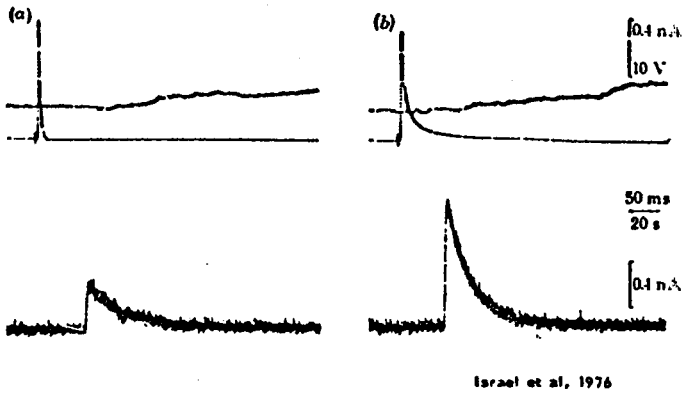
Israel et al, 1976

Fig. 20. Release of ATP after single nerve impulses. (a) lower trace: oscilloscope record of electrical response (post-synaptic) to a single stimulus applied to the nerve; upper trace: intensity of the p.m. current due to light emission resulting from the reaction of ATP with the firefly extract. (b) Responses to single and triple stimulation of the nerve. (Different preparation). Upper trace: light emission; lower trace: electrical responses. Time constant of the light recording system was 110 ms. (From Israel *et al*, 1976).



Israel et al, 1976

Fig. 21. Effect of curare on the release of ATP. (a), control. (b), after about 80 min in 5×10^{-4}M curare. (c), concentration of curare increased to 10^{-3}M. Note marked diminution of light signal indicating depression of ATP release post-synaptically. (From Israel *et al*, 1976).



Israel et al, 1976

Fig. 22. Effect of eserine, an anticholinesterase, on ATP release. (a), control. (b), same preparation 15 min after eserine (10^{-4}M). Slow time base on the lower traces shows the augmentation of ATP release in the presence of an anticholinesterase. (From Israel *et al*, 1976).
(Forrester, Harper & MacKenzie, 1975, J. Physiol. London.)

The second possibility requires a radical change of our views concerning the nature of the excitable cell membrane. For many years a group of workers, led by Ling, have maintained that there is simply not enough energy available to run all of the postulated "pumps" in the cell membrane (Minkoff & Damadian, 1973). Ling has proposed an association-induction hypothesis which regards the cell membrane as having a minor role to play in maintaining the intracellulor environment. It is proposed that the physical state of the cell is maintained by protein-ATP complexes occurring at various *cardinal sites* and that the high intracellular potassium and low intracellular sodium levels result from adsorption of these ions to the protein without consuming energy (Ling, 1978).

Elucidation of the ATP release mechanism may advance our understanding of the relationship between a cell and the external environment.

Acknowledgement: It is a pleasure to thank Professor I.A. Boyd, Professor A.R. Lind, Dr. M.O. Hassan, Dr. C.A. Williams, Dr. R.G. Evans and Dr. Hiltrud Mueller for their collaboration and support without which much of this work would not have been possible.

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Commentary - paper 20.

Although the concept of ATP coming through, or from, depolarizing membranes was still controversial, evidence was steadily accumulating that this was a normal response to oxygen demand. A significant paper was produced by Paddle & Burnstock (1974, reference in following paper) showing that ATP appeared in the coronary sinus effluent from Langendorff perfused guinea pig hearts. These isolated heart preparations do not perform the normal amount of work since the left ventricle does not beat against a constant 'afterload.' The following paper describes the appearance of ATP from a working heart preparation in which the left ventricle beat against a controlled pressure. Obviously the viability of such a preparation comes into question, much in the same way that the sartorius muscle preparation had been criticised. A considerable portion of the results and discussion sections dealt with the aspect of adequate oxygenation. Probably most crucial is the fact that a considerable proportion of oxygen in the perfusate was not being taken up by the myocardium, indicating satisfactory oxygenation for the ventricular work being done.

Finally, in this paper a model for the roles of adenosine and ATP in the local control of myocardial blood flow is given. Recently some doubts have been cast upon the role that adenosine might play because of experiments with theophylline and aminophylline. These compounds completely inhibit the vasodilatory action of adenosine on the coronary arteries and other tissues, but do not inhibit the dilatory action of hypoxia or ATP.

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APPEARANCE OF ADENOSINE TRIPHOSPHATE IN THE CORONARY SINUS EFFLUENT FROM ISOLATED WORKING RAT HEART IN RESPONSE TO HYPOXIA

BY M. G. CLEMENS* AND T. FORRESTER

From the Department of Physiology, Saint Louis University School of Medicine, 1402 South Grand Boulevard, Saint Louis, Missouri 63104, U.S.A.

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SUMMARY

1. A working rat heart preparation was used to study the release of adenosine-5′-triphosphate (ATP) into the coronary sinus effluent in response to hypoxia.

2. The left ventricle was set to pump against an hydrostatic pressure of 65 cm water; the left atrial filling pressure was kept constant at 10 cm water. The power output of the heart at these pressures was estimated to be approximately one half of the maximum power development.

3. Samples for ATP assay were collected (*a*) 30 sec before onset of hypoxia, (*b*) 60–90 sec after onset of hypoxia, (*c*) 5 min after restoration of oxygenated buffer solution. Respective concentrations of ATP were (nM ± S.E.) 0·63 (±0·18), 4·70 (±0·39) and 0·63 (±0·06). The total amounts of ATP detected were (p-mole/min) 5·9 (±0·9), 46·1 (±6·0) and 5·5 (±1·2) respectively.

4. Viability of the hearts was judged to be satisfactory on the following grounds. Alterations in left atrial filling pressure produced typical Frank-Starling responses of the left ventricle. Oxygen extraction from the perfusate increased in response to increased workload. Coronary blood flow increased immediately upon introduction of hypoxic conditions and mechanical recovery from hypoxia was always complete within 5 min of restoring oxygen.

5. In view of the marked extracellular ATPase activity it is concluded that significant vasodilatory concentrations of ATP are released into the myocardial extracellular space in response to hypoxia. A scheme is proposed describing the possible role of adenine nucleotides in the local control of myocardial blood flow.

INTRODUCTION

Adenosine-5′-triphosphate (ATP) has long been known as a powerful dilator of coronary arteries (Fleisch & Weger, 1937; Folkow, 1949). It is 4 times more potent in this respect than adenosine monophosphate and adenosine (Winbury, Papierski, Hemmer & Hambourger, 1953; Wolf & Berne, 1956). Much evidence has accumulated

* Present address: Yale University School of Medicine, Department of Surgery, 333 Cedar Street, New Haven, Connecticut 06510, U.S.A.

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to indicate that ATP is released from electrically active frog skeletal muscle (Abood, Koketsu & Miyamoto, 1962; Boyd & Forrester, 1968), human skeletal muscle (Forrester & Lind, 1969; Forrester, 1972; Parkinson, 1973) and nervous tissue (Holton, 1959; Abood *et al.* 1962; Kuperman, Volpert & Okamoto, 1964; Pull & McIlwain, 1972; Burnstock, 1972). A particularly clear demonstration of ATP release from (or through) an electrically excited membrane has been provided by Israel, Lesbats, Meunier & Stinnakre (1976). They monitored the post-synaptic release of ATP from electric organ of *Torpedo* directly, using the sensitive luciferin-luciferase assay technique. In a similar way the release of ATP from brain synaptosomes depolarized by elevated extrasynaptosomal potassium or veratridine has been directly measured (White, 1977). Some indirect evidence has led to the suggestion that ATP may cross the cardiac cell membrane (Fedelešová, Ziegelhöffer, Krause & Wollenberger, 1969; Ziegelhöffer, Fedelešová & Šiška, 1971) and also skeletal muscle membrane (Chaudry & Gould, 1970) as the intact molecule.

Paddle & Burnstock (1974) have demonstrated the presence of ATP in the coronary sinus effluent from isolated guinea-pig hearts in response to hypoxia. A similar finding was noted by Stowe, Sullivan, Dabney, Scott & Haddy (1974). These hearts were perfused in the Langendorff mode and hence the amount of work performed by the musculature was very low and difficult to assess. The source of ATP was undetermined, but Paddle & Burnstock discussed the possibility that 'purinergic' nerves might give rise to such concentrations in the coronary sinus effluent. Release of ATP from isolated heart cells in response to very brief periods of hypoxia has been recently measured (Forrester & Williams, 1977), indicating that one source of ATP was the hypoxic myocardial cell.

The purpose of the present experiments was to test the coronary effluent for ATP from a *working* heart preparation. Such a preparation was developed by Neely, Liebermeister, Battersby & Morgan (1967) and is particularly suited for the study of myocardium beating against a normal load. Perfusion of the coronary vascular bed also becomes similar to the situation *in vivo*. The work of the heart can be readily calculated and the metabolic state can be assessed by measurement of the oxygen extraction by the myocardium.

A preliminary account of some of these results has already appeared (Clemens & Forrester, 1979).

METHODS

ATP assay. The ATP concentration of the coronary sinus effluent was determined by the firefly luminescence technique described by Strehler & McElroy (1957). Two modifications of this procedure were used in different series of experiments. In all Ca^{2+} paradox experiments the assay was carried out as modified by Forrester (1972). In the experiments dealing with hypoxia the procedure described by Silinsky (1974) was adopted in order to assay very low concentrations and also to allow for any alterations of fluid composition in its intravascular passage through the myocardium. Silinsky showed that this method gives reproducible results despite differences in firefly enzyme sensitivity and levels of inhibition by buffer constituents. The firefly luminescence was continuously monitored before, during and 15 sec after addition of the sinus effluent sample by automatic pipette; this makes it unnecessary to assume that the magnitude of the background signal is a constant. Aliquots of 1 ml. of coronary sinus effluent were removed from the collecting tube and placed in a chilled microfuge tube kept on ice. Samples were stored at 0 °C until assayed for ATP. Several vials of firefly lantern extract (Sigma FLE 50) were each reconstituted with 5 ml. deionized distilled water; all vials were pooled to give an homogeneous suspension of extract.

Throughout the duration of assays the pooled suspensions were kept on ice to prevent any loss of activity: 0·4 ml. of extract was pipetted into quartz microcuvettes which were placed in a cuvette carrier and then into the light-tight sample housing of the photomultiplier (P.M.) tube. After positioning the cuvette in front of the tube windows and rendering the housing light-tight, 1400–1600 V DC were delivered across the P.M. tube. The background light signal was recorded. 0.25 ml. of sinus effluent was then added to the firefly extract through an injection port in the lid of the housing. This results in an immediate fall in light signal intensity, presumably caused by

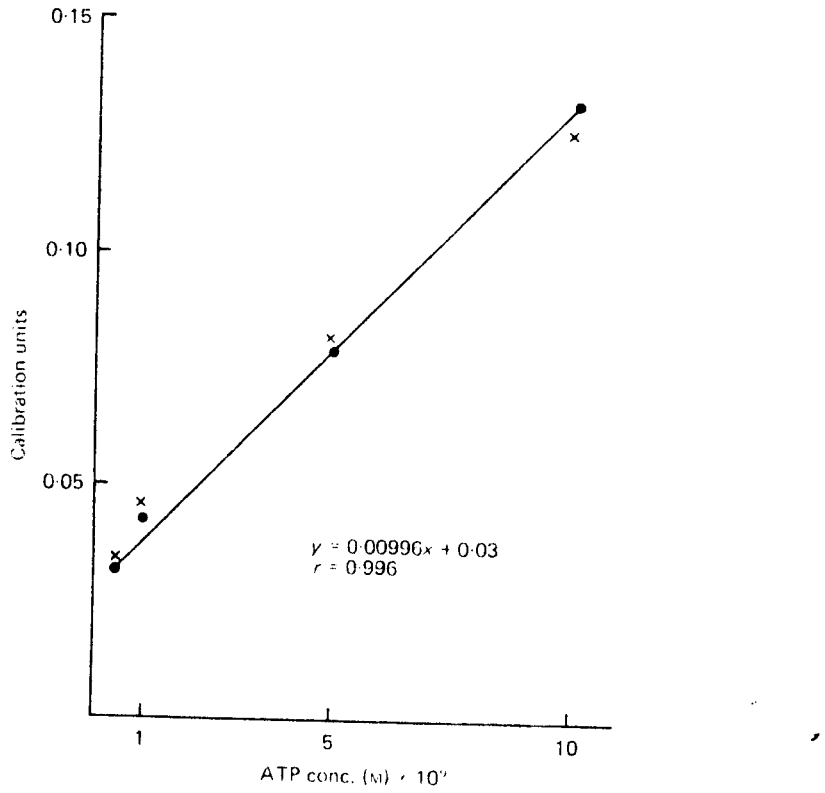

Fig. 1. Dose–response relationship for ATP using firefly assay. (●), values before assay of test solutions; (×), values after assay procedure (about 4 hr). Calibration units = $(S_{10}-S_i)/S_B$, where S_{10} = height of signal 10 sec after injection of sample, S_i = height of inhibited signal and S_B = height of background signal. Abscissa scale shows range between 10^{-9} and 10^{-8} M.

some inhibition of the luciferin-luciferase reaction. Then follows a rise in light signal caused by the ATP-dependent light production (see Silinsky, 1974, Fig. 2). The actual signal intensity was obtained by subtracting the level of inhibition immediately following sample injection from the light emission signal 10 sec after injection. If any variation in background signal occurred during an assay run, all values were standardized by dividing by the intensity (height) of the background signal. Calibration standards with ATP concentrations of 0·5, 1·0, 5·0 and 10 nM were run at the beginning and end of each assaying session. Sample concentrations were determined from a standard dose–response curve (Fig. 1). Concentrations down to 5×10^{-10} M can be reliably detected by this method.

Working heart preparation. A modification of that described by Neely *et al.* (1967) was used. This model differs from the Langendorff perfused heart in that the left ventricle is required to perform work against a controlled afterload (Fig. 2). The left atrial filling pressure is set by the height of

an overflow chamber. Fluid is hydrostatically propelled into the atrium and passes through the opening of the mitral valve, resulting in left ventricular diastolic filling and subsequent systolic ejection against a column of fluid set at a pressure determined by the height of the upper overflow chamber. The apparatus contained parallel overflow chambers and perfusion lines on both the atrial and aortic sides (not shown in Fig. 2). The atrial and aortic perfusion systems could be rapidly changed by stopcocks situated close to the heart. Both atrial overflow chambers and one aortic chamber were filled by a peristaltic pump from reservoirs kept in a water-bath set at 38 °C. The overflow from each chamber was returned to its respective perfusate reservoir. The overflow from the aortic chamber could be collected to quantify aortic output. Glass microfibre fillers with an effective retention of 1·6 μm were placed in each perfusion system to remove any particles (i.e. $CaCO_3$ precipitate) that might compromise coronary flow. In the Ca^{2+} paradox experiments the pulmonary artery was cut and the coronary sinus effluent allowed to run down the epicardial surface of the heart for sample collection. In the hypoxia experiments and in all experiments in which O_2 consumption was measured, the pulmonary artery was cannulated to avoid contamination by damaged tissue or room air. The cannula was secured directly over a fraction collector for collection of coronary sinus effluent.

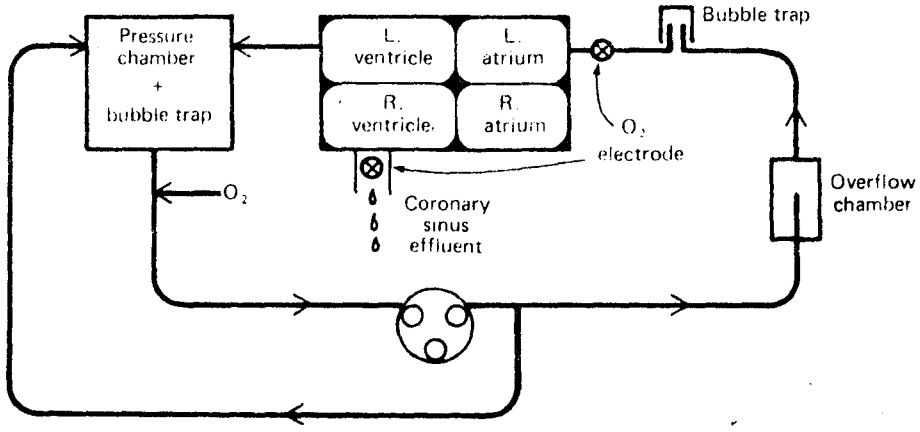

Fig. 2. Simplified scheme of working heart preparation modified after Neely *et al.* (1967). Left ventricular afterload set by height of overflow chamber. Coronary sinus effluent collected from cannulated pulmonary trunk. O_2 electrodes (⊗) placed in cannulae to left atrium and from pulmonary trunk. O_2 delivered to perfusate just below pressure chamber + bubble trap.

The perfusion solution used for all experiments was a modified Krebs-Henseleit bicarbonate buffer of the following composition (mM): NaCl, 118; KCl, 4·7; $MgSO_4$, 1·2; K_2HPO_4, 1·2; $CaCl_2$, 2·5; $NaHCO_3$, 25; glucose, 11·1. The buffer was equilibrated with 95 % O_2:5 % CO_2 and the pH adjusted to 7·4 ± 0·01 with 1 N-NaOH.

Measurement of left ventricular work. The mechanical work performed by the left ventricle was determined from the *cardiac output* and *aortic pressure*. The cardiac output was measured by collecting the output from the aortic overflow chamber in a graduated cylinder for 60 sec intervals. To this amount was added the coronary flow to give total cardiac output. Aortic pressure was monitored with a Statham pressure transducer displayed on a Grass polygraph. Heart rates were determined by visual counting of pulse pressure waves at a rapid chart speed. Since preload and afterload were held constant, aortic pulse pressure was normally used as an indication of left ventricular 'contractility'. In some experiments where heart rate altered, a pressure-rate index (Kobayashi & Neely, 1979) was used to eliminate error from the Frank-Starling effect of increased diastolic filling time during bradycardia. Left ventricular rate of work production (power) was calculated using the formulae of Kannengiesser, Opie & Van der Werff (1979). This method computes *pressure* power and *kinetic* power by the following equations:

$$\text{pressure power} = 0{\cdot}002222 \times P_S \times CO,$$

$$\text{kinetic power} = \frac{1}{432 \times 10^7} \times \frac{\rho CO^3}{A^2} \times \frac{T}{T_E},$$

where P_S = peak systolic pressure, CO = cardiac output, ρ = density (1 gm/ml.), A = cross-sectional area of aortic cannula, T = cardiac cycle time and T_E = ejection time.

Measurement of coronary flow. The output of the right ventricle was taken as an indication of approximately 95% of the total coronary artery flow. It does not take into account that portion of coronary flow draining directly into the left ventricle via Thebesian veins. This was collected from a cannula securely placed in the cut end of the pulmonary artery in timed fractions with a Gilson escargot type fraction collector.

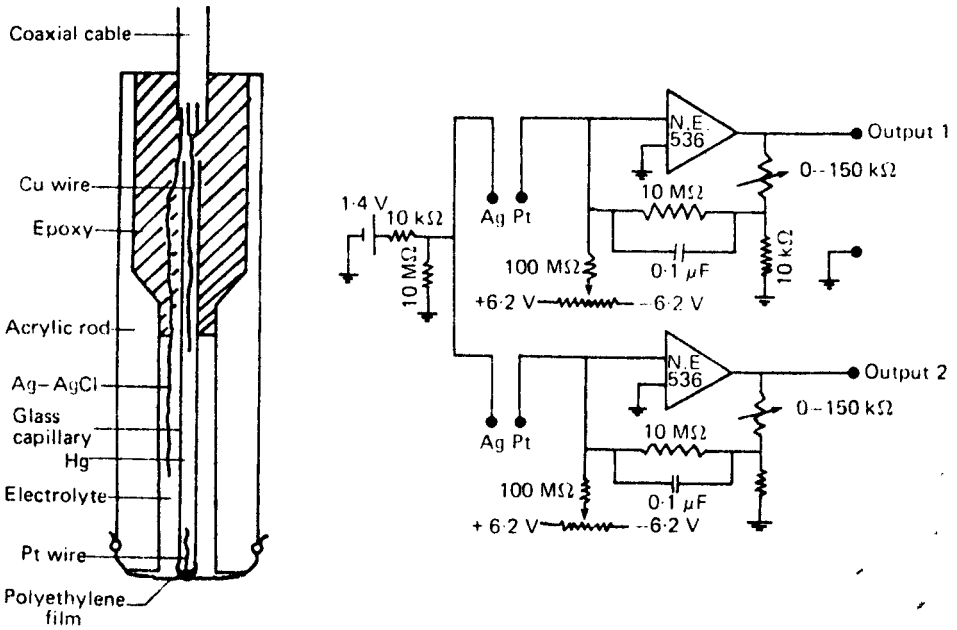

Fig. 3. Construction and circuitry of O_2 electrode (see text).

Oxygen electrode construction. A modification of the method of Clark (1956) and Staub (1961) was used. The housing contained both the platinum cathode and the Ag/AgCl reference anode (Fig. 3). Approximately 1 cm of 25 μm diameter Pt wire (Medwire Inc.) was stripped of its Teflon coating and placed in the end of a glass capillary tube. The Pt wire tip was then sealed into a glass bead by heating in a burner flame. The capillary tube was then filled with mercury and a fine copper wire then inserted, making electrical contact with the platinum wire through the mercury. This copper wire was then soldered to the centre strand of a coaxial cable. Approximately 5 cm Ag wire which had previously been coated with AgCl by electrolysis was then soldered to the outer sheath of the coaxial cable. This whole assembly was then inserted into the milled core of an acrylic rod so that the glass bead containing the platinum wire protruded about 1 mm beyond the tip of the rod. The base portion of the rod was then filled with acrylic softened with ethylene dichloride. Extreme caution was taken to ensure no electrical communication between the central and outer strands of cable. The glass bead portion was then gently abraded on no. 400 emery paper wetted with a 3 M solution of KCl until the resistance between the central strand and any probe placed in the KCl solution was no longer infinite. The cavity in the tip of the acrylic rod was then filled with saturated KCl solution and the tip covered with polyethylene film (Glad Wrap-Union Carbide). With the electrodes filled with electrolyte, a resistance between the strands of cable of about 60 kΩ indicated intact electrical connexion between the Pt cathode and the Ag/AgCl anode, with no low

resistance short circuits in the base of the electrode. Only the O_2-sensitive tip of the electrode came in contact with the perfusion fluid which flowed past the electrode tip. The output of the electrode was amplified by a two-channel operational amplifier current-to-voltage circuit similar to that described by LaForce (1967). It consisted of a field effect transistor operational amplifier (input impedance 10^{12} Ω) with zero set by a 'bucking' current at the input, and the output set by a variable resistance in the feed-back loop. The cathode is polarized to −0·7 V by a 1·4 V H_g cell and a voltage divider circuit (Fig. 3). The amplifier circuit produced an output of 0·2–0·8 V for 95 % O_2 saturated with water vapour at 37 °C.

Measurement of O_2 consumption. The consumption of O_2 by the working heart was determined from the difference in p_{O_2} between buffer entering the heart (arterial perfusion line) and that of the coronary sinus effluent. O_2 tension was constantly monitored by the Clark-type platinum O_2 cathode described above. This was mounted in a flow-through chamber kept at 38 °C with a water jacket. The current resulting from the reduction of O_2 at the cathode was amplified and converted to a voltage signal by the laboratory constructed high impedance (10^{12} Ω) electrometer amplifier (Fig. 3). The amplifier output was calibrated with standard gases and recorded using a potentiometric recorder (Fisher Recordall). $\dot{V}_{O_2}$ was calculated by multiplying the difference between inflow p_{O_2} (arterial) and coronary sinus p_{O_2} (venous) by the solubility of O_2 in water at 37 °C ($2{\cdot}7 \times 10^{-5}$ ml. O_2 . ml. H_2O^{-1} . $mmHg^{-1}$). This gives the total volume of O_2 consumed per ml. coronary flow. Total oxygen consumed per minute is obtained by multiplying by the coronary flow per minute. $\dot{V}_{O_2}$ for each heart was expressed as ml. min^{-1} . 100 g wet wt^{-1}.

Setting up the heart preparation. Sprague-Dawley rats (150–300 g, either sex) were given 500 u. Na heparin I.P. 15–30 min before sacrifice. The rats were anaesthetized with Na pentobarbitone (0·4–1·0 ml., depending on size of rat and body fat content). As soon as the rat no longer showed an abdominal stretch reflex, a skin incision was made and the heart was exposed by cutting out a sternal flap. The heart was lifted manually and freed with one scissor cut. This freed portion consisted of both atria and ventricles plus thymus and portions of lung tissue. The heart was immediately dropped into a beaker of ice-chilled Krebs-Henseleit bicarbonate buffer solution. Time from initial thoracotomy incision to placing heart in cold buffer was usually 10–15 sec. After the heart had cooled and contractions ceased, it was then cleaned from excess tissue and the aortic and pulmonary stumps identified and isolated. The aorta was cannulated (polyethylene flared cannula) with a ligature placed around the aorta proximal to the origin of the brachiocephalic artery and perfusion of the coronary artery bed begun immediately at 37 °C (Langendorff mode) from the overflow chamber set 65 cm above the heart. The chamber was supplied with buffer by a peristaltic pump. Once the heart resumed spontaneous beating at a stable rate it was prepared for atrial cannulation. After further cleaning, the inferior vena cava and the boundary between left and right atrium were identified to avoid damage to the right atrium. A small hole in the left atrium was produced by cutting the junction between one of the pulmonary veins and the left atrium. The left atrial cannula was then inserted. It was usually necessary to rotate the aorta on its cannula to position the left atrium to receive the cannula. This prevented excessive torsion on the aorta which might obstruct the openings of the coronary arteries. The atrium was then secured to the cannula with several ligatures which included any remaining lung tissue and associated pulmonary veins. Competency of the cannulation was verified by opening the stopcock in the atrial perfusion line and observing distension of the left auricular appendage and a sudden increase in aortic pulse pressure. When the pulmonary artery was cannulated competence of the left atrial cannulation was further verified by the absence of perfusate leaking from the heart. Each heart was allowed to beat for at least 15 min in the non-working mode before left atrial filling pressure was set at 10 cm H_2O.

RESULTS

When the perfusate of an isolated working rat heart was changed from an oxygenated buffer to one made hypoxic by bubbling with 95 % N_2 : 5 % CO_2, there was a typical rapid decline of aortic pulse pressure and often a bradycardia (Fig. 4). Occasionally there was a complete loss of pulse pressure. In all cases cardiac output was severely compromised to the extent that the aortic overflow chamber had to be supplemented by a parallel reservoir to maintain a constant coronary perfusion

pressure. Upon reversion to an oxygenated perfusate after a 90 sec period, the mechanical activity steadily improved to the control level. This was the case even in hearts that had completely lost the aortic pulse during the hypoxic period.

Samples of coronary sinus effluent for ATP assay were taken (1) in the 30 sec control period before the onset of hypoxic perfusion, (2) 60–90 sec after the onset of hypoxia, and (3) 5 min after the restoration of oxygenated buffer (Fig. 4*A*). In four

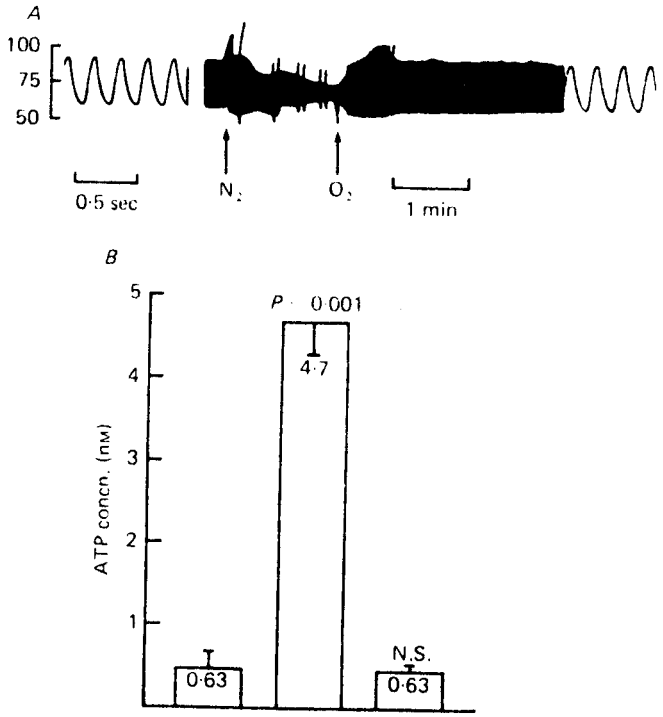

Fig. 4. *A*, recording of aortic pulse pressure from a working heart preparation exposed to hypoxia for a period of 90 sec. Samples for ATP assay collected 30 sec before hypoxia, 60–90 sec after onset of hypoxia and 5 min after restoration of oxygenated buffer solution. Note change in time scale. Vertical scale, mmHg *B*, concentrations (±S.E.) of ATP in the coronary sinus effluent from four hearts. *P* value indicates significance from control and recovery samples. N.s., not significantly different from control (one way analysis of variance test used).

hearts, ATP was detected in the coronary effluent during the 30 sec before the onset of the hypoxic period. A mean concentration of 0·63 (±S.E. 0·18) nM-ATP was detected in the coronary effluent during the period of 30 sec before the onset of hypoxia; samples collected during the last 30 sec of the hypoxic period contained ATP, 4·7 (±S.E. 0·39) nM. Restoration of oxygenated buffer after the 90 sec hypoxia resulted in a decline in ATP concentration and by 5 min the level of ATP had returned to control levels of 0·63 (±S.E. 0·06) nM (Fig. 4*B*).

Total amount of ATP released. The total amount of ATP released into the coronary sinus effluent is equal to the concentration of ATP multiplied by the coronary sinus flow. The coronary flow was measured throughout the periods of sampling and hypoxia and Fig. 5 shows the increase in flow produced during hypoxic perfusion.

In eight hearts the mean coronary flow was significantly increased during hypoxia ($P < 0.05$). In two hearts the coronary flow during hypoxia actually decreased. However, in those cases the aortic pulse pressure became unrecordable; there was failure of ventricular emptying and distension of both ventricles was evident. It is likely that this resulted in a combination of increased intramural pressure from ventricular distension, and increased coronary venous pressure from the deficiency in right ventricular emptying. In four hearts, the total amounts of ATP before, during and after the hypoxic period were (p-mole/min): 5·9 (±S.E. 0·9), 46·1 (±S.E. 6·0) and 5·5 (±S.E. 1·2), respectively.

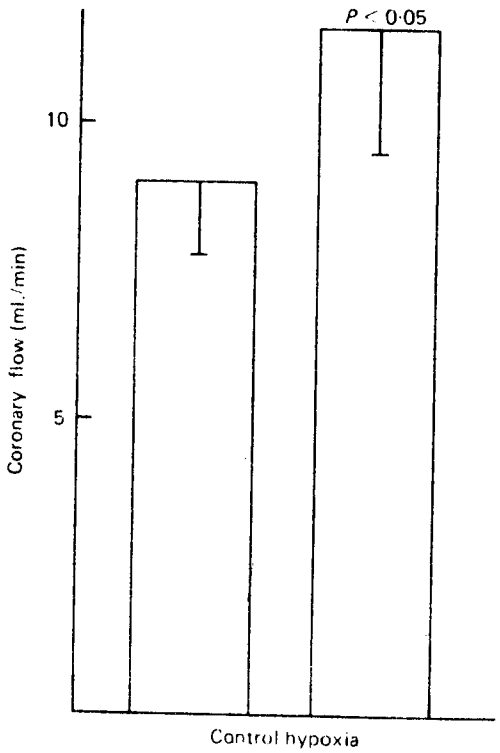

Fig. 5. Coronary flow (±S.E.) of eight working heart preparations during oxygenated and hypoxic periods. Significantly greater flow in hypoxia ($P < 0.05$, Student paired t test).

It is noteworthy that in four preliminary experiments in which the pulmonary artery was not cannulated, but rather the coronary effluent was allowed to drip from the heart through the cut pulmonary trunk, the ATP contents during control, hypoxia, and recovery periods were (p-mole/min) 26·8 (±S.E. 6·6), 63·6 (±S.E. 10·3) and 25·3 (±S.E. 10·0), respectively. This resulted in an increased total loss of ATP; nevertheless, the *difference* in ATP content between control and hypoxic coronary effluent samples is virtually the same for both sets of experiments (40·2 p-mole/min *vs.* 42·9 p-mole/min for uncannulated).

Assessment of heart viability

While the correlation between the rise of ATP concentration in the sinus effluent and the development of hypoxia seems clear-cut, nevertheless the question of cellular damage, and hence leakage of ATP through incompetent membranes, must be addressed. All hearts used in this study fully regained control levels of aortic pulse pressure, but this cannot discount the possibility of membrane damage during the hypoxic period. It may simply indicate that the damage was *reversible* and that membrane reconstitution was dependent upon a renewed supply of O_2.

Various experiments were carried out which together indicate that these heart preparations were at an acceptable level of viability when compared to those of other workers.

TABLE 1. Response to incremental change in left atrial pressure

Preload (cm H_2O)	S_p (mmHg)	CO (ml./min)	T_c/T_e	W_P	W_K	Power (mW)
10	88 (82)	19·2 (22·3)	1·86 (2·2)	3·75 (4·06)	0·0116 (0·025)	3·76 (4·09)
15	90 (87)	27·8 (30·6)	2·40 (2·2)	5·56 (5·92)	0·059 (0·066)	5·62 (5·98)
20	92 (90)	37·1 (39·3)	2·40 (2·2)	7·58 (7·86)	0·14 (0·139)	7·72 (8·00)
10	85 (85)	19·8 (22·0)	3·99 (2·2)	3·74 (4·16)	0·033 (0·024)	3·77 (4·18)

Second heart results in parentheses. Diameter of aortic cannula = 1·68 mm.

S_p = peak systolic pressure; CO = cardiac output; T_c/T_e = ratio of cardiac cycle time to ejection time; W_p = pressure power; W_k = kinetic power.

Display of Frank-Starling mechanism. The preparations rapidly responded to a change in left atrial filling pressure. Table 1 shows the results from two preparations in which the left atrial pressure was rapidly changed in increments of 5 cm H_2O. The pulse pressure and stroke volume increased to new levels within 3–4 beats (heart rate = 200/min). It can be seen that while the systolic pressure was only slightly altered, the cardiac output faithfully reflected the change in preload, with kinetic work (W_K) being greatest at the greatest value of cardiac output.

Measurement of myocardial O_2 consumption. The O_2 consumption of two working heart preparations was measured with the left atrial filling pressure set at 10 and 15 cm H_2O and the aortic pressure set at 65 cm H_2O. Advantage was taken of the fact that the hearts are set up in the Langendorff (non-working) mode of perfusion before insertion of the left atrial cannula and imposition of an afterload on the left ventricle (see Methods). Thus a comparison between the O_2 consumption in the non-working and working mode could be made on the same hearts. Table 2 gives a summary of the results obtained from two hearts first set up in the non-working mode and then set to work at two left atrial pressures, 10 and 15 cm H_2O. The coronary sinus p_{O_2} ranged from 123 to 209 mmHg, indicating an O_2 extraction much less than the maximum that can be achieved, since *in vivo* coronary sinus p_{O_2} is usually less than 20 mmHg (Keele & Neil, 1971). Also, the hearts were able to increase the O_2 extraction in response to an increase in left atrial preload of 5 cm H_2O. It is thus likely that at the lower workload (10 cm H_2O), at which the ATP experiments were performed, the hearts were adequately oxygenated.

TABLE 2. Response in two hearts to change in left atrial pressure at zero and 10 and 15 cm H_2O

L. atrial filling pressure (cm H_2O)	Non-working (Langendorff)	Working	
	0	10	15
Coronary flow (ml. min^{-1})	7·2 (8·6)	11·4 (11·7)	12·2 (11·4)
Coronary sinus p_{O_2} (mmHg)	170 (209)	157 (152)	123 (140)
$\dot{V}_{O_2}$ (ml. min^{-1}. 100 g^{-1})	8·3 (10·3)	12·9 (16·1)	14·9 (19·4)

Coronary flow during perfusion with aqueous buffer. In the control period of perfusion the coronary flow was 9 ml./min. Despite this high control flow, however, the introduction of hypoxic buffer perfusion increased the flow to 11·6 ml./min. (Fig. 5). The quantitative aspect of the adequacy of oxygen supply to the working heart will be discussed later (see Discussion, p. 153).

Computation of the power output. Two experiments were performed to calculate the power output of hearts working at different preload levels. The left atrial filling pressure (cm H_2O) was set at the usual 10, then increased to 15, 20, and back to 10. The two equations derived by Kannengiesser *et al.* (1979) were used to compute the power outputs (see Methods, p. 147). Pressure can be approximated either as the average systolic pressure (Neely *et al.* 1967) or as the peak systolic pressure (Kannengiesser *et al.* 1979), as done here. The former gives an underestimation of the pressure power whereas the latter gives an over-estimation.

These results can be compared with those obtained by Kannengiesser *et al.* (1979) who found a mean power output (mW) of 13·3 (±0·5) and 14·0 (±0.8) after 2 and 9 min respectively. The peak systolic pressure in their experiments was 163 as against 85 mmHg in the present work. The significance of this comparison is that ATP has been identified in the coronary sinus effluent from hearts which are probably not exerting their full power output. It is noteworthy (see Discussion) that the working heart preparation produced almost eight times the concentration of ATP in the sinus effluent in response to hypoxia than that demonstrated by Paddle & Burnstock (1974) in the non-working heart, and yet still has the capacity for further power output.

Exhibition of Ca paradox. In 1966 Zimmerman & Hülsmann described the occurrence of sarcolemmal damage when Ca was restored to a heart previously deprived of Ca. This they termed the 'Ca paradox'. Experiments in this work have shown that deprivation of Ca from the working myocardium with subsequent restoration abolished irreversibly the ability of the heart to contract. In six heart preparations working at the usual load (preload, 10 cm H_2O; afterload, 65 cm H_2O), switching from the normal perfusion buffer to Ca-free buffer with 1 mM-EGTA caused loss of mechanical activity within 30 sec. No activity was possible after restoration of Ca in 10 min. In two hearts ATP, 7 and 10 nM, appeared in the coronary sinus effluent after 30 and 60 sec of Ca-free perfusion, respectively. These findings indicate that ATP may be lost from the cell (or membrane) during the period of Ca restoration. The main point to be made is that these heart preparations responded like other myocardial preparations to Ca-free perfusion, probably indicating similar healthy membrane structure.

DISCUSSION

ATP has been detected in the coronary sinus effluent from the working heart preparation; upon rendering the heart hypoxic the concentration in the effluent increased eightfold. The total amounts released during the hypoxic period were calculated to be 46·1 p-mol/min. Ruigrok, Boink, Spies, Blok, Maas & Zimmerman (1978) report tissue ATP levels of 18·8 μmole . g dry wt^{-1} for oxygenated isolated rat hearts; assuming 0·2 g dry wt. per heart, the total tissue ATP would amount to approximately 4 μmole. The value of 46·1 p-mole thus represents a loss of less than 0·001 % of the total tissue ATP per minute. These rates are far below the reported rates of *de novo* synthesis of adenine nucleotides in rat heart (Zimmer, Trendelenberg, Kammermeier & Gerlach, 1973).

It is not possible to attribute the source of ATP to any single tissue in a perfusion experiment such as the one performed in this work, although the formed elements in the blood can be discounted since perfusion was with a saline solution. Apart from the myocardial cell, ATP could be released from nerves, vascular endothelium, vascular smooth muscle or some other unknown source. Paddle & Burnstock (1974) suggested 'purinergic' nerves as a possibility, although to date there is no evidence for the existence of such nerves associated with heart muscle. Pearson & Gordon (1979) have demonstrated selective release of adenine nucleotides, including ATP, from cultured vascular endothelial and smooth muscle cells in response to 'potentially damaging stimuli' such as trypsin and physical trauma. While this release of nucleotides was not accompanied by perceptible cell damage, it is likely that these stimuli are considerably more severe than 90 sec hypoxia. ATP is known to be released along with catecholamines (Douglas, Poisner & Rubin, 1965), and hypoxia may exacerbate this release from adrenergic nerve terminals. The previous finding that ATP is released from isolated adult rat heart cells in response to very brief periods of hypoxia (Forrester & Williams, 1977) clearly indicates the myocyte as one definite source. So although the proportion of ATP released into the coronary sinus effluent from the myocyte is unknown at the present time, it is probable that the contribution is a significant one.

The coronary flow rate in the present experiments was markedly elevated at 500 ml. 100^{-1} g . min^{-1} in the control period, suggesting that these preparations might be hypoxic from the outset. The rate of perfusate flow required to satisfy the myocardial O_2 demand can be readily assessed. Since blood is approximately 3·6 times more viscous than aqueous solution, and flow is inversely proportional to the first power of the viscosity (for straight tubes), for any given vascular resistance and perfusion pressure the flow should be at least 3·6 times greater for an aqueous solution than for blood. If we assume an *in vivo* O_2 consumption rate of 15 ml. min^{-1} . 100 g^{-1} for an atrial filling pressure of 10 cm water, and 70 % O_2 extraction from blood (Rushmer, 1976), then a coronary blood flow of 1·7 ml. min^{-1} would be necessary to supply the oxygen requirements. In fact, the mean coronary perfusion flow during the control periods was 9·6 ml. min^{-1}. The vascular resistance required for this flow rate of *aqueous medium* would give a *blood flow* of 2·3 ml. min^{-1}. Other observations demonstrate that the hearts were satisfactorily oxygenated throughout the control periods. First, introduction of hypoxic conditions caused an immediate increase in

coronary flow. Secondly, the coronary sinus p_{O_2} ranged from 123 to 209 mmHg, indicating that a considerable proportion of oxygen in the perfusate was not being utilized. Thirdly, the hearts were able to increase O_2 extraction in response to an increase in left atrial preload (Table 2). It is probable that at the low workload (10 cm water) imposed, the hearts received sufficient O_2 throughout the control period. Thus, although the coronary vasculature is more dilated when perfused with aqueous buffer solution, the extent of this dilatation is moderate and allows further dilatation to take place in the face of increased O_2 demand.

The amounts of ATP appearing in the coronary sinus effluent from working heart can be compared to the values found by Paddle & Burnstock (1974) using the non-working guinea-pig heart preparation. Although the guinea-pig heart is probably larger than the rat heart, for simplicity the same size will be assumed. A further assumption is that extracellular ATPase activity remains constant in both working and non-working preparations. The mean flow rate for the non-working preparations was 5·8 ml. min^{-1}; the mean concentration of ATP in the coronary effluent before hypoxia (90 sec duration) was 0·225 nM and after hypoxia was 0·67 nM (Paddle & Burnstock, 1974, Table 1). Thus the average total amount of ATP increased from 1·3 to 3·9 p-mole . min^{-1}. In the working heart the average total amount in the coronary effluent increased from 5·9 to 46·1 p-mole . min^{-1} for the same duration of hypoxia. Thus, the working heart releases more ATP than the non-working heart in the oxygenated state, a strong indication that the release of ATP is coupled in some way to O_2 demand. It is evident that the imposition of hypoxia upon the working heart provides a strong stimulus for release of ATP.

When the role of ATP in the dilatory response is considered, it becomes necessary to assess the actual amounts that are released into the extracellular space. Paddle & Burnstock (1974) showed that only 1% of infused ATP was recovered in the sinus effluent. Thus we can suppose that the concentrations detected in the effluent from hypoxic myocardium represent only 1% of the actual amounts present in the perivascular space. This assumes no change in the extracellular ATPase activity with increased myocardial work or hypoxia and also no account is taken of the surface ATPase activity of the myocardial cells (Williamson & DiPietro, 1965). McCruden (1970) demonstrated that the threshold of coronary vasodilatation in response to ATP lay between 6×10^{-8} and $5{\cdot}6 \times 10^{-7}$ M entering rabbit coronary arteries; no allowance could be made for any attenuation of ATP by the vascular endothelium. This would indicate that only a threshold effect would occur with $4{\cdot}7 \times 10^{-7}$ M concentration perivascularly. However, recent work has shown that cat pial vessels have a threshold response to ATP at 10^{-10} M applied to the adventitial side (Forrester, Harper, MacKenzie & Thomson, 1979). Obviously $4{\cdot}7 \times 10^{-7}$ M would profoundly affect the calibre of the resistance vessels, provided the sensitivity of the two types of vessel to ATP were the same.

The eightfold rise in the amounts of ATP released from the working heart during the hypoxic period probably signifies a major role for ATP in the vasodilatory response of the coronary arteries to hypoxia. The levels of ATP found in the effluent during the control periods indicate that ATP was being continuously released, even when the myocardium was oxygenated. This may contribute to the typical high 'resting' flow rate found in myocardium. Continuous release of ATP has already been

observed in the soleus muscle (Forrester & Hamilton, 1975) which also has a high resting flow rate.

The comparative roles of ATP, ADP, AMP, and adenosine in the control of coronary flow are unclear at the present time. Although adenosine has been implicated in the dilator response of coronary arteries to hypoxia (Berne, 1964), the

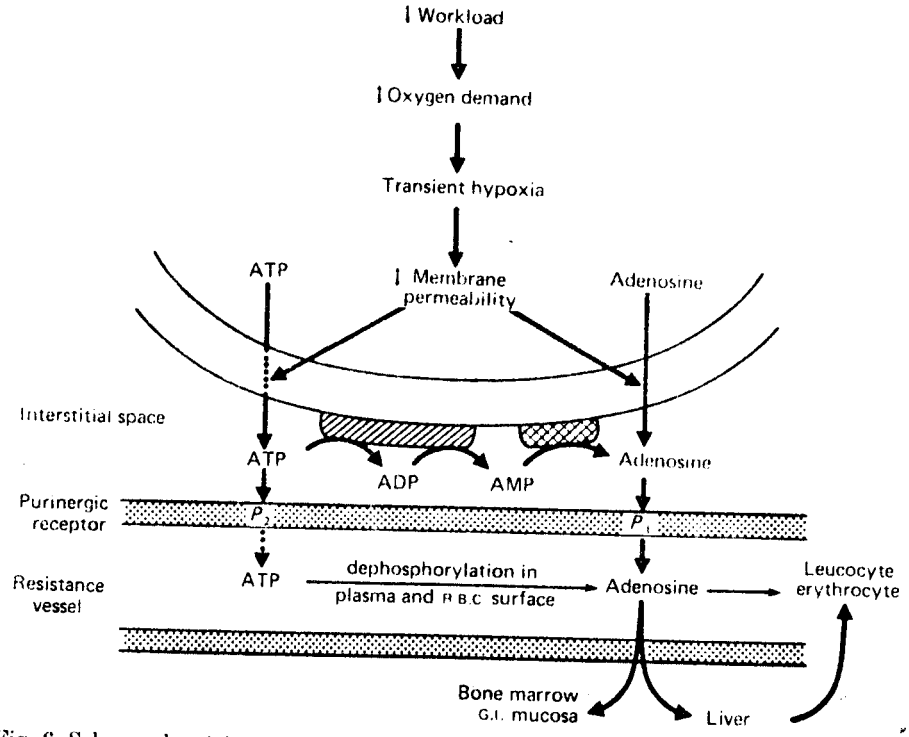

Fig. 6. Scheme showing proposed link between an increase in myocardial workload and dilatation of local resistance vessels. Increase of membrane permeability to adenosine and ATP is triggered by some unknown means through the effect of transient hypoxia on the cell membrane. Although permeability of membrane to adenosine is greater, the potency of ATP as a vasodilator in the coronary circulation is greater than that of adenosine (Wolf & Berne, 1956). Interstitial space contains ectoATPases which dephosphorylate (▨) (▩) ATP to adenosine (Pearson & Gordon, 1979). Not depicted are ATPases which face outward from the vascular smooth muscle. Adenosine and ATP act upon purine receptors P_1 and P_2 respectively (Burnstock, 1978). Residual ATP is slowly dephosphorylated in the blood plasma and more rapidly degraded (or taken up) by the erythrocyte. Adenosine is finally distributed to tissues unable to synthesize the purine ring moiety *de novo* (Pritchard *et al.* 1975). Not shown is the further degradation of adenosine to inosine and hypoxanthine in the interstitial and vascular compartments.

hyperaemia associated with myocardial hypoxia is not blocked by aminophylline, whereas the action of adenosine is (Afonso, Ansfield, Berndt & Rowe, 1972). Furthermore, Giles & Wilcken (1977) demonstrated that the vasodilatory action of ATP upon the coronary circulation is not blocked by aminophylline.

The recent characterization of outward facing 5′-nucleotidase in the perfused rat heart (Frick & Lowenstein, 1976), the demonstration of surface ATPase activity in

rat myocardial cells (Williamson & DiPietro, 1965) and the ecto-ATPase activity described in porcine vascular endothelium and smooth muscle (Pearson & Gordon, 1979) allow the following proposal with regard to the role of adenine nucleotides in the local control of myocardial blow flow (Fig. 6).

Adenine nucleotides and adenosine can all act as vasodilators, ATP being the most potent compound. Inosine and hypoxanthine do not have any vasodilatory action (Wolf & Berne, 1956). The presence of extracellular ATPase activity suggests that ATP released from hypoxic myocardium is rapidly broken down by stepwise dephosphorylation to adenosine (see discussion by Pearson & Gordon, 1979). That the vasodilatory action of adenosine, but not that of hypoxia or ATP, is blocked by aminophylline makes it more likely that the principal vasodilator involved is ATP. Degradation products can be taken up locally by the myocardial cell or distributed to those cells that cannot synthesize the purine ring *de novo*, such as erythrocytes, leucocytes, bone marrow cells and cells of the gastrointestinal mucosa. Such distribution can take place either directly in the bloodstream or via the liver (Pritchard, O'Connor, Oliver & Berlin, 1975).

Such findings once again emphasize the fundamental problem of how ATP is released from the hypoxic cell. It is plausible to regard the intrinsic membrane protein as a source of ATP (see Discussion in Forrester & Williams, 1977). This would mean that ATP in the first instance came *from* the membrane rather than through it and implies some compartmentalization of ATP in the cytoplasm for purposes of replenishment. In any event, elucidation of the release mechanism may further our understanding of the behaviour of heart cell membranes exposed to low O_2 tension.

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Commentary - paper 21.

The second Satellite Symposium on 'Mechanisms of Vasodilatation' was held in Antwerp, July, 1980. This paper is the response to an invitation from the organizing committee. The problem with adenosine and its action being blocked by methyl xanthine derivatives, together with the developing purinergic nerve theory proposed by Burnstock, led to the unusual (and challenging) title - 'Adenosine or Adenosine Triphosphate?' which was given to this author. Upon reviewing the evidence for both compounds it is concluded that perhaps adenosine has a role to play in post-exercise hyperaemia, while ATP may be important in ongoing exercise hyperaemia. The great potency of ATP as a vasodilator and the mechanisms of swift extracellular degradation indicate a powerful extracellular system which is kept under strict control by exogenous nucleotidases.

ADENOSINE OR ADENOSINE TRIPHOSPHATE?

T. Forrester

Department of Physiology, Saint Louis
University Medical Center, Saint Louis
MISSOURI 63104

INTRODUCTION

It has long been suspected that the mechanisms for local blood flow control may vary from one type of tissue to another, and it is slowly emerging that local control mechanisms may basically differ within the same type of tissue. For example, the mechanisms for dilatation in cardiac muscle may be quite separate from those in operation for skeletal and smooth muscle. Another variation of mechanism within muscle types may be termed 'temporal,' that is, the mechanisms responsible for exercise hyperaemia may differ from those giving rise to reactive or post-exercise hyperaemia in the same muscle. At the present time we can only speculate as to the different mechanisms involved. Thus the question posed in the title must be supplemented by a more specific question: 'for what purpose?'

The immediate dilatation seen in skeletal muscle blood vessels in response to even the mildest exercise would seem to indicate that exquisite forms of response and adjustment are present. The potency of ATP and adenosine in dilating the blood vessels of heart and skeletal muscle is well recognized. It is therefore the purpose of this review to evaluate the roles of adenosine and ATP in the exercise hyperaemia of skeletal and cardiac muscle.

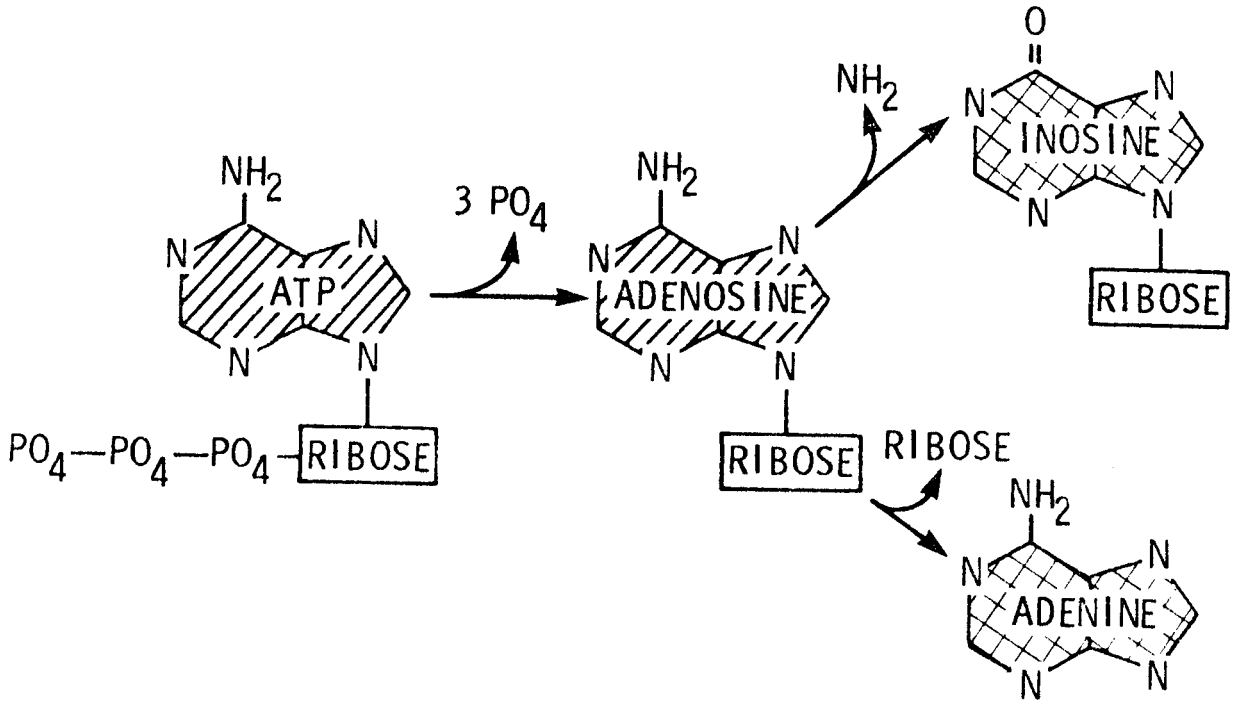


Fig. 1. Degradation of ATP to inosine and adenine via adenosine. Loss of the amino group or the ribose moiety from the purine ring abolishes action of the molecule as a vasodilator. ATP and adenosine are powerful vasodilators of the coronary and skeletal muscle blood vessels.

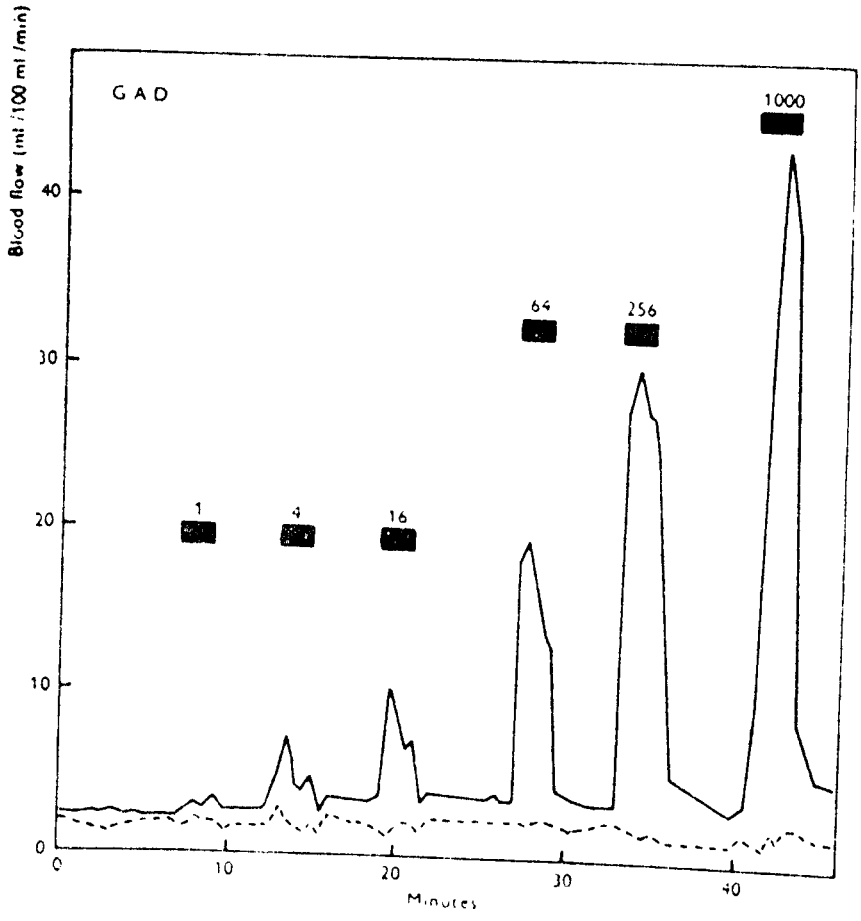
HISTORIC

Development of the Concept

Dilatation of resistance blood vessels by the action of 'metabolites' released from local tissues is a fundamental concept that is traditionally attributed to W.H. Gaskell (49)*. One of the earliest statements suggesting the involvement of purine compounds in this process is to be found in a paper by Barcroft & Dixon (11). Presenting studies on the metabolism of the puppy dog heart they stated: 'In our experiments the carbon dioxide levels may be only an index of other metabolic products such as lactic acid and purine bodies which have a similar vasodilatory action.' Quite how the concept arose of purine body release from active myocardium is presently unclear. More than twenty years later Drury & Szent-Gyorgyi (34), investigating the properties of muscle extracts applied externally to the heart and blood vessels, found that certain purine purine compounds were completely inactive, whereas others of similar structure were extremely potent. Fig. 1 shows the structure-function relationship of the adenine series of purine compounds. Loss of the amino group at position 6 in the purine ring, or loss of the ribose moiety from position 9, virtually abolishes exogenous activity of the molecule as a vasodilator. These findings were soon verified and extended (101, 103, 128, 55, 14, 38). Earlier Zipf (136, 137, 138) had suggested that the

*In fact two years before Gaskell's paper Latschenberger & Deahna (Pflugers Arch. 12, 157) had suggested that metabolites such as weak acids could be involved in the local dilatation process.

Fig. 2. The effect on forearm blood flow of graded doses of magnesium ATP injected over 2 min periods into the brachial artery of a normal subject. Dotted line is control obtained from the contralateral arm. Thick bars, times of perfusion with dose in ug/min. Note that 16 ug/min increases blood flow by about three times. (From Duff, Patterson & Shepherd, 1954, J. Physiol. London).



vasodilator substances in the blood returning from ischaemic or hyperaemic tissues may be adenylic acid or adenosine.

A general account of the actions of exogenous adenosine triphosphate (ATP) was given by Green & Stoner in 1950 (57). This was the result of an urgent enquiry during World War II into the cause of irreversible wound shock. Extracellular purine compounds, especially ATP, could obviously reach the extracellular compartment and the bloodstream following acute trauma; this in turn could precipitate a widespread and long-lasting peripheral vasodilatation. At this time Buchthal, Deutch & Knappeis (19) showed an effect on frog skeletal muscle after close arterial injection. Also at this period an interesting paper by Emmelin & Feldberg (37) appeared in the British Journal of Pharmacology describing the cardiovascular effects of intravenously injected ATP; however the rationale for this experiment was not explained. Perhaps ATP was then being regarded as a potential therapeutic agent. In 1954 the action of intra-arterial ATP on the blood vessels of the human forearm was described by Duff, Patterson & Shepherd (35) Fig. 2. The vessels were greatly sensitive to ATP, especially the magnesium salt, and also responded to the injection with a hyperaemia which matched that to be found in muscles exercising vigorously. When compared to other vasodilators (histamine, acetylcholine) magnesium ATP infusion proved to be as painless as it was potent.

Application of purine compounds to the heart continued. It became clear that the adenine compounds, especially ATP, were capable of dilating the coronary resistance vessels to a near

maximum and to an extent which matched naturally occurring hyperaemia (132, 133). Other studies on the exogenous action of adenine compounds on cardiac tissue soon followed (e.g. 126, 72).

Why were these investigators applying exogenous ATP to various tissues? Was the idea of 'purine body' release implicit in these accounts? Perhaps these questions can be answered during the discussion period.

Release of Purine Compounds

In the period from the 1930s to the publication of their classical papers in 1952 Hodgkin & Huxley (62) provided a quantum leap forward in our understanding of the permeability of cell membranes to charged particles. Their work supported the concept of the membrane as a bilipid barrier with 'gates' or 'pores' which could open and close. Indeed it has now been computed that one segment of the sodium channel is a pore 3 Å by 5 Å in dimension and another part, facing outwards from the membrane, is 9 Å by 10 Å (60, 61). Thus the contemplation of any highly charged molecule passing across a cell membrane becomes a difficult one and usually when evidence suggests such a transferance this brings into question the competence of either the membrane or the investigator. Nevertheless in the face of this theoretical obstacle Holton & Holton (64) detected an increase in the absorption spectrum at 260 nm from fluid which had perfused stimulated sensory nerves. They tentatively suggested that ATP might be the transmitter substance released antidromically from sensory nerves to cause local vasodilatation ('axon reflex'). The full paper was published six years later (65) with positive

identification of the substance as ATP. Release of ATP along with catecholamines was described later (33).

No such permeability problems existed for adenosine, however. The cell membrane seemed highly permeable to adenosine and its breakdown products (27, 63, 82, 70) and a flux figure has been calculated for skeletal muscle membrane, suggesting a 'facilitated diffusion' process (46). There is some evidence to suggest that ATP can penetrate hypoxic myocardial cell membrane as the whole molecule (135).

THE ADENOSINE HYPOTHESIS

Adenosine and the Coronary Circulation

It is largely due to the work of R.M. Berne and his colleagues that adenosine is at present regarded as a significant natural local vasodilator in the coronary circulation. In 1960 Jacob & Berne (70) described the rapid penetration of the myocardial cell membrane by adenosine. They predicted that the outward movement of the molecule would occur with similar ease. Release of inosine and hypoxanthine in response to myocardial hypoxia was found by Berne in 1963 (13). The assumption was made that these were the degradation products of adenosine which had been released from the myocardial cell during a period of hypoxia. The enzymic activity required for such extracellular degradation had already been defined (27, 10). Both the prediction and assumption were soon verified by Richman & Wyborny (100) who showed that if adenosine deaminase activity was inhibited by 8-azaguanine adenosine was easily detected in the effluent from hypoxic heart. Katori & Berne (76) verified this result and also provided important data

Table 1. Distribution of purine derivatives appearing in the coronary sinus effluent from non-working guinea pig hearts in the presence of 10^{-3}M 8-azaguanine.

	Anoxia	Severe Hypoxia
adenosine (nmoles)	55 ± 15	64 ± 27
inosine (")	167 ± 56	42 ± 50
hypoxanthine (nmoles)	82 ± 30	61 ± 25
Totals	304	167

Severe hypoxia indicates levels of oxygen tension around 40 mm Hg.

(Katori & Berne, 1966)

suggesting that as the oxygen demand of the myocardium increased, so also did the levels of released adenosine. Table 1 gives some data summarized from Katori & Berne (76). Samples from isolated guinea pig hearts perfused in the Langendorff mode were analysed for adenosine, the deaminated derivative inosine and the purine ring compound hypoxanthine. All experiments were performed in the presence of 8-azaguanine. The total purine amounts were greater in the sinus effluent from totally anoxic hearts, it is interesting that much more inosine appeared in the samples from the anoxic tissue. Perhaps the degree of hypoxia affected the removal of the ribose moiety from the purine ring (inosine----→hypoxanthine). Unknown effects from a mM concentration of 8-azaguanine may also have been in operation.

Improved sensitivity for adenosine detection enabled investigators to discard the use of 8-azaguanine. Soon adenosine was detected in the pericardial space (104) and in the coronary sinus effluent during reactive hyperaemia (106). Quantitatively there seemed to be enough to produce a useful degree of vasodilatation in response to the stress. In 1977 Wiedmeier & Spell (130) answered the 'hen and egg' question: is dilatation responsible for adenosine release or is adenosine release responsible for vasodilatation? They showed no release when dilatation was evoked without an oxygen demand.

Actions of methylxanthines and dipyridamole. Thus far the evidence for adenosine being a principal vasodilator in the coronary circulation was satisfactory, but some problems have arisen out of experiments using the adenosine antagonist drugs theophylline and aminophylline. In 1970 Afonso (3) and Afonso

& O'Brien (4) showed that the methylxanthine compound aminophylline inhibited the coronary vasodilator action of exogenous adenosine when given either intravenously or directly into the coronary artery. Thus a crucial experiment was developed to validate the adenosine hypothesis. If hypoxia-induced release of adenosine was responsible for relaxation of the vascular smooth muscle, would administration of aminophylline inhibit hypoxia-induced vasodilatation? Afonso et al (5) found that aminophylline did not significantly influence coronary vasodilatation induced by hypoxia. This result was confirmed by Wadsworth (127). The competitive nature of this inhibitory action was demonstrated by Bunger et al (21) using theophylline on the guinea pig heart. Methylxanthines have been found to be adenosine competitors in many other tissues (109, 30, 89, 6, 85, 110). A complicating feature of methylxanthine infusion was emphasized by Curnish et al (28). They made the point that since these compounds increased the myocardial oxygen consumption and demand - 'it is conceivable that with large doses of aminophylline attenuation of reactive hyperaemia is masked by the overriding effect of high concentrations of endogenous vasoactive metabolites.' Perhaps ATP belongs in this latter class since Giles & Wicken (54) have clearly shown that the action of ATP on the coronary arteries is unaffected by aminophylline.

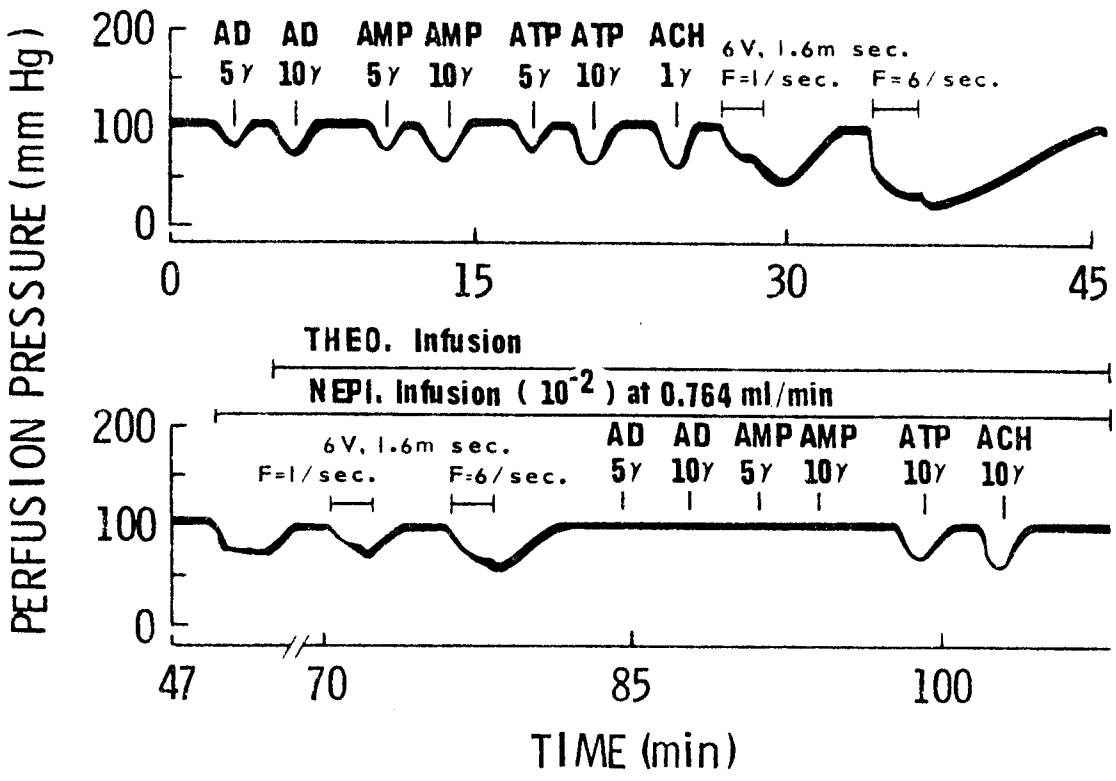
Potentiation of the action of adenosine by the drug dipyridamole has been frequently described (84, 15, 73, 92, 94, 107, 75). Since dipyridamole can potentiate hyperaemia it would seem plausible that adenosine therefore contributed to the hyperaemic process. However, dipyridamole can also

potentiate the action of ATP (108), so it is not really possible to discriminate between the relative roles of adenosine and ATP with the use of this drug.

Adenosine and Skeletal Muscle Circulation

Early observations on the vasoactivity of purine compounds in the skeletal muscle vascular bed were more concerned with the actions of AMP ('adenylic acid'), ADP and ATP than with adenosine (38, 39, 111, 74). However, the coronary action of adenosine led into investigations as to whether adenosine might also play a part in skeletal muscle blood flow regulation. Adenosine was reported to be a powerful dilator of these vessels (59) and Scott *et al* (113) provided some evidence that adenosine as well as ATP and AMP could be responsible in part for dilatation in skeletal muscle.

The usual approach of venous effluent analysis has been used and adenosine has been readily identified in the effluent in response to muscle stimulation and contraction (32, 16, 17). Some problems are associated with these observations. For example, Gerlach (50, 51, 52) had shown that in skeletal muscle, in contrast to heart muscle, adenine nucleotide degradation occurs *via* IMP formation. Only traces of adenosine are produced. IMP is not vasoactive. This did not suggest a role for adenosine in exercise hyperaemia. In answer to this dilemma Rubio *et al* (105) defined specific sites of adenosine production in skeletal muscle. Nucleotidase activity was found in endothelium and in localized zones within muscle cells in close proximity to blood vessels. It was suggested that adenosine is produced only in the vicinity of the blood vessels, but elsewhere in the muscle ATP is converted to IMP as Gerlach



Tabaie, Scott and Haddy (1977)

Fig. 3. Effects of ATP, AMP, adenosine, and stimulation on the resistance of perfused isolated gracilis muscle of dog. Upper trace shows that ATP is slightly more potent than adenosine as a vasodilator. Stimulation produces two phases of dilatation, exercise hyperaemia and post-exercise hyperaemia. Lower trace shows that theophylline mostly abolishes the post-exercise component, but the exercise hyperæmia is largely unaffected. The action of adenosine is abolished by theophylline whereas the action of ATP is not, suggesting that adenosine has a role to play in post-exercise hyperaemia while ATP may be involved in exercise hyperaemia. (From Tabaie, Scott & Haddy, 1977).

had demonstrated. Bockman et al (17) showed that in blood-perfused dog hindlimb at constant flow the venous plasma adenosine concentrations did not increase during muscle contraction (stimulated at 2 - 4Hz). They also found that the adenosine content of the muscle was not significantly elevated after five minutes of stimulation, although an increase was noted after ten minutes contraction.

Action of methylxanthines. As in the coronary circulation the methylxanthine derivatives block the vasodilatory action of adenosine on skeletal muscle blood vessels (121, 66). Fig. 3 shows an average representation of results obtained by Tabaie, Scott & Haddy (121) using the blood-perfused isolated dog gracilis muscle. In the presence of 10^{-3}M theophylline the action of infused adenosine was completely blocked, but the effect on the blood flow of stimulating the muscle at 1 Hz for 30 seconds was not. On closer inspection two components can be seen in the dilatation response produced by stimulation. There is an exercise hyperaemia and a pronounced post--exercise hyperaemia. It can be seen that while the exercise component is not much affected, the post-exercise component is almost completely blocked. The base-line flow in those experiments was adjusted to control levels with the use of norepinephrine. Measurements of contractile force and venous oxygen tension indicated that the attenuation of dilatation did not result from a decrease in muscle activity. It can also be seen that the action of ATP is completely unaffected by theophylline infusion.

Recently Honig & Frierson (66), using dog gracilis muscle,

verified the result with theophylline and also showed that the adenosine-potentiating drug dipyridamole did not potentiate exercise hyperaemia. However, in about half of the muscles tested dipyridamole did slow the recovery of resistance after muscle contraction had ceased. They suggested that adenosine may have a role to play in prolonging the recovery of skeletal muscle blood flow after exercise.

RELEASE OF ADENOSINE TRIPHOSPHATE

Despite the fundamental problem of membrane permeability, much evidence has accumulated for the release of ATP from many different types of tissue. There follows a brief account of this literature.

Release from Nerve

Following the pioneer work of Pamela Holton (65), release of ATP from frog sciatic nerve was indirectly shown by Abood et al (1). Kuperman et al (78) verified this work. The conclusion at that time was that the ATP was released from electrically excitable tissue during the phase of depolarization. Pull & McElwain (99) showed that adenine nucleotides were released from electrically stimulated brain slices. When hypoxia was applied along with electrical stimulation the release was greatly augmented. They suggested that most of the nucleotide material may have been released in the form of ATP before rapid degradation by extracellular ATPases. This observation was repeated (79).

T.D. White (129) directly monitored release of ATP from synaptosomes prepared from rat brain using the sensitive luciferin/luciferase system. Both veratridine and high extra-

cellular potassium induced release. This work supported the concept that release occurred during the phase of membrane depolarization.

Release from Skeletal Muscle

Abood *et al* (1) were the first to demonstrate the outflux of ATP from activated skeletal muscle. Release was later demonstrated in isolated frog sartorius muscle, the identification using the highly sensitive perfused frog heart preparation and the luciferin/luciferase reaction (18). This work could not be confirmed by Dobson *et al* (32). One difference between the two experimental protocols is that the drug neostigmine was used by Boyd & Forrester (18) to augment the appearance of acetylcholine in the bathing solution. Neostigmine is known to have a direct action on the membrane of skeletal muscle (77) and also in the presence of neostigmine motor nerve fibres may repetitively fire in response to one stimulus, so that the number of impulses delivered to the muscle may have been underestimated. Dobson *et al* aerated the bathing solution through the periods of soaking and stimulation. Bubbling tends to destroy small amounts of ATP. The perfused frog hindlimb was used by Forrester & Hassan (43) and ATP was detected in the effluent in response to stimulation of the sciatic nerve. This suggested that the source was not damaged cells. Forrester & Hamilton (42) studied the release of ATP from blood- and Krebs-perfused cat soleus muscle. ATP levels in the plasma were 0.3 μM during stimulation at 10 Hz (control: 0.07 μM). Corresponding values for muscles perfused with Krebs solution

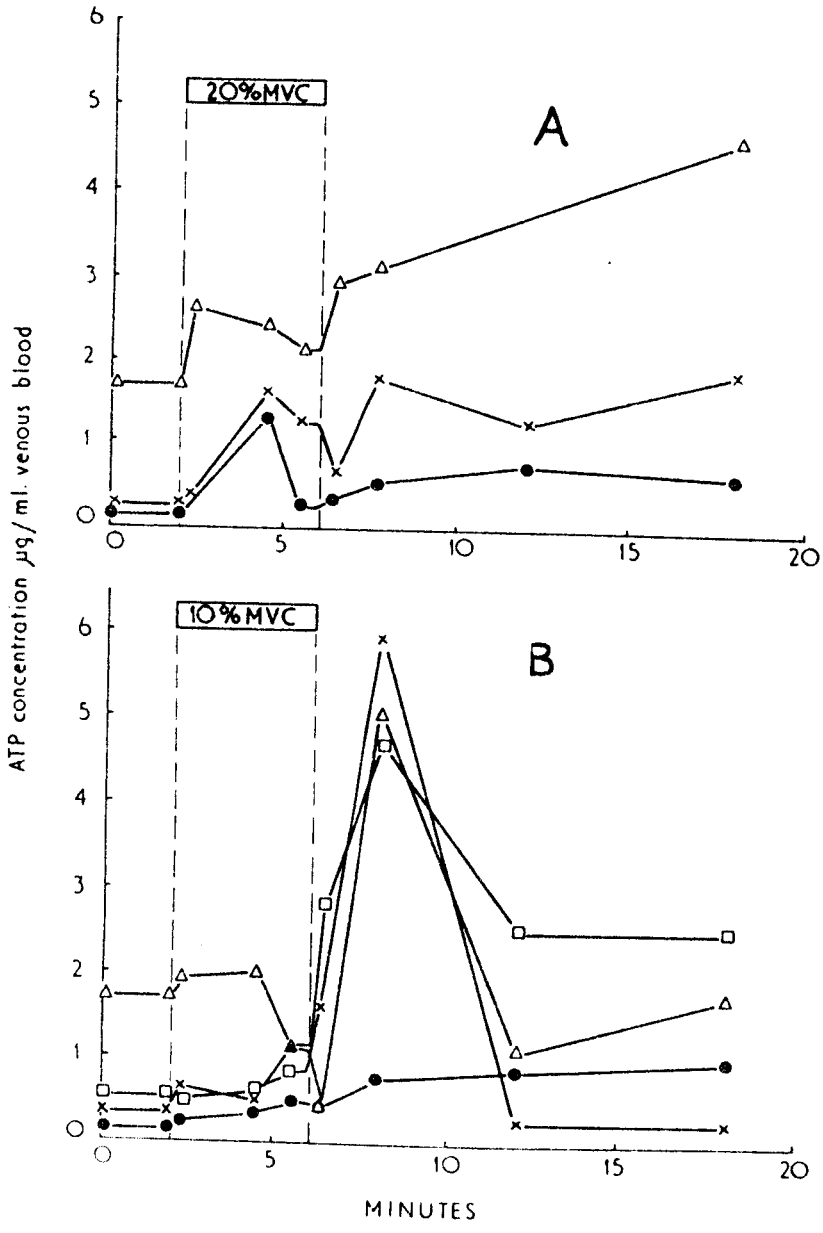


Fig. 4. ATP concentration (µg/ml) in the venous effluent from human forearm muscle before, during and after 20% (A) and 10% (B) maximum voluntary contraction. Different symbols represent different subjects. Rectangles show duration of contraction. Note the slow rise in concentration during the post-contraction phase in the 20% MVC (a tension eventually resulting in fatigue) in contrast to the sharp rise and fall in the non-fatiguing 10% MVC. (From Forrester & Lind, 1969).

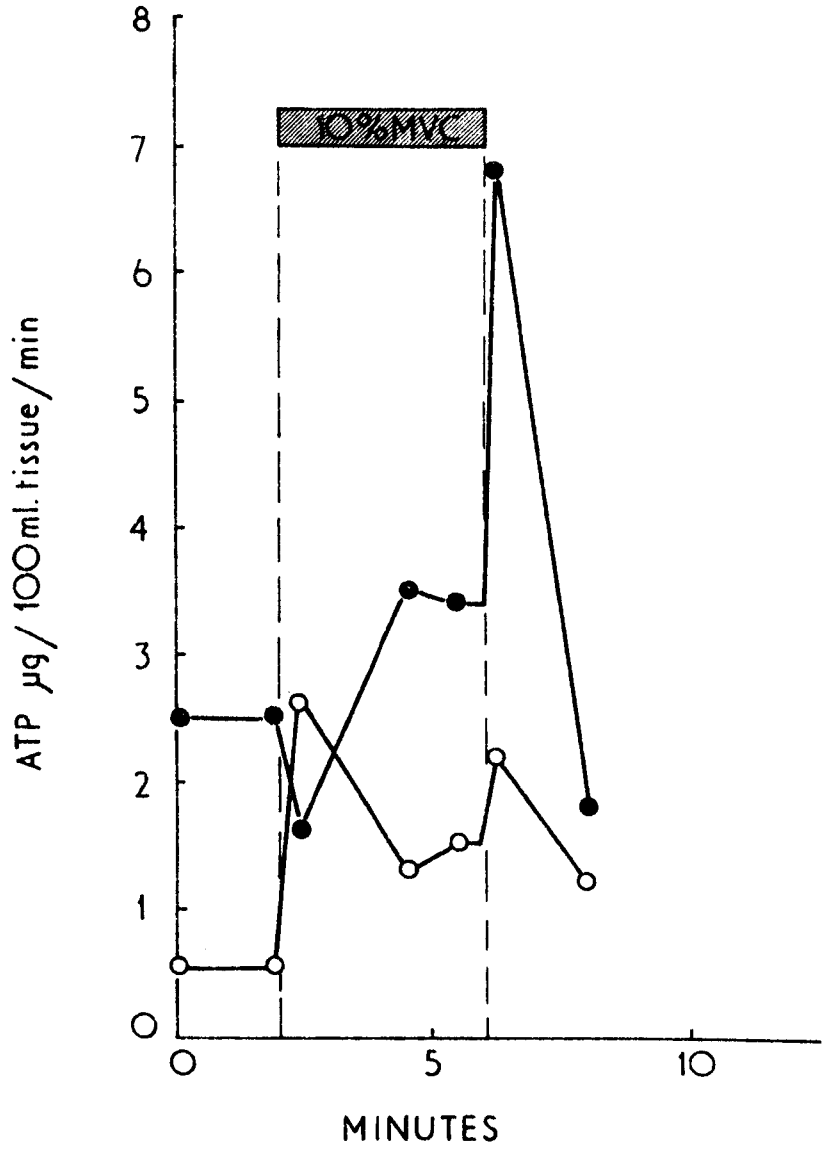


Fig. 5. Amounts of ATP measured in arterial (open circle) and venous (solid circle) blood of one subject before, during and after a 10% MVC. Note the crossover in first samples after onset of exercise. Arterial levels (from brachial artery) remain constant and below venous levels indicating that the ATP was added to the blood in its passage through the exercising muscle. (From Forrester & Lind, 1969).

were 1.8 μM (control: 0.9 μM). It was thought that the lower levels of ATP detected in the plasma could be due to surface ATPase activity of blood elements and the higher levels in the Krebs perfusate might have resulted from the release from hypoxic muscle.

Identification and assay of ATP in fresh human plasma (40) led to measurements of ATP coming from contracting human forearm muscles (45). Figs. 4 and 5 show that the concentrations were elevated and that it was added to the blood in its passage through the muscle. These results naturally led to the enquiry: is ATP released in sufficient quantities to satisfy the vasodilator requirements of active skeletal muscle? Previous studies on the effect of infused ATP on the circulation through human forearm musculature (35) provided an opportunity to compare the amounts of ATP released in vivo to the amounts infused to cause dilatation (see Fig. 2). The main difficulty with a quantitative study of this sort is the fact that an increase in blood flow through exercising muscle has the effect of washing out and diluting the ATP to an unknown extent. One way to avoid this difficulty is simply to occlude the arterial supply to the muscles during the period of exercise, and then gradually restore the flow, which will then pass through the line of least resistance, in this case the dilated vessels in the muscle bed. Human subjects gripped a bar isometrically at 5% of their maximum voluntary contraction in the presence of venous and arterial occlusion. This exercise is a mild form and normally gives rise to an exercise hyperaemia of only three times the resting flow (81).

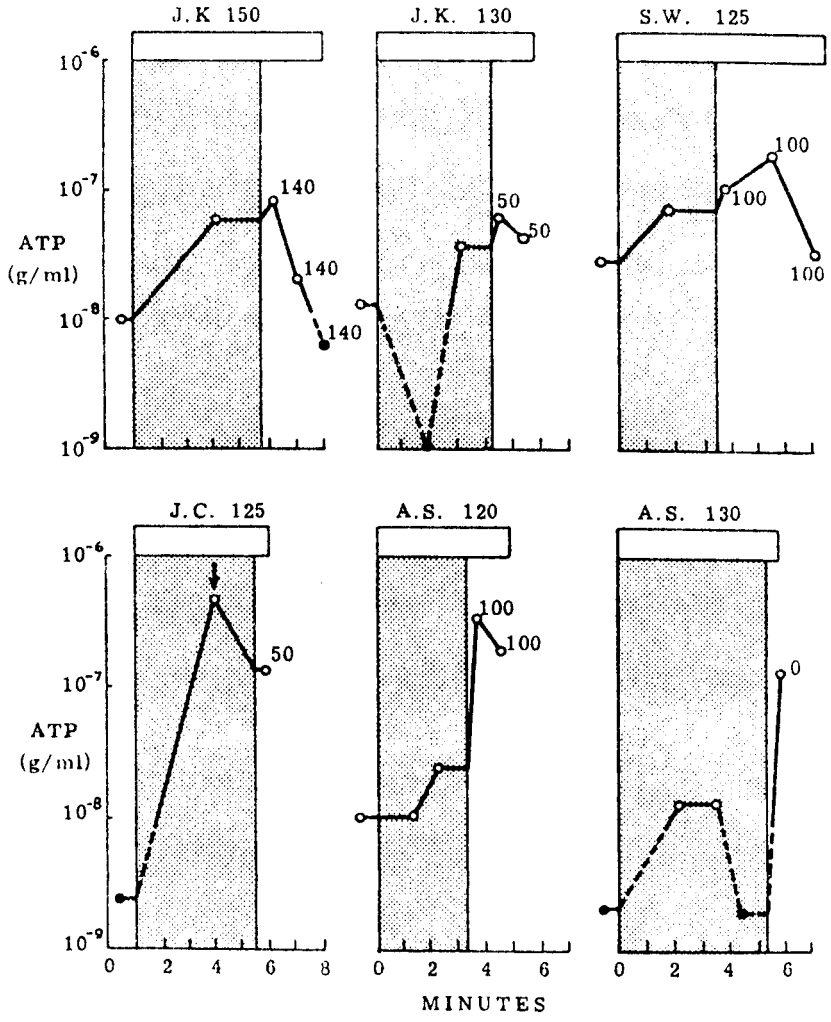


Fig. 6. Concentrations in venous plasma before, during and after arterial occlusion and exercise; shaded areas, time of arterial occlusion. Resting systolic blood pressure given above each figure. Filled symbols. samples below assay threshold (dashed lines). Vertical arrow in lower left shaded area, cuff pressure lowered to obtain sample. Figures above post-occlusion samples indicate cuff pressure when sample was taken. (From Forrester, 1972).

Duff et al (35) showed that a threefold increase in forearm flow was produced by infusing ATP, 16 g/min. (30 nmoles/min) into the brachial artery (Fig. 2). Fig. 6 shows the results obtained in six experiments on four subjects. Note the logarithmic ordinate scale, indicating a ten- to hundred-fold increase in the concentration at the end of the four minutes of exercise. These concentrations represent only a small fraction of the ATP originally present because the rapid degradation in the bloodstream could not be avoided. This factor would also certainly have diminished the effective concentrations of ATP infused by Duff et al (35). Allowing for the blood ATPases it was computed that 7.5 - 10.5 g/min. (14 - 20 nmoles/min) were released (41). These amounts compare well to the 16 g/min infused by Duff et al to produce the same threefold increase in flow (Fig. 2). Detection of ATP in the venous effluent from exercising muscle was also performed by Chen et al (25) using dog hindlimb. They analysed femoral venous blood for ATP and AMP before and during sciatic nerve stimulation at 6 and 25 Hz. Table 2 gives a summary of their results. It is clear that despite an increase in flow which would dilute the ATP levels, there is significant increase in ATP concentration in the venous effluent.

A fair criticism of this type of experiment is that the blood elements, especially blood platelets, contribute to the ATP levels detected in the plasma. Indeed this was the case in the work of Forrester & Lind (45) where platelets were shown to contribute up to half of the ATP in plasma from resting forearm muscle. Nevertheless with the use of non-

Table 2. Analysis of femoral venous plasma for ATP and AMP before and during sciatic nerve stimulation at 6/sec (S) and 25/sec (Tetany).

(N=10)	Control		S	Tetany	Post-tetany
	FA	FV	FV	FV	FV
ATP ng/ml plasma	206	165	289*	436*	484*
AMP ng/ml plasma	60	52	85*	188*	158*
FV flow ml/min	-	153	375*	233*	413*

FV, femoral vein flow, *, $P < .01$ compared to control.

(Chen, Selleck & Scott, 1972

wettable containers and cannulae the background levels of ATP can be reduced below the threshold for assay by firefly luminescence (41). This point was pursued by Bockman et al (16). They found a great variation in the amounts of ATP in venous effluent from resting skeletal muscle and concluded that changes in these concentrations could not be interpreted as significant when associated with exercising muscle. In a second series of experiments they stimulated dog hindlimb tetanically at 25 Hz. Three preparations were perfused at systemic pressure and four were perfused at a constant flow, chosen to be 2 - 3 times that of control flow. The ranges of ATP concentrations measured are shown in Fig. 7. Although no precise paired data were given, it is clear that in both cases there was a tendency for the range of ATP concentrations to increase in association with muscle contraction. It is of particular interest that the ATP concentrations found in plasma from stimulated muscle with a constant flow are higher than those found with a constant pressure. If indeed all of the ATP comes from the blood elements, then it is unclear why there should be any difference in range, whether the muscles are perfused with constant pressure or flow, or stimulated, or resting. Perhaps Bockman et al are suggesting that muscle contraction itself damages the blood elements in some way, in which case bouts of severe, prolonged whole body exercise would soon give rise to thrombocytopenia and possibly haemolysis. Also of significance is their finding that one hour after the muscle contraction the range of ATP in venous plasma was greatly increased to 0.09 - 2.97 nmoles/ml. Further studies are needed for clarification.

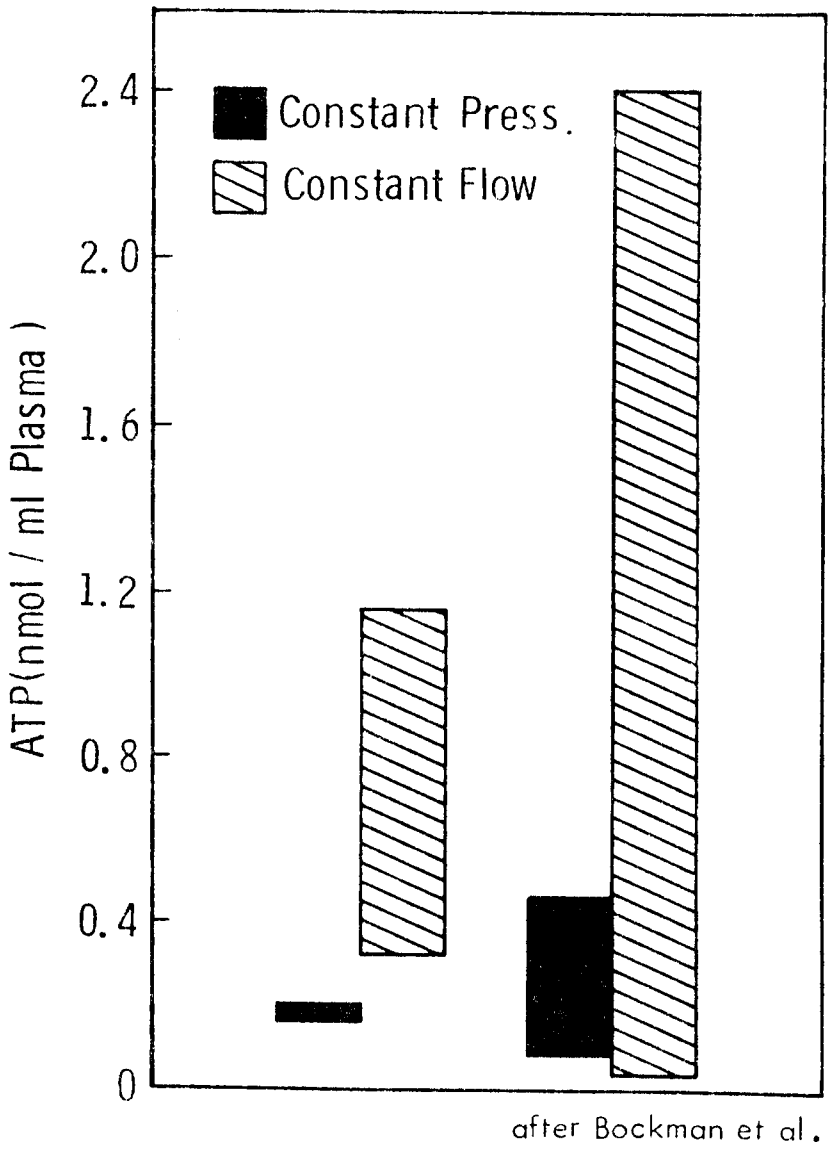


Fig. 7. Range of ATP concentrations detected in the venous effluent from perfused dog hindlimb preparations. Three hindlimbs were perfused at systemic pressure; four preparations were perfused at constant flow. Columns at left indicate range before stimulation, columns at right represent ATP ranges during and immediately following two minutes of tetanic contraction. (From Bockman, Berne & Rubio, 1975).

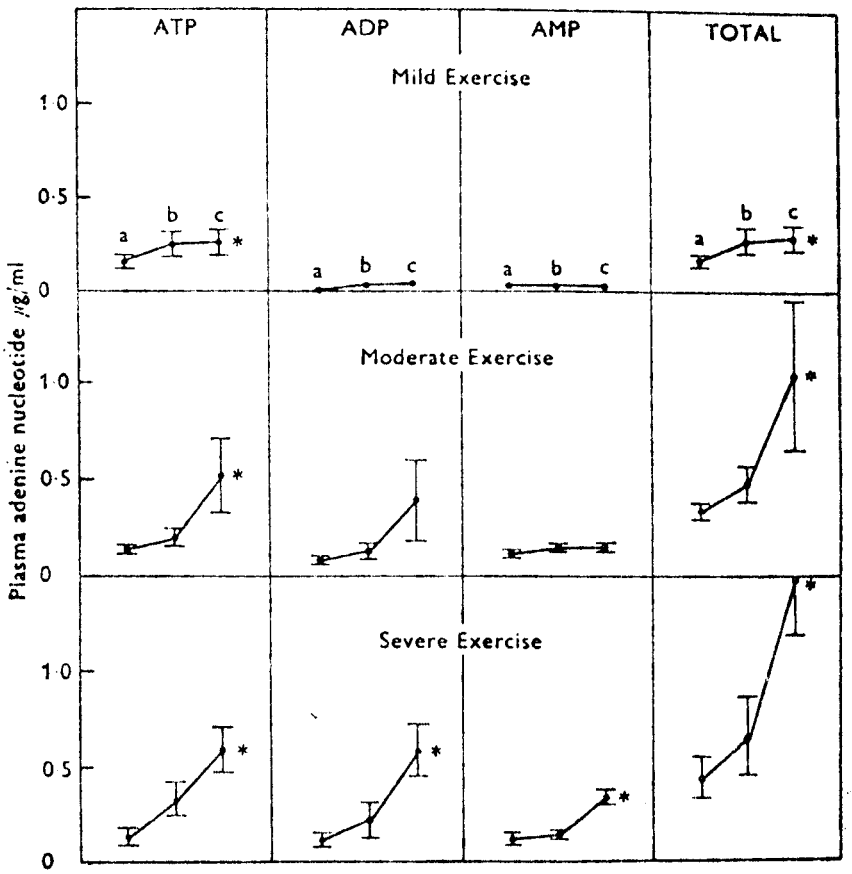


Fig. 8. Plasma adenine nucleotide levels before (a), 1 min after (b) and 5 min after (c) periods of mild, moderate and severe rowing for 5 min. Each point is the mean of 5 samples ± S.E.M. *$P < 0.05$ (sign test). (From Parkinson, 1973, J. Physiol. London).

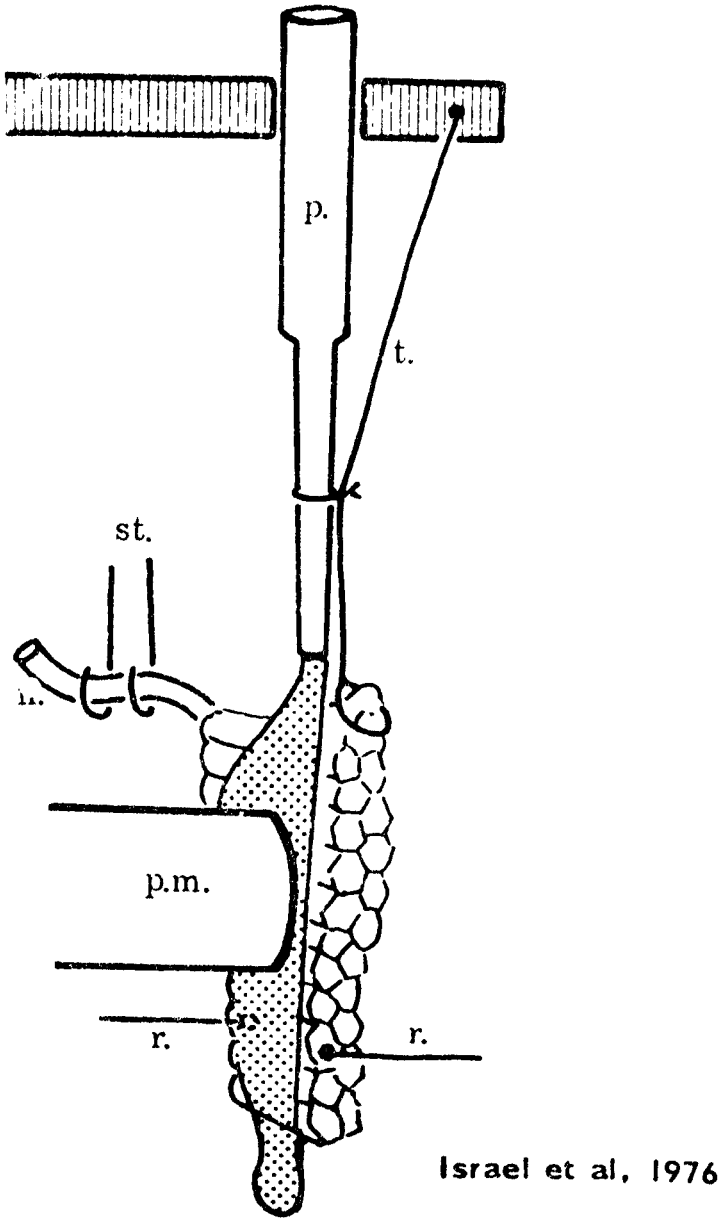


Fig. 9. Experimental set-up to measure post-synaptic release of ATP from electric organ of Torpedo marmorata. Firefly extract is perfused over the surface of the organ from a pipette (p). Thread (t) stabilizes pipette and tissue. Nerve (n) stimulated through silver-silver chloride electrodes (st). Light signal from firefly extract recorded by photomultiplier tube (p.m.). Post-synaptic electrical response recorded with platinum electrodes. (From Israel, Lesbats, Meunier & Stinnakre, 1976, Proc. Royal Society, London).

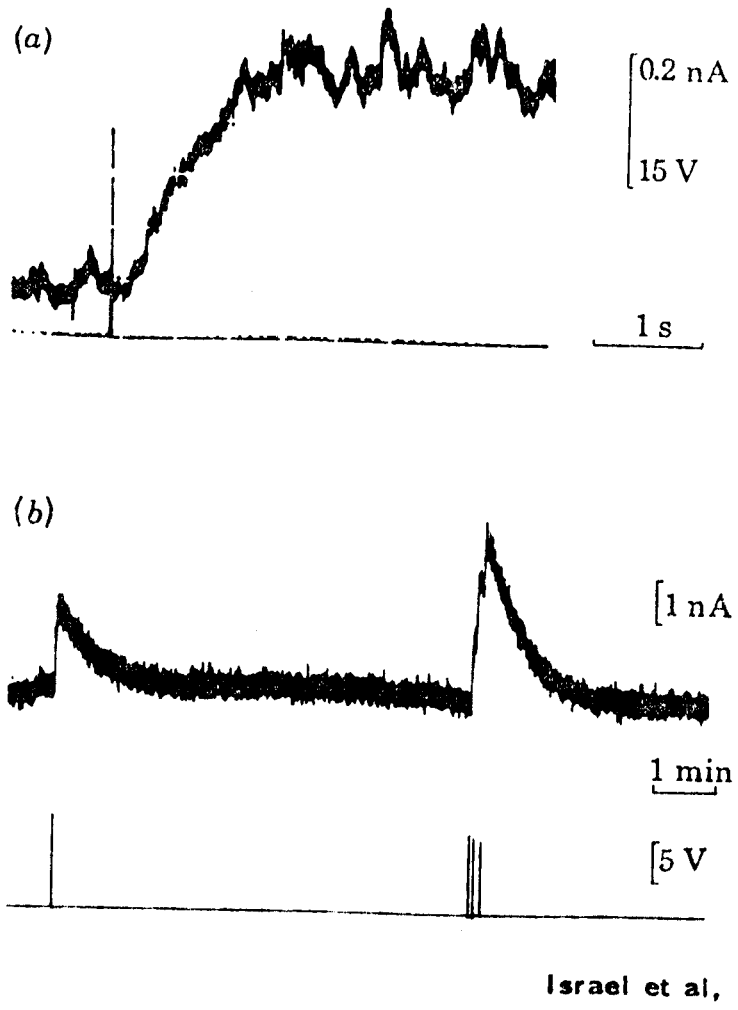


Fig. 10. Release of ATP after single nerve impulses. (a) lower trace: oscilloscope record of electrical response (post-synaptic) to a single stimulus applied to the nerve; upper trace: intensity of the p.m. current due to light emission resulting from the reaction of ATP with the firefly extract. (b) Responses to single and triple stimulation of the nerve (different preparation). Upper trace: light emission; lower trace: electrical responses. Time constant of the light recording system was 110 msec. (From Israel et al, 1976).

In 1973 a short report by Parkinson (93) indicated that adenine nucleotides appeared in human plasma five minutes after whole body exercise (Fig. 8). The implications of this are unclear at present, but it may indicate that there exists a highly dynamic situation regarding nucleotide levels after exercise, possibly the result of ongoing nucleotide redistribution in the body to tissues which cannot synthesize the purine ring *de novo*.

Release from the Neuromuscular Junction

In 1975 Silinsky & Hubbard (115) found ATP to be released from the stimulated nerve-muscle preparation of rat hemi-diaphragm. Since curare blocked the release they concluded that the nerve terminal was the source. These results were further extended by Silinsky (114).

Israel *et al* (68) have demonstrated very elegantly the release of ATP in response to single nerve impulses applied to the electric organ of *Torpedo marmorata*. A diagram of the experimental set-up is shown in Fig. 9. The electric organ can be regarded as a gigantic motor endplate without the underlying skeletal muscle element. It is supplied by a cholinergic motor nerve. Israel *et al* stimulated the nerve and recorded the post-synaptic response from the electric organ. At the same time they arranged for the surface of the organ to be superfused with extract of firefly lanterns. Fig. 10 shows the response of the superfusing firefly extract to single stimuli applied to the motor nerve. A discrete light signal is recorded for each nerve impulse, even in the case of three stimuli delivered in rapid succession. The post-synaptic source of this ATP release was demonstrated by the

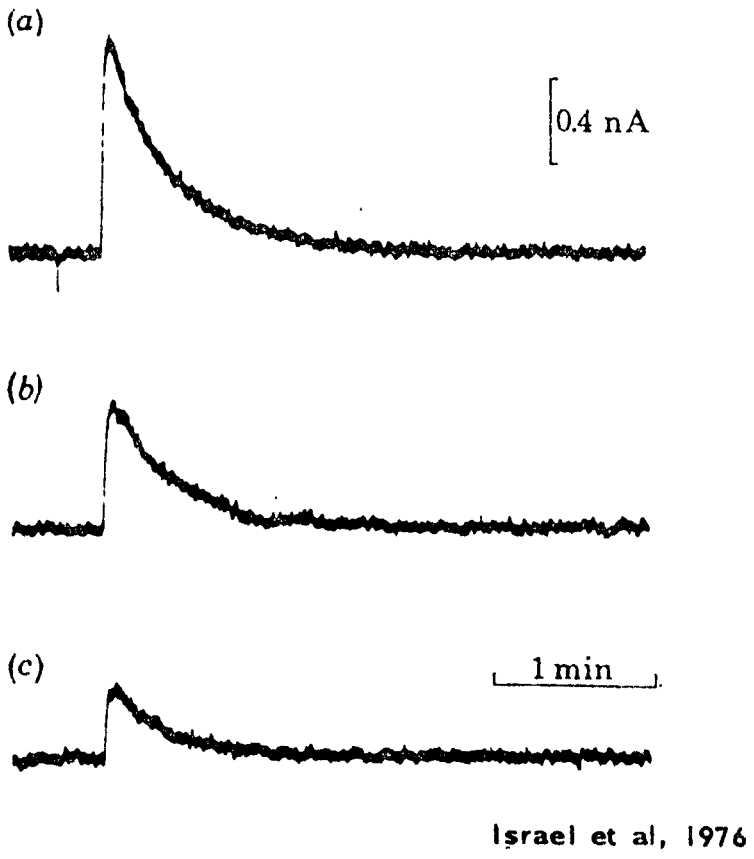


Fig. 11. Effect of curare on the release of ATP. (a), control. (b), after about 80 min in 5×10^{-4}M curare. (c), concentration of curare increased to 10^{-3}M. Note marked diminution of light signal indicating depression of ATP release post-synaptically. (From Israel et al, 1976).

fact that the light signal was diminished when curare was applied to the synaptic junction *via* the superfusate (Fig. 11). Since eserine had the effect of enlarging the light signal, the question was whether the post-synaptic ATP release was related to activation of the acetylcholine receptor, or was caused by depolarization of the post-synaptic membrane. This point was neatly resolved by first blocking the receptor with curare and then superfusing a high concentration of potassium to depolarize the post-synaptic membrane. A light signal was produced by this maneouvre, indicating that ATP release was associated with membrane depolarization rather than with activation of the cholinergic receptor. This finding supported the earlier conclusions of Abood *et al* (1).

The actions of released nucleotides and adenosine upon neurotransmitter release at synaptic junctions has been reviewed in a recent symposium (53).

Release of ATP from Myocardial Tissue

Non-working heart preparations. An increase in the levels of ATP in the coronary sinus effluent of Langendorff-perfused hearts (guinea pig) exposed to a 90 second period of hypoxia was demonstrated by Paddle & Burnstock (90). They found that a reduction in the perfusion pressure (constant flow) by more than 50% was accompanied by an increase in ATP concentration in the perfusate from 0.21 ($\pm$0.05) to 0.64 ($\pm$0.09) pmol/ml. (Fig. 12). When 0.1 M ATP was perfused into the coronary circulation only about 1% was recovered, suggesting that binding, degradation or perhaps uptake into cells was

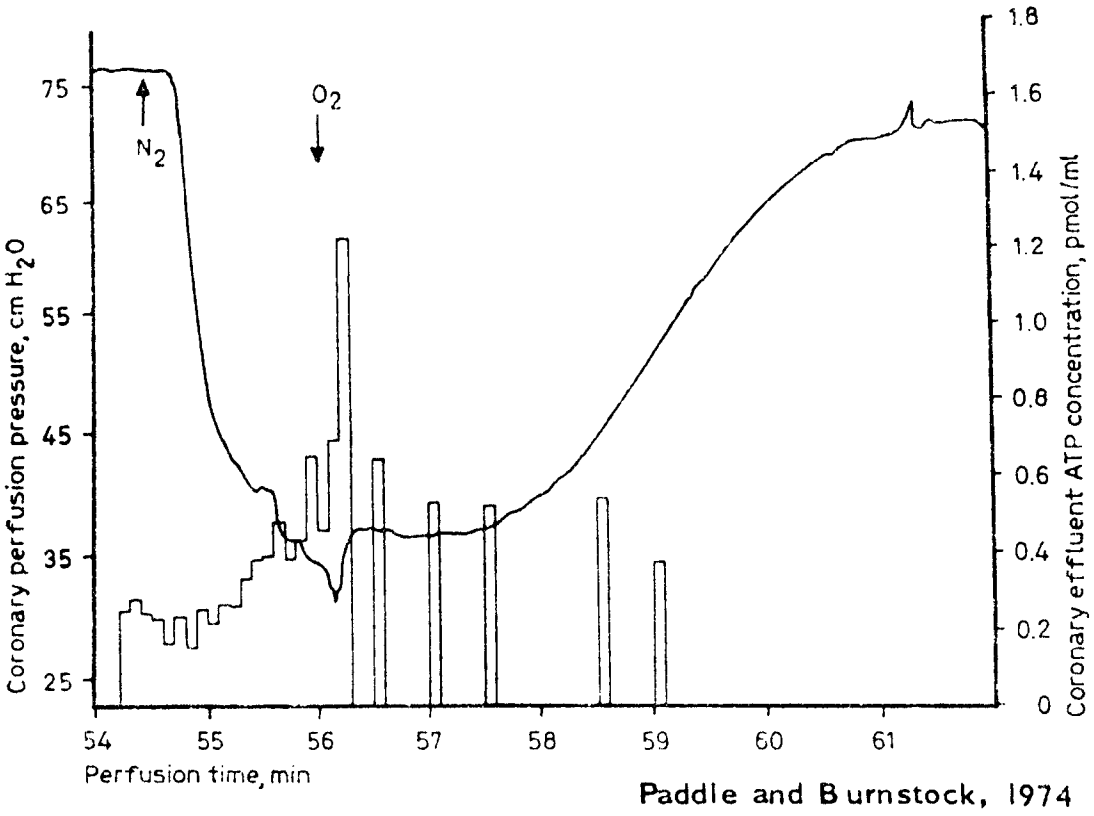


Fig. 12. Effects of a single period of hypoxia on the coronary perfusion pressure and effluent ATP concentration. Flow rate was constant at 4.8 ml/min. Perfusion pressure recorded continuously. Effluent ATP concentrations are average values during six second collection periods. (From Paddle & Burnstock. 1974).

taking place. This verified the earlier finding by Baer & Drummond (9). In any event it was probable that much more ATP was released than was detected in the coronary sinus effluent. Interpretation of the source was partially clarified. Adrenergic and cholinergic nerves were ruled out since guanethidine and hyoscine did not significantly alter the vasodilatory response to hypoxia, nor did they have any effect on the release of ATP. The authors suggested that purinergic nerves were possibly responsible for the release. That same year a note appeared by Stowe et al (119) who found 2.00 pmol/ml ATP appearing in the perfusate from a similar preparation in response to 180 seconds of hypoxia.

Isolated myocyte preparation. The use of adult rat heart cells (47) avoided many problems associated with whole heart preparations. The high ratio of surrounding fluid volume to cellular volume provides excellent conditions for oxygenation; no alteration in surrounding fluid volume occurs, thus avoiding the difficulty of having to estimate ATP concentrations in fluid coming from a vascular bed which is continuously dilating in response to hypoxia. The actual estimation of ATP remains free from both contaminating elements, such as the blood platelets, and degrading elements. The firefly luminescence test for ATP was applied to the cell suspension medium before, during and after the application of hypoxia. It was found that ATP, 0.34 M/mg protein was released from oxygenated cells, while 1.28 M/mg protein was released within 30 seconds of rendering the cells hypoxic (Fig. 13). Fig. 14 shows the compiled results. When the hypoxic cells were restored to the oxygenated state the levels

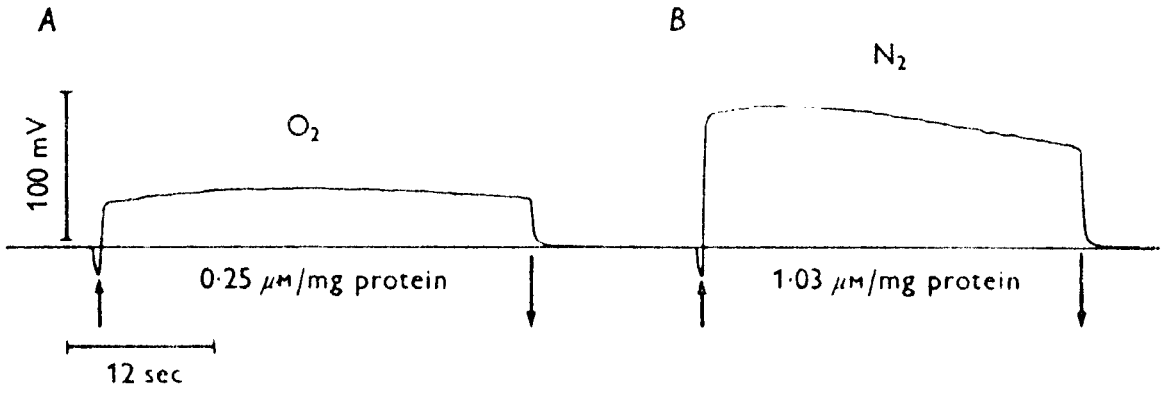


Fig. 13. Emission of light from firefly extract on addition of myocardial cells resuspended in either: A, oxygenated or B, nitrogen-equilibrated buffer solution. pH 7.4. Each signal represents amount of ATP extruded after cells had been in contact with the respective buffer solution for 30 sec at 37°C. (From Forrester & Williams, 1977, J. Physiol. London).

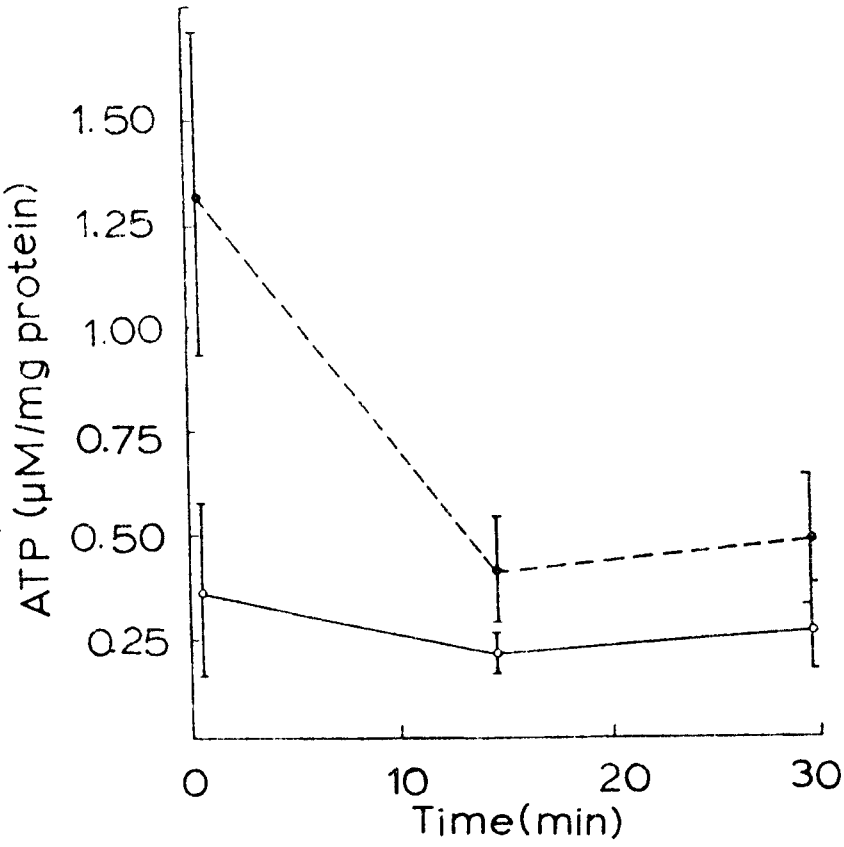


Fig. 14. Effect of a period of 30 seconds hypoxia on ATP concentration in isolated myocardial cell suspensions (rat heart). Lower graph, control in oxygenated solution. Difference between points at one minute incubation is highly significant (0.001 p 0.01). Each point is average ±SE (n = 6). From Forrester & Williams, 1977).

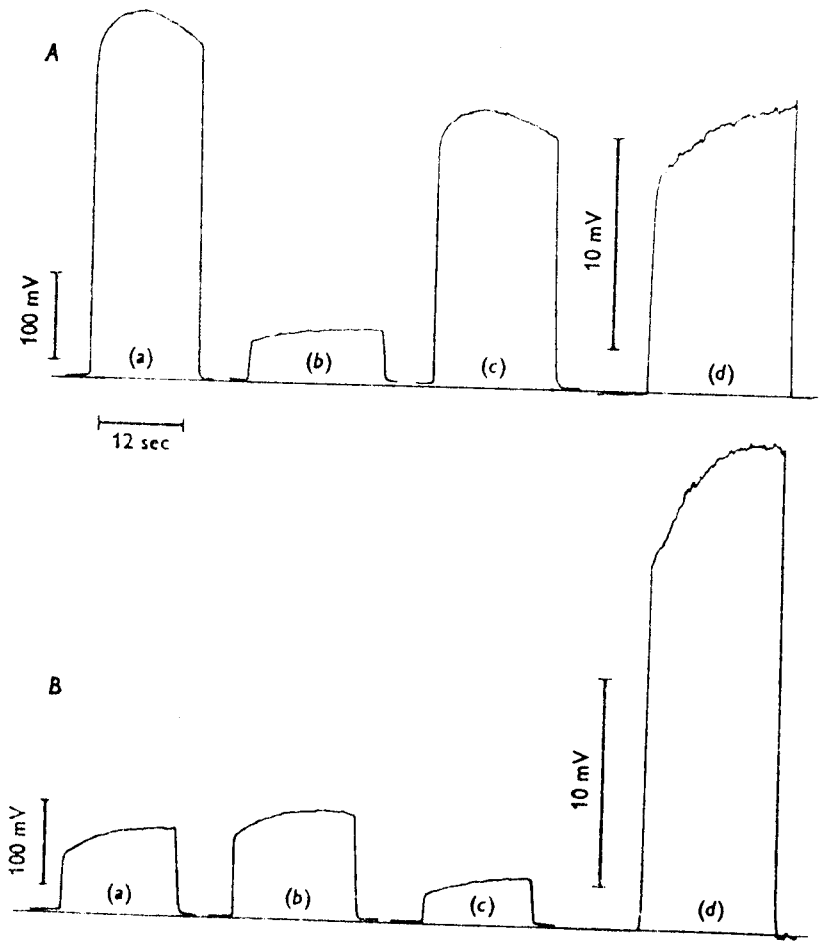


Fig. 15. Emission of light from extract in response to a cell suspension exposed alternately to hypoxic and oxygenated buffer solution. A (a), signal after exposure of cells to hypoxic buffer; (b), response after cells had been returned to oxygenated medium; (c), response when returned to hypoxic buffer; (d), response when finally returned to oxygenated madium; note increase in amplification scale. B, (a) - (d), paired oxygenated controls matching the solutions tested in A. (From Forrester & Williams, 1977, J. Physiol. London).

of ATP reverted to the previous control levels (Fig. 15). The amounts of ATP released were calculated to fall well within the range necessary for near maximum dilatation of the coronary vascular bed. Thus it was concluded that one source of ATP was the myocardial cell. The rapidity of the release in response to hypoxia could not be fairly estimated, since it took several seconds to assess the ATP levels after application of hypoxia. However, it was evident that the response was extremely fast, perhaps indicating that the role of ATP as a vasodilator starts at the very onset of myocardial hypoxia. This would be appropriate in view of the fact that the myocardium can store little oxygen and dilatation of coronary vessels is thus immediately required.

The viability of such preparations is difficult to assess. Certainly no ATP was detected from cell suspensions obtained from hearts which had been left asystolic for ten minutes. The insulin receptor was intact (47) and it has been shown that respiration of these cells increases in the presence of an uncoupler but is completely inhibited by a respiratory poison , indicating normal function of the mitochondrial oxidative apparatus (98). Moustafa et al (86) have reported that a transient increase in cyclic AMP occurred in response to isoprenaline when cells were pretreated with theophylline, while calcium reduced the levels of cyclic AMP, presumably by (normally) inhibiting adenyl cyclase. Powell et al (97) were able to measure the trans membrane potentials in myocytes similarly isolated. When they were incubated at 37°C in a Krebs buffer solution with 0.5 mM $CaCl_2$ the majority of cells had resting potentials more negative than - 70 mV within a few

milliseconds of microelectrode penetration. Action potentials with overshoots were recorded in chloride-free or high calcium solutions. It was suggested that these cells can adopt a low potassium conductance in the standard incubation medium, resulting in a low recorded transmembrane potential. The cells were able nevertheless to assume more negative potentials under appropriate conditions (exposure to chloride-free, low sodium, high calcium or high manganese media) and still retain the ionic mechanisms responsible for the generation of active currents.

Scanning electron microscopy (96) of these cells has shown their surface to have regular arrays of T-tubules at intervals consistent with sarcomere lengths for contracted cells. T-tubules had openings both ovoid and circular in shape. Intercalated discs appeared exactly similar to those observed in whole tissue.

Working heart preparation. When isolated hearts are perfused retrogradely through the cannulated aortic stump in the Langendorff mode the work performed by the myocardium is inestimable and perfusion of the myocardium does not really resemble the state *in vivo*. The working heart prearation developed by Neely *et al* (88) was used to assess the ATP concentrations in the coronary sinus effluent when the left ventricle was made to work against a normal afterload. The perfusion of the vascular bed is thought to be more normal under such circumstances. The hearts were exposed to 90 seconds of hypoxia and the ATP levels in ghe sinus effluent were measured just before the introduction of hypoxia, at the end of the hypoxic period and 5 minutes after oxygen was restored to the preparation. Fig. 16 shows the results obtained (26). The point of main

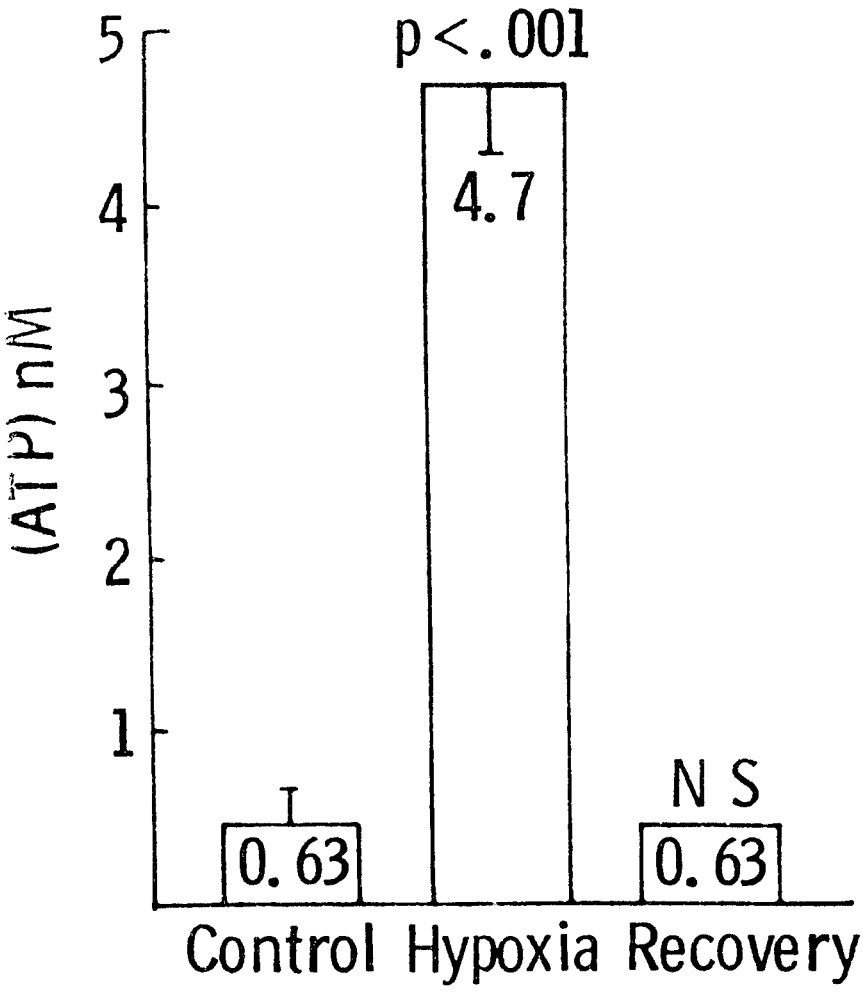


Fig. 16. Concentration of ATP in the coronary effluent of isolated working hearts perfused with oxygenated buffer (control), during the last 30 sec of hypoxia (hypoxia) and after 5 min recovery with oxygenated buffer (recovery). Values are means of four experiments ± S.E.. N S = not significantly different from control. (From Clemens & Forrester, J. Physiol, London, in Press).

TABLE 3. *ATP in coronary sinus effluent from working and nonworking hearts*

Reference	Coronary effluent Concentration ATP (nM)	Heart preparation
Paddle and Burnstock (92)	0.63	Nonworking guinea pig
Stowe et al. (121)	2.00	Nonworking guinea pig
Clemens and Forrester (28)	4.70	Working rat

interest is that a working preparation produced about eight times the amount of ATP into the sinus effluent that the non-working preparation did (90). Of course the obvious criticism is that these working hearts are not adequately supplied with oxygen via the Krebs perfusate, however the viability of the hearts was judged to be satisfactory on the following grounds: alteration in the left atrial filling pressure produced typical Frank-Starling responses of the left ventricle; oxygen extraction from the perfusate increased in response to increased workload; coronary blood flow increased immediately upon introduction of hypoxic perfusion and the mechanical recovery from hypoxia was always complete within 5 minutes of restoring oxygen to the preparation. Calculation of the oxygen extraction showed figures comparable to those calculated for blood-perfused hearts. Table 3 gives a comparison of the concentrations found in the coronary sinus effluent from working and non-working hearts. In view of the rapid degradation of ATP in the coronary vascular bed (9, 90), it is likely that ATP concentrations at the arteriolar level may approach one hundred times those detected in the coronary effluent. These amounts (5×10^{-7}M) would certainly have an immediate and profound effect on coronary vascular smooth muscle.

Release of nucleotides from endothelium and smooth muscle.

Recently Pearson & Gordon (95) found that ATP and other nucleotides were selectively released from vascular smooth muscle and endothelial cells in culture. Whether the released nucleotide was a response to damage or whether it represented a specific secretory mechanism is unresolved. Extracellular

conversion of ATP--ADP--AMP--adenosine was also observed in these cell preparations. Extracellular conversion to adenosine occurred much more rapidly in smooth muscle cells.

MODE OF ACTION OF ATP AND ADENOSINE

It is well recognized that ATP can bind to proteins and the possibility that an -SH group is involved has been proposed (120, 12). Binding of ATP to renal membranes is associated with calcium (91) and ATP interacts with isolated sarcoplasmic reticulum (23) producing a conformational change (80). There is much evidence that exogenous ATP acts on the cell surface (29, 124, 24) and effects on cell volume (102) indicate an alteration in membrane permeability. An ATP-binding protein has been isolated from membranes of nerve endings (2). Since ATP has a rigid structure (7), that is, it is resistant to bending or distortion, it is plausible that a matching receptor structure exists on the membrane protein moieties (117). These observations lend support to the purinergic receptor (P_2) hypothesis put forward by Burnstock (22).

Analysis of the effects of ATP on the electrical properties of membranes has been pursued by many investigators. External application to intestinal smooth muscle (taenia coli) abolishes spike activity and produces hyperpolarization of the membrane if the concentration is high enough (20, 69, 8, 123, 71). In this respect ATP was found to be the most potent of the adenine nucleotides and adenosine. Goto *et al* (56) analysed the actions of these compounds in bullfrog atrial muscle and showed that while ATP and ADP produced an enhancement of calcium inward current and augmented the associated

phasic tension development, AMP and adenosine elicited a negative inotropic effect. The tonic tension (calcium independent) was inhibited by all the adenine compounds. The adrenergic receptor was excluded as a mediator of these effects since β-blocking drugs had no influence upon their actions. Other effects on smooth muscle have been noted (118).

Much evidence suggests that exogenous adenosine affects cellular metabolism through activation of adenylate cyclase, elevating the levels of cyclic AMP (112, 118, 67, 22). A rise in cyclic AMP levels in response to exogenous ATP was demonstrated by Sattin & Rall (109); this could be a significant mechanism in the local control of cerebral blood flow and metabolism (44). That adenosine can penetrate the cell membrane so easily would suggest that its actions are quite separate in nature from exogenous ATP, although evidence has been assembled for the existence of a specific adenosine (P_1) receptor site (22).

Takata & Kuriyama (122) have recently shown that ATP markedly hyperpolarized the smooth muscle membrane of the coronary artery of guinea pig, but found that there was very little response with AMP and none with adenosine. In the presence of low calcium concentrations the ATP-induced hyperpolarization was significantly reduced. They classified the process into two components, a fast phase which was less sensitive to calcium and a subsequent slow phase which was calcium sensitive. ATP and adenosine relaxed the smooth muscle in the presence of high potassium or acetylcholine. It was proposed that ATP mainly increases the potassium

conductance and in part the sodium conductance. The interactions described with potassium and acetylcholine may be of great significance when considering the problem of 'metabolite' control of local blood flow. Cholinergic vasodilator nerves and potassium released from active skeletal muscle and cardiac muscle may well interact effectively to regulate flow.

DISCUSSION

Evidence has been reviewed to assess the roles of ATP and adenosine as local vasodilators in contracting heart and skeletal muscle. Although ATP is the more potent agent a fundamental problem remains as to how it is released from an hypoxic cell. The present view would suggest that it is released during the phase of membrane depolarization, but whether it comes through or off the membrane is unclear. Elucidation of this mechanism may advance our understanding of the relationship between a cell and its external environment. That cellular damage is the real cause of ATP released from hypoxic cells is a valid criticism. However, it need not be regarded as an 'all-or-none' phenomenon; tissues and cells can suffer degrees of damage, and hypoxia itself could be classified as a very early and mild type of reversible cell damage. In other words a form of 'early warning system' is in operation.

The major problem with adenosine and exercise hyperaemia is the action of the antagonist methylxanthine drugs. Active hyperaemia in heart and skeletal muscle is largely unaffected by theophylline, which blocks the action of adenosine in these

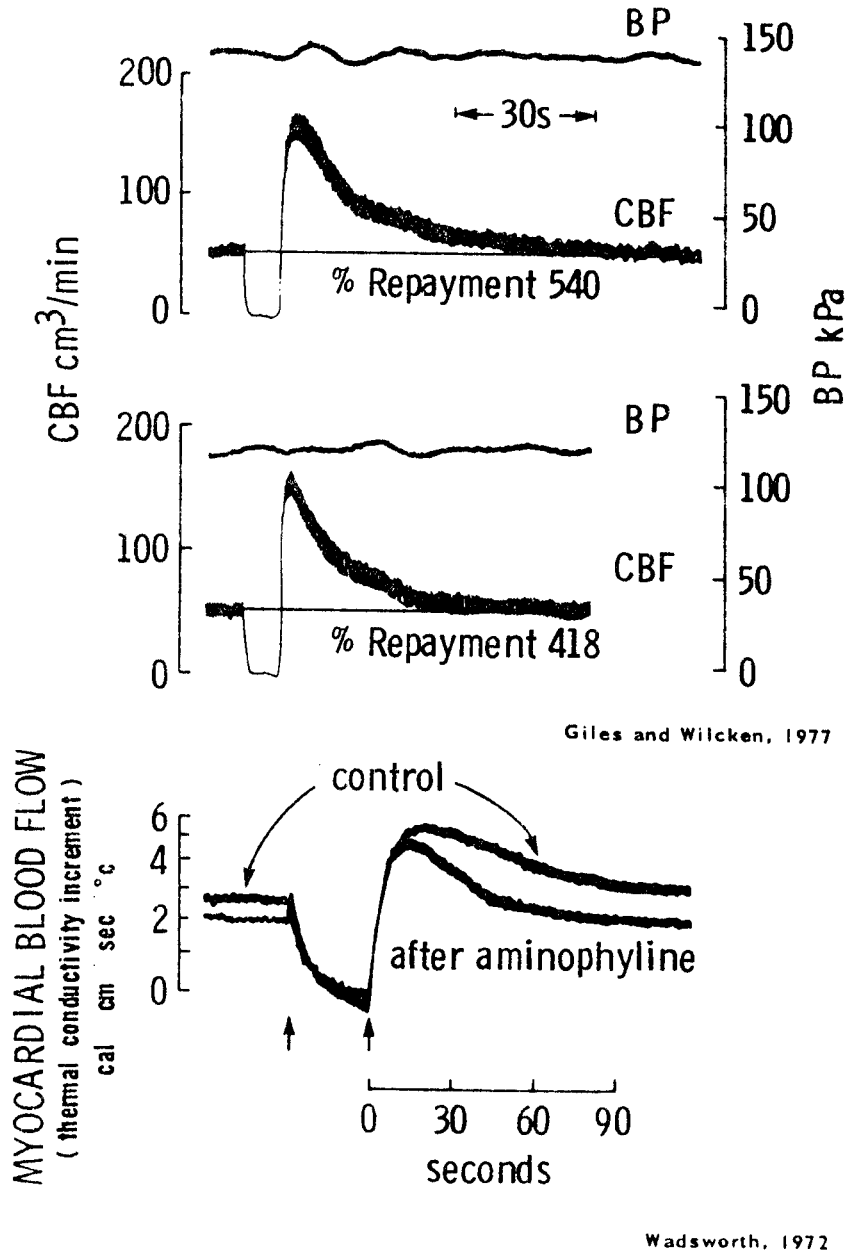


Fig. 17. Upper figure shows reactive hyperaemia after 8 seconds coronary arterial occlusion in heart of the greyhound. Middle figure shows the response after aminophylline, 200 ug/min, continuously infused into the coronary artery. The period of reactive hyperaemia has been shortened by aminophylline. Lower figure, shows reactive hyperaemia after 30 seconds occlusion of the left anterior descending artery in the cat. The lowesttrace shows the abbreviated reactive hyperaemia 15 minutes after injection of aminophylline, 7 mg/Kg. (From Giles & Wilcken, 1977 and Wadsworth, 1972).

vascular beds. The action of ATP in these beds is unaffected by theophylline. A role for adenosine seems evident in post-occlusion hyperaemia of cardiac muscle. Fig. 17 shows the effect of aminophylline on post-occlusion hyperaemia of myocardium in two separate studies (127, 54). In both cases there is a clear diminution of flow in the post-occlusion period. The adenosine contribution to this flow has presumsbly been eliminated by the aminophylline. The view has been put forward that adenosine helps to prolong dilatation after severe exercise of skeletal muscle (59, 66).

Orientation of membrane enzymes. Degradation of extracellular ATP to ADP, AMP and IMP by an enzyme system on the outer surface of intact frog skeletal muscle has been observed and the properties of the enzymes involved (ATPase, adenylate kinase and AMP deaminase) have been extensively studied (36, 83). It has also become evident that 5'nucleotidase has its active site facing outwards from the membrane in skeletal muscle (134), and in many other tissues (58, 31, 125). Extracellular enzyme activity of this type has also been observed in cardiac tissue (131, 48). This presents a difficulty with the concept of adenosine being formed inside the muscle cell as a response to hypoxia. No less a problem exists for ATP in this respect. If extensive extracellular ATPase activity is present in skeletal and cardiac muscle, does this indicate that ATP is anticipated to arrive extracellularly?* Is this part of a much wider system of nucleotide and nucleoside dispersal in the body

*One wonders how the neurotransmitter theory for acetylcholine would have fared if the cholinesterases had been discovered and characterized before the discovery of acetylcholine release.

for redistribution of the purine ring moiety to certain tissues which do not have the intrinsic ability to synthesize the ring de novo? Obviously much more work has to be done to investigate these possibilities.

It would be unlikely for there to be just one metabolite involved in exercise hyperaemia. Variations in control mechanisms will occur between muscle types and temporally within the same muscle. To add further difficulties to an already complex field, the question of species variation must also be kept in mind. Dogs are different from humans and experimental findings in one can only serve to guide our ideas about the other. For example, it has been shown that there is no 5'nucleotidase present in pigeon or turtle hearts (87). Also, the application of 10 mM adenosine to the coronary arteries of turtle hearts has no effect (87).

Burnstock (22) has proposed the existence of two types of 'purinergic' receptor, P_1 for adenosine and P_2 for ATP. The P_1 receptor is blocked by methylxanthine compounds. On this basis it would appear that the P_2 receptor predominates in the vascular beds of cardiac and skeletal muscle. The source of much of the adenosine detected in the effluent from these tissues may be ATP originally released and very quickly degraded to adenosine.

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