S T U D I E S O N

T H E M E T A B O L I S M O F C E L L S

I N V I T R O.

A Thesis Submitted for the Degree of Doctor of Philosophy in the Faculty of Science

by

Enid S. Pearson, B.Sc.

Biochemistry Department

University of Glasgow.

January 1959.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

CONTENTS.

	Page Number
Introduction	1.
Techniques	
Tissue Culture Methods	19.
Chemical Methods	31.
Results	
Adaptation Experiments	46.
Studies with Insulin	57.
Discussion	76.
Summary	96.
Acknowledgements	99.
Bibliography	100.
Appendix	108.
Publications	111.

INDEX TO TABLES.

Table number	Number of preceding page.
1 - 4	49.
5 , 6	51.
7 , 8	54.
9 - 12	56.
13	57.
14 - 17	58.
15 - 20	59.
21 - 23	60.
24 - 27	63.
28 - 32	64.
33 , 34	65.
35	66.
36 , 37	68.
38 , 39	70.
40	71.
41	73.
42	75.

INDEX TO FIGURES.

Figure number	Number of preceding page
1 - 5	49.
6 - 13	54.
14 , 15	56.

INTRODUCTION.

Introduction.

A Brief History of Tissue Culture.

The first convincing experiments in tissue cultures were those of Harrison who in 1907 maintained frog's nerve tissue alive and active in a hanging drop of frog's lymph. This established the fact that animal cells could continue to survive and function in a situation apart from the living animal. In effect Harrison's achievement led the way to direct observation of the living cell and to a consideration of the action of outside agents on it.

This pioneer work was extended by Carrel and Burrows (1910) who some three years later reported the successful cultivation of adult tissues of dogs, cats and frogs. They managed to maintain in culture cartilage, bone and pieces of thyroid, spleen and kidney. When they reported the growth of fowl sarcoma *in vitro* they were one of the first to realise the possible value of these methods in the study of the cancer problem. Burrows (1910) applied the plasma - clot technique to the cultivation of chick embryonic material. It was believed that the fibrin net-work supplied an essential framework for the cells: the material to be cultivated was embedded in a plasma clot on a coverslip and inverted over a depression slide. The handling of these cultures was a skilled process and contamination a major hazard. It is a tribute to the care of these early workers that they could report the pulsation of chick heart fragments after three months in tissue cultures (Carrel, 1912) and their continued growth after sixteen years (Carrel, 1928).

This step forward to the production of 'permanent' cell lines was brought about by the discovery that embryonic tissue extract promoted rapid cell growth which continued indefinitely if an adequate medium was supplied (Carrel, 1913 a. Carrel, 1913 b). In the following years there were reports of several other 'pure' cell strains most of which were avian in origin. These included cultures of cartilage, (Fischer, 1922 a) iris epithelium (Fischer, 1922 b) and thyroid cells (Ebeling 1925).

The discovery of the growth promoting effect of embryo extract led naturally to a study of cell nutrition. It was claimed that by measuring the halo of cells which had migrated out from the original explant, the amount of growth could be determined to within an error of ten per cent. (Ebeling, 1921). This technique was the basis of a systematic investigation by Ebeling (1924) into the effect of various amino acids on the growth of fibroblasts. He found that mixtures of the thirteen amino acids available to him, brought about no increase in cell mass. The list of amino acids did not include methionine and tyrosine which some thirty-three years later were shown to be essential for the growth of chick heart fibroblasts. (Morgan and Morton, 1957 a). Other early work on cell nutrition used pepsin digests of embryo, egg white, rabbit brain and ox blood. From these studies it was concluded that cells obtained their nitrogen from proteoses and not from the smaller split products of protein digests. (Carrel and Baker, 1926). These studies were unprofitable because the battery of pure amino acids, vitamins, coenzymes and nucleic acid components available today, was

unknown to the early workers. However they saw clearly the potential use of the tissue culture method and emphasized that it was not an end in itself but a technique to be used to investigate the properties of the cell. Harrison, more than anymore else, realised that the initial period of wonderment at the novelty of the results and the manual dexterity involved was over, and that it was time to apply the method to the formidable problems in numerous fields of research. (Harrison, 1928). He stated clearly that tissue culture provided 'a means for the exact study of growth in pure cell lines, the conditions influencing the process, and the chemical nature of the medium required.' But Harrison was ahead of his time, and it was nearly thirty years before adequate techniques made this exact study possible. At the conclusion of an address to the Tenth International Zoological Congress in 1927 he said, 'The ideas underlying the method are sound, but in its application vision of the great problems awaiting solution and their precise formulation for experimental test must hold sway over mere technical skill'. His words are perhaps even more apt today.

Since the beginning of tissue culture there have been signs of the inherent divisions of the subject and subsequently it split into cell culture and organ culture. The 'pure' cell lines of Carrel and others, established by serial transfer, were probably mixed cell populations. The first report of a clone strain was that of Sanford, Earle and Likely (1948) who isolated from a culture of mouse connective tissue a single cell in a fine capillary. From this was grown the strain-L cells, clone 929, which are now widely used as experimental material.

Simpler methods of single cell isolation have been developed and many reports of additional clone strains have appeared recently. (Puck, Cieciura & Fisher, 1956. Puck, Marcus and Cieciura, 1956). These cell cultures are characterised by their rapid and unorganised growth. In contrast to this small pieces of embryonic rudiments can be maintained and developed in culture by using conditions in which growth is relatively restricted and cell division at a low level. In this was a degree of organisation can be achieved and the development of the organ rudiment may proceed normally. The progressive maturation of embryonic nerve cells in isolated ganglia, the elaboration of intercellular products, such as myelin, and the histogenesis of bone and skin have been observed by these methods of organ culture.

Great interest was aroused by the fact that under certain conditions the normal morphology of a tissue could be maintained in vitro. Attempts were made to discover the nature of the factors required for this continued process of differentiation. The problem is in many ways linked to the study of the organisation of the developing embryo and for this reason embryonic material has been extensively employed in the study of differentiation. The reverse process, which is sometimes called de-differentiation, is observed in the emergence of a typical cell culture from explanted material. In this case there is no attempt to preserve the structural intergrity of the tissue and with successive sub-culture the dominance of one cell type is soon apparent. These results suggest that the maintainance of different cell types within a tissue depends on their juxtaposition. The effect of one

cell type upon another may be a physical one, or it may be mediated by some diffusible substance. Several experiments have been designed to test these theories and will be considered subsequently.

Both types of cultures, organ cultures and cell cultures, have the same basic nutritional requirements. Before considering the specific factors necessary for tissue organisation it is important to survey the range of compounds necessary for survival and growth. These may be called the non-specific factors in cell nutrition.

Cell Nutrition.

(a) Non-Specific Factors.

Looking back over the years of research that have led to an understanding of the nutritional requirements of the cell, it is possible to pick out certain discoveries that have been vital to this progress. The development of clone strains of cells has been of major importance. In this way a standard test material, easily maintained for an indefinite period, was provided.

The next difficulty to be overcome was that of supplying a sufficient number of replicate cultures for precise study. It was discovered that cell suspensions of L-cells would grown directly on glass or perforated cellophane subtrates and this dispensed with the need for a plasma clot. (Earle, Evans and Schilling 1950. Evans and Earle 1947). Shortly afterwards it was noted that uniform cell suspensions could be prepared from a variety of tissues using trypsin as a dispersing agent. (Moscona, 1952). These suspensions were used to seed culture vessels.

More recently it has been demonstrated that constant agitation of cells in a fluid medium allows the cells to grow in suspension (Owens, Gey and Gey, 1953. Earle, Schilling, Bryant and Evans, 1954). This further expands the size of cultures which can be conveniently grown.

Side by side with these advances in culture technique, research was proceeding to seek chemical methods of evaluating cell growth and metabolism. Willmer (1942) used a modification of the method of Berenbl Chain and Heatley, (1939) for the microdetermination of nucleo-protein phosphorus as a measure of cell growth. This method was extended to include the fractionation of the nucleic acid into ribonucleic acid (RN and deoxyribonucleic acid (DNA) (Davidson, Leslie and Waymouth, 1949). (Hull and Kirk, 1950). Davidson and Leslie (1950) found a correlation between the rate of protein synthesis and the rate of accumulation of ribonucleic acid phosphorus. Similarly Healy, Fisher and Parker (1954) established a relationship between the deoxyribonucleic acid phosphorus content and the cell count of a culture. With the more widespread use of radioactive isotopes, a further relationship was found between the growth of tissue cultures and the incorporation of radioactive metabolites into their nucleic acids. (Lu and Winnick, 1954). The results prove the validity of accepting the increase in nucleic acid (DNA) content of a culture as a measure of its growth. Other procedures for the analysis of cells have been evolved which permit the estimation of lipid, carbohydrate, RNA, DNA and protein nitrogen in small tissue samples. (Paul, 1958). These methods are applicable to the study of metabolic problems in tissue cultures

The accurate determination of growth by chemical methods necessarily involves the destruction of the tissue, which, in many instances, is not desirable. For this reason Waymouth (1956) developed a hematocrit method for nutritional studies with strain L-cells. She found the packed cell volume to be in consistent agreement with the total cell count.

With the development of these techniques the way was opened for the elucidation of the specific nutritional requirements of tissue culture cells. The last few years have witnessed a rapid advance in this direction. One of the first to employ the new techniques was Eagle who demonstrated that twelve amino-acids are essential for the growth of strain L-cells and HeLa cells. (Eagle, 1955 a, 1955 b). These are arginine, cystine, histidine, lysine, leucine, iso-leucine, methionine, phenyl alanine, threonine, tryptophan, tyrosine and valine. It is to be remembered that Eagle's medium was supplemented with whole or dialysed human or horse serum. However, omission of any one of the twelve amino acids arrested cell growth which was resumed on restoration of the missing acid.

Morton, Pasieka and Morgan, (1956) developed a 'nutritional depletion technique' for the study of the requirements of chick heart fibroblasts. The freshly explanted tissue was cultivated for an initial period in simple salt solution to lower the nutritional reserves of the cells. In this way a more sensitive system for the assay of nutritional requirements was obtained. These workers were able to use a completely synthetic medium to map out the amino acid requirement

of chick fibroblasts. (Morgan and Morton, 1957 a).

This work has been extended to other cell types and in general there is a similarity in the pattern of essential and non-essential amino acids for a variety of cells. In a review article on tissue culture nutrition Morgan (1958) emphasizes that complex inter-relationships between amino acids may not be detected by these methods whereby single amino acids are omitted individually from a synthetic medium. An illustration of this complexity is found in his paper on sulphur metabolism. (Morgan and Morton, 1957 b).

A very important part of the artificial environment of tissue culture cells is in the form of carbohydrate which supplies a source of energy for the cells. The custom of early workers, to incorporate glucose at the approximate blood level of 100 mg / 100 ml. was maintained in many of the recently formulated synthetic media. A departure from this tradition was made in Waymouth's nutrient solution (1955) which contained 500 mg. glucose / 100 ml. This proved satisfactory and it can be concluded that carbohydrate concentration is not a critical factor in cell nutrition. However, an investigation into the utilisation of added sugars by chick fibroblasts showed that only a limited number of sugars were metabolized by the cells. In their work Harris and Kutsky (1953) used a basal medium containing serum and embryo extract which had been previously dialysed. They found only D-fructose, D-glucose and D-mannose to be utilised by the chick cells. Eagle (1958) reports that ribose permitted the growth of a number of cell lines but only

within a limited concentration range.

In deciding the composition of a synthetic medium it was common practice to include all the known vitamins. Newer techniques made possible a study of the cell's requirements for individual vitamins. Eagle (1955 c) was able to demonstrate specific vitamin deficiences in the strain L and HeLa cells and to reverse these signs of deficiency with the appropriate vitamin. Other workers confirmed that choline, folic acid, nicotinamide pantothenic acid, pyridoxal, riboflavin and thiaminewere essential for the survival of strain L-cells and showed in addition that inositol and vitamin B12 were beneficial to the growth of these cells. (Evans et al., 1956).

Work on the fat soluble vitamins has centred on vitamin A. Fell and Mellanby (1952) demonstrated that hypervitaminosis A arrested the growth of embryonic limb bones although there was no evidence of cell degeneration. However cultures of chick heart fibroblasts showed increased cell density and size of outgrowth in the presence of excess vitamin A in the culture medium. (Lasnitzki, 1955 a).

There is a trend in the formulation of many synthetic media to include a variety of co-factors and conjugated forms of known nutrients. This follows the assumption that the cells cannot synthesise these quickly enough from the smaller precursor compounds. However it has been shown by experiment that many co-enzymes possess less growth promoting ability than the parent vitamin. It is possible that the size of these co-enzymes precludes their entry to the cell. (Eagle, 1956 a).

In the remaining areas of cell nutrition, which include a study of the effects of nucleic acid components, lipids and minerals on cell metabolism, the information is less complete and sometimes contradictory. For example, Ehrensvard, Fischer and Stjernholm (1949) found adenine and guanine to have no effect on the growth of chick heart fibroblasts whereas Morgan, Morton and Parker (1950) found these two purine bases to be beneficial to the survival of fibroblasts. Work in this field has included studies on the purine and pyrimidine bases and other nucleic acid components. It is being further extended with the use of ^{14}C labelled compounds: Thomson, Paul and Davidson (1956) have used ^{14}C - formate to study the metabolic stability of DNA in fibroblast cultures.

Morton, Morgan and Parker (1950) employed 'Tweens' as a source of fatty acid in synthetic feeding mixtures. They found that levels above 0.05% were toxic to the cells but lower levels would maintain healthy cultures. More recently Evans et al., (1956) have omitted the group of unsaturated fatty acids from a medium for the cultivation of L-cells. This has not proved harmful.

The basis of all media is an inorganic salt solution which corresponds closely to the composition of the body fluids. The study of the exact mineral requirements has not been completed. Harris (1954) studied the behaviour of chick heart tissue explanted in the absence of external sources for a CO_2 / bicarbonate system. At the normal pH range of 7.2 - 7.8 outgrowth was completely inhibited but, on addition of 0.05% sodium bicarbonate there was a return to the

normal growth pattern. The salt requirements of mouse fibroblasts and HeLa cells have been assayed by Eagle (1956 b) who found that these mammalian cells have no requirement for bicarbonate. He found sodium, potassium, calcium, magnesium, chloride and phosphate ions to be essential for growth and survival.

From time to time reports have been published of factors found to be stimulating to cell growth but whose exact chemical nature is unknown. Carrel (1913 a,b,) early discovered embryo extract to be such a factor and subsequently many workers have attempted to isolate the active component from it. Harris and Kutsky (1954) isolated a nucleoprotein fraction from chick embryo which, when combined with the dialysate, exhibited growth promoting properties. Following the example of Carrel (1928), but obtaining different results, Sanford studied the effect of ultra filtrates of embryo extract on the proliferation of cells in vitro. (Sanford et al., 1952). She found the activity of this fraction comparable to that of whole embryo extract. Further fractionation of the ultra filtrate resulted in loss of activity. Leibermann and Ove (1957) have recently attempted the purification of a serum protein required by appendix cells in tissue culture. These cells continued to multiply when fed Eagle's medium, supplemented with a dialysate of an auto-claved solution of commercial peptone, and a glyco protein containing hexose, hexosamine and sialic acid. The true chemical nature of the growth promoting factors in embryo extract and serum, remains a mystery.

In some measure, a precise knowledge of the nutritional requirements of tissue culture cells has been obtained. The picture is as yet incomplete but, at the present time much attention is being focussed on the previously neglected details of it. The knowledge gained, however, both in nutrition and chemical techniques applicable to cell cultures, had made it possible to use these cultures for accurate biochemical experiments.

(b) SPECIFIC FACTORS.

It is now relevant to consider the factors required for the organisation of tissue in vitro. Much light has been thrown on this subject by the study of organisation and differentiation in the developing embryo. Grobstein (1953) has obtained some important results, working with sub-mandibular salivary gland rudiments in vitro. This system consists of epithelial and mesenchymal components which can be cultured separately, in suitable recombination or in combination with other tissues. When the two components are cultured together the epithelial component undergoes branching to form an extensive system of ducts and terminal buds. In combination with mesenchyme from other sources this did not occur, showing that morphogenesis of the sub-mandibular gland is dependent on some special property of the capsular mesenchyme. In further experiments Grobstein pre-cultured mesenchyme before combining it with epithelial rudiments. The result showed that the morphogenetic effect persisted during culturing for a few days, but declined with successive sub-culture. Mesenchyme, which had been killed by heat treatment, failed

to promote morphogenesis but to some extent preserved the structural organisation of the epithelial component. This suggests that at least two factors are concerned in tissue organisation, an unstable diffusible substance and the actual physical structure of the inductor tissue. In more recent work Grobstein (1958) has cultured dorsal spinal cord and metanephrogenic mesenchyme on either side of membrane filters of different thickness. Epithelial tube formation was induced in the mesenchyme across filters up to 80 μ in thickness. An intermediate layer of cellophane between two filter layers blocked all transmission: when the cellophane was perforated by a small hole tubules formed in the mesenchyme immediately over or close to the hole. These results give support to the idea that large molecular materials are involved in the induction. This theory is supported by Niu and Twitty (1953) who cultivated ectodermal and mesodermal tissue from the species Triturus torosus. Young gastrula ectoderm cultivated by itself showed no evidence of histological or cytological differentiation. However striking changes occurred when the explant was grown in medium in which a mesodermal explant had been developing for the previous week. The ectodermal piece, in the absence of physical contact with the mesodermal tissue underwent marked histological differentiation. Later investigations suggested that a nucleoprotein in the medium might be effective in induction. (Niu 1956).

Some simple nutritional factors may also, on occasions, provide

a stimulus to differentiation. The development of chick ectoderm can be influenced in a radical manner by the presence of high vitamin A concentrations in the culture medium. Under these conditions a metaplastic change occurred: there was a complete suppression of keratinization and the ectoderm differentiated into mucus secreting, ciliated epithelium similar to that of normal nasal mucosa (Fell and Mellanby, 1953). In the absence of vitamin A the cells differentiated into squamous keratinizing epithelium. It is interesting to note that vitamin A deficiency in vivo causes the normal epithelium of the respiratory tract to be replaced by stratified keratinizing epithelium.

The possibility that physical factors may affect tissue organisation has been reinforced by the work of Leighton who used a sponge matrix for tissue culture. In this way organised aggregates of cells were formed in the outgrowths of explants from chick embryo and from mammary adenocarinome. (leighton 1951). More recently the growth patterns of a number of animal tumours have been studied by this method. (Leighton, 1954).

RELATIONS TO PRESENT STUDY.

These results indicate some of the factors which can effect tissue differentiation in vitro. Of particular interest to the present study is Grobstein's finding that the morphogenetic effect of caspular mesenchyme declines with successive sub-culture. This suggests that the loss of some diffusible material, vital to tissue organisation, parallels the process of de-differentiation in explanted material.

Some attempts have been made to define the biochemical indices of this change. Jones, Featherstone and Bonting, (1956) found that primary explants of chick embryo intestine cultivated for eight days in vitro lost more than half their cholinesterase activity, expressed per unit weight. Addition of acetyl choline to the medium eliminated the loss of enzyme activity. It seems that growth in the absence of the substrate resulted in loss of enzyme activity. It is possible that this is a general phenomenon occurring in a number of enzyme systems on transfer from in vivo to in vitro conditions. A further report of enzyme induction has been made by DeMars with strain HeLa cells (1958). He found that when the cells were grown in the presence of high concentrations of glutamic acid there was a notable increase in the activity of the glutamyl transferase enzyme system, as compared with cells grown in the presence of glutamine. It is thought that the transferase enzyme protein catalyses synthesis of glutamine. When the cells have adaptively formed this enzyme system, they can grow indefinitely with low concentrations of glutamic acid. Part of the present study is an attempt to define the possible changes occurring in carbohydrate metabolism and oxygen uptake during the initial period of de-differentiation in explanted chick embryonic material. An estimation was also made of the rate of incorporation of radioactive phosphorus into various phosphorus fractions. In this way it was hoped to define more precisely the metabolic changes accompanying the emergence of a uniform cell culture from explanted embryonic tissue. This work has been reported in recent papers.

(Paul and Pearson, 1957 a, 1957 b).

One large and varied group of compounds which are lost on transfer from *in vivo* to *in vitro* conditions are the hormones. For a long time it has been realised that tissue cultures provide several new approaches to the problem of hormone action. They make possible the study of the effect of a single hormone on viable material isolated from complicating factors present in the whole animal. Despite these advantages the method has thrown surprisingly little light on the subject. The most interesting results have described morphological changes in explanted material. Gaillard (1955) prepared organ cultures of parathyroid gland and cultivated them in immediate contact with the inner periosteum of explants of actively growing parietal bone from mouse embryo. A strong resorption of bone was observed in these circumstances. Estrone was shown to produce squamous metaplasia in explants of six week old mouse ventral prostate gland. (Lasnitski, 1954). In the presence of testosterone propionate similar material retained its normal histogical structure but in glands from older mice hyperplasia of the alveolar epithelium was observed (Lasnitski, 1955 b). These results indicate the parallel between the *in vivo* and the *in vitro* effect of hormones.

There have been few investigations into the changes in cell metabolism caused by hormones. Early work attempted to relate hormone action and cell growth. Von Haam and Cappel (1940 a) failed to observe an increased area of growth or an increased mitotic index in mouse embryo cultured in the presence of various sex hormones.

Continued studies showed that adrenaline and adrenal cortical hormones, similarly, had no effect. Insulin or thyroxin, in low concentrations, increased cell growth. (Von Haam and Cappel, 1940 b). Gill (1938) found adrenaline to have no effect on the release of glycogen from embryonic chick liver or muscle in vitro. About the same time Verne and Verne-Soubiran (1939) reported that neither anterior pituitary hormone, thyroxin, follicle-stimulating-hormone nor testosterone influenced lipid metabolism in tissue cultures of fibroblasts. More positive results have been obtained in studies with insulin. The initial studies were carried out by Gey and Thalhimer (1924) who measured the area of outgrowth in chick fibroblast cultures over a period of days. The increase was found to be greater in insulin-containing medium than in control medium. In addition to their studies on cell growth, von Haam and Cappel reported an increased uptake of glucose by fibroblasts in insulin-containing medium. The early results suggested that in the presence of insulin there is an increased rate of synthesis of the majority of cellular components. More recent papers have described the increase in more exact terms. Leslie and Davidson (1951) reported a greater amount of lipid phosphorus, ribonucleic acid phosphorus and protein nitrogen in explants of chick embryonic heart grown in the presence of the hormone. This was confirmed in a later paper by Leslie and Paul (1954) who also noted an increased synthesis of deoxyribonucleic acid (DNA) under these circumstances. This enhanced synthesis of DNA was accompanied by an increased glucose utilization and by a pronounced

fall in the pyruvate content of the medium. Using an organ culture technique, Sidman (1956 b) demonstrated that insulin increased the synthesis of glycogen and lipid in explants of brown adipose tissue from rats.

The second part of the study aimed to define the action of insulin in terms of the chemical metabolism of the cell. The project was aided by the recent advances in cell nutrition and chemical techniques, which have been discussed. The results will be considered in the light of current theories on insulin action.

T E C H N I Q U E S.

Techniques.

Animal cells in tissue culture demand specialized conditions for maintainance and growth. Not the least among these conditions is that all glassware and other equipment used in culturing these cells, should be sterile and non-toxic to the cells. It has been shown, for example, that some rubber stoppers contain factors which will inhibit growth (Parker, Morgan and Morton, 1951) and the same inhibitory effect has been noted with inadequately washed glassware. For this reason all new glassware was tested for toxicity and the following cleaning procedure employed as a routine.

Cleaning of Apparatus.

Solutions.

A. Sodium metasilicate (x 100).

360 g. sodium metasilicate

40 g. 'Calgon'

This was dissolved in 4 litres of water and filtered through glass wool.

It was diluted $\frac{1}{100}$ with tap-water for use.

B. N Hydrochloric acid.

It was diluted $\frac{1}{100}$ with tap-water for use.

C. De-ionised water.

General Glassware.

The glassware was scrubbed, then boiled in a domestic boiler containing solution A. After cooling it was rinsed with tap-water and placed overnight in a large bath of solution B. Before oven drying, the glassware was rinsed several times with tap-water and finally with de-ionised water.

Pipettes.

Pipettes were similarly treated, except that they were steeped in solution A and not boiled in it.

Stoppers.

All rubber stoppers were cleaned by boiling in a 5% (w/v) solution of sodium carbonate after which they were rinsed thoroughly with water.

Sterilisation.

For tissue culture purposes it is necessary to sterilise the culture vessels and the various media employed. This can be achieved by heat or by filtration. The most convenient method is by dry heat, 90 minutes at 160°C, but it is limited to materials which suffer no damage at this elevated temperature. Glassware was sterilised by this method but rubber bungs, syringes and most salt solutions were sterilised in an autoclave maintained at 15 lb. pressure for 20 minutes. The heat labile components of the medium were sterilised by filtration. For large volumes a Seitz asbestos filter was used while for smaller quantities a porcelain filter (Berkefeld W) or a sintered glass filter (3) was employed.

TISSUE CULTURE MEDIA.

The basis of all media is a balanced salt solution (BSS) which provides inorganic ions, a suitable osmotic pressure, a source of energy and a buffer system to control pH. This salt solution is made up in doubly glass distilled water. Hanks' BSS (1948), which employs a phosphate buffer system and does not require gassing with a CO_2/ air mixture, was used routinely. When it was necessary to adjust the pH accurately Earle's BSS (1943) with added sodium bicarbonate was used and the complete medium equilibrated with 5% CO_2 / air mixture. It was customary to include 0.02% phenol red in the salt solution to indicate the approximate pH. Tables of the constituents of these salt solutions are in the appendix.

In addition to the salt solution and a supply of oxygen, certain amino acids and vitamins are required, supplemented with serum protein, if the tissue is to grow. It has been found that omission of the protein and use of a completely synthetic mixture results in poor growth. However, all the requirements of the cell can be met using Eagle's medium supplemented with 20% horse serum. (Eagle, 1955 a). Alternatively a preparation of chick embryo extract can replace the constituents of Eagle's medium. Other chemically defined media, considered previously, were not utilized in this work. A description of Eagle's medium is appended.

Horse blood was collected under aseptic conditions directly into centrifuge bottles. It was allowed to clot and the serum

separated by centrifugation. It was then filtered through a Seitz type filter and a portion of the filtrate tested for sterility and toxicity. The serum was stored in screw-cap prescription bottles at -10°C until required. It was judged to be sterile if an aliquot produced no growth in a standard Difco thioglycollate broth tube after seven days incubation. The serum was tested for toxic components by including it in the medium fed to test-tube cell cultures. These were examined microscopically over several days for signs of growth inhibition.

Chick embryo extract was prepared in the following manner with 15 -day old embryos. Under aseptic conditions the embryo was taken from the egg and, after removing the eyes, was transferred to a universal container where it was pulped and diluted with an equal volume of BSS. This mixture stood for at least half an hour before the supernatant fluid was separated by centrifugation. The extract was transferred to a second container, tested for sterility in the manner described for horse serum, and stored at-10°C. Before use the EE was again centrifuged.

To minimise the risk of contamination, penicillin (50 units/ml) was included in the medium. At this level the antibiotic suppresses bacterial growth without influencing the growth of tissue culture cells. (Metzger et al., 1954).

Methods of Culture.

Most tissue culture work employs either primary explants or established

established cell strains as experimental material. The first is usually derived from embryonic tissue, although there are instances of successful cultures arising from adult material. (Perry, 1956). The two most useful methods of obtaining cultures of primary explants for biochemical purposes are the chopped tissue method and the trypsinization procedure. A modified organ culture method will also be described.

Chopped Tissue Technique.

The material used in the initial experiments to be described was chopped embryonic tissue. With some care this system will provide reproducible cultures for the quantitative assay of various indices of growth. One of the early uses of this technique was for the cultivation of viruses. (Maitland & Maitland, 1933). These workers grew vaccinia virus in suspensions of minced kidney fed on serum and Tyrode's solution. (Tyrode, 1910). The technique was employed by Morgan et al., (1950) as a test system in cell nutrition studies. Biochemical investigations were made on chopped embryonic material by Leslie and Paul (1954).

In the present studies the embryonic tissue was cut by means of a McIlwain tissue chopper, which is pictured opposite. (McIlwain and Buddle, 1953). It was set to give a uniform cubic explant of 0.5 mm. side. The metal cutting platform was unscrewed and sterilised by dry heat. Razor blades and 7 cm. filter paper were similarly prepared. The rest of the instrument was swabbed with ethanol to minimise

contamination. Just before use the machine was re-assembled; the razor blade, handled by sterile forceps, was fixed in position by a screw clamp. Sterile filter paper, dampened with BSS was placed on the platform to such a depth that the blade cut evenly into, but not through, the top sheet.

Hearts were removed aseptically from 15 - day embryos, the atria and large vessels cut off and the ventricles halved longitudinally. These halves were placed cut side down on the sterile filter paper and chopped in two directions at right angles to give cubes of the required dimensions. The diced tissue was then suspended in growth medium and dispensed in suitable portions to the culture vessels. It was important at this stage to keep the fragments in even suspension, to insure that replicate inocula were obtained. Alternatively the explants may be handled individually, and a certain number planted in each flask. This technique can be applied to a wide variety of tissues.

In organ culture it is advisable to maintain the tissue at or near an air interface, as it requires a high O_2 tension. Several ingenious methods have been devised for this purpose. In the watch glass method (Fell and Robinson, 1929) the organ is grown on the surface of a plasma- embryo extract clot in a watch glass placed in the middle of a ring of wet cotton wool in a Petri dish. Trowell (1954) maintained rat lymph glands by growing them on lens paper supported on a tantalum gauze table standing in nutrient fluid. By using a brand of lens paper that floats indefinitely Chen (1954,a)

dispensed with the tantalum. The technique was refined still further by Shaffer (1956) who made histological processing of the tissue very simple by using as a support a cellulose-acetate fabric soluble in acetone. It is interesting to remember that in 1912 Carrel was using pieces of silk veil to support plasma clots for the growth of chick embryo fragments. Shaffer made the fabric water-repellent by silicone coating. It was essentially a modification of this technique which was employed in the present investigation using brown fat from rats.

A five to six week-old rat was killed by stunning and cutting the carotid artery. The brown fat lying between the shoulder blades was quickly removed under aseptic conditions. It was then trimmed free of adhering tissue and chopped into uniformly small explants.

3 cm. squares of nylon net were autoclaved; the edges of these squares were subsequently dipped into molten paraffin wax. One at a time a predetermined number of explants were placed on the nylon rafts, floating in BSS. The rafts were then transferred to separate 50 mm. Petri dishes containing 3 ml. of growth medium. Each dish was covered with a circle of polythene, secured with an elastic band, and finally the Petri dish lid was positioned. The dishes were incubated at 36°C in the hot room which provided more satisfactory conditions for these cultures than did a small incubator. This method is sometimes named the floating raft technique.

Trypsinization technique. (with fresh tissue).

This technique, employed to obtain replicate cultures from chick embryo, was based on the work of Moscona (1952). Using a 3% trypsin solution, under controlled conditions, he was able to obtain suspensions of discrete viable cells from limb buds and the mesanephros of early chick embryo. Further cultivation of these cells showed that they continued to differentiate and attained a remarkable degree of histological organisation. An important point in the preparation of the trypsin solution was his use of calcium and magnesium free saline which has been shown to reduce the stability of the intercellular materials and hence to the mutual adhesiveness of the cells.

To obtain a culture of muscle cells, our starting material was decapitated, eviscerated chick embryo (15 days) from which the wings, feet and skin had been removed. The remaining tissue was finely chopped with sterile scissors, washed with calcium and magnesium free saline (see appendix) and suspended in about 50 ml. of 0.5% trypsin (Difco), also made up in the special salt solution. The container was placed in a roller drum at 38°C for about fifteen minutes then the tissue was more thoroughly disaggregated by sucking it up and down a wide bore pipette. The trypsin was separated by centrifugation and discarded, while the digest was suspended in growth medium and dispensed to suitable vessels. As the resulting cultures contained much bone debris, they were again treated with trypsin after two days growth and the suspension thus obtained

filtered through a fine steel gauze, fitted to the end of a 50 ml. syringe. The filtrate, now free of debris, was centrifuged, resuspended in medium and re-inoculated into clean flasks to provide a fairly homogeneous culture of muscle cells.

Established Cell Strains.

Most of the work to be described has been carried out with established cell strains which have been maintained in culture for a number of years and which, in theory, could be maintained indefinitely. Such a strain is Earle's L-cells, clone 929, which have been grown from a single cell isolated in 1948 from a culture of mouse connective tissue. (Sanford et al., 1948). This was achieved by taking a dilute suspension of cells and sucking it into a capillary tube which was then examined microscopically. A portion of the tube, containing only one cell, was broken off and implanted in a plasma clot. Presently and outgrowth of cells from the end of the tube was observed and the population was transferred to a larger vessel. In this manner a clone strain of cells was obtained. This ensures that all the cells are of the same general type although they are not necessarily genetically homogeneous. Some work was also carried out with HeLa cells which are derived from human epithelial cancer cells.

A third type of cell strain investigated, was that derived from the buffy coat of human blood in this laboratory. Centrifuge tubes, Pasteur pipettes, serum needles and syringes were coated with silicone

and sterilised. Blood was then removed by venepuncture and 10 ml. transferred to each centrifuge tube stored in ice. Under sterile conditions the tube was centrifuged at 3,000 r.p.m. for 10 minutes in an M.S.E. refrigerated centrifuge. Using a siliconed pipette the supernatant plasma was transferred to a second tube and stored in ice. Meanwhile one drop of $\frac{1}{10}$ dilution of EE was added to the packed cells, stirred carefully on the surface and centrifuged at 3,000 r.p.m. for two minutes. This tube was then incubated and after about 15 minutes the contents had clotted firmly. The clot was washed with BSS, cut into explants and implanted in a plasma clot of the autologous plasma. After some weeks it was possible to treat the explant with trypsin and obtain a cell suspension from which a uniform culture of cells was obtained.

All these cell strains grow as a uniform layer directly on the glass of the culture vessel and can therefore be fed with the liquid media previously described. Any type of non-toxic glass container with a flat surface which can be conveniently sterilised and sealed will serve as a growth vessel. The choice of vessel depends on the requirements of the experiment, but for routine handling large penicillin flasks are employed. The diagrams in the appendix show some of the glassware which is commonly used.

As the cells grow, the nutrients in the feeding solution are metabolised to acid end products, which accumulate in the medium with a consequent lowering of pH. This is indicated by a colour change in

the phenol-red, present in the feeding solution. The cells are maintained by replacing the exhausted medium with fresh medium. When overcrowding of the cells becomes apparent half of them are transferred to a clean flask. The cells are detached from the glass surface by physical agitation or with the aid of a 0.5% solution to trypsin made slightly alkaline with sodium bicarbonate. In this way a cell suspension is obtained. The suspension is centrifuged, the supernatant liquid discarded and the cells are resuspended in fresh medium before being inoculated into the growth vessels. By accurate pipetting it is possible to obtain a large number of replicate culture.

For experimental purposes it is sometimes desirable to know the number of cells being handled. This is estimated by diluting a portion of cell suspension to a suitable degree with a 1% solution of crystal violet in 0.1 M citric acid. A small drop of the dilute suspension is allowed to flow into a Fuchs-Rosenthal haemocytometer. The floor of the chamber is marked off in areas of $\frac{1}{16}$ sq. mm. and the depth is known to be 0.2 mm. It is possible, under the microscope, to count the number of cells lying in a certain area (usually 5 sq. mm.) and from this to compute the concentration of cells per cu.cm. This assumes that all the cells in the 0.2 mm. depth of the chamber are visualised.

Setting - up an Experiment.

Although the experiments to be described differ in detail, the

initial dispensing of cells and selection of controls was performed according to a standard procedure throughout the series. This method ensured as nearly reproducible cultures as possible, and an un-biased selection of control cultures.

The cells were prepared as an even suspension in a suitable volume of medium and 0.5 ml. of suspension was added to the flasks in a given order. A further 0.5 ml. was then added in the reverse order to give a total inoculum of 1 ml. in each flask. It was essential to keep the cells well mixed for they tended to sediment very quickly in the dispensing medium. Usually, half the cultures set up were used as controls and the remainder as tests. These two groups were selected by the use of a table of random numbers.

In general, two types of medium were employed, Eagle's medium and embryo-extract containing medium. The concentration of glucose was 200 mg./100 ml. unless otherwise stated, and when insulin was present it was in the concentration of 2 units/ml. medium. Pure insulin was supplied by Boots Pure Drug Co. Ltd., as an $\frac{N}{50}$ acid solution containing 200 units/ml. Control medium was prepared by adding an equivalent amount of $\frac{N}{50}$ Hydrochloric acid in place of insulin. Any necessary pH adjustment was made with 6.6% (w/v) sodium bicarbonate solution.

Methods of Chemical Analysis.

Analysis of the Medium.

In many experiments it was desirable to analyse the medium at intervals after their commencement and to compare the concentration of various metabolites then present, with their initial levels in the medium. Usually the medium was freed of cells by centrifugation and stored in the deep freeze until analysis. These methods have been used by Leslie and Paul (1954) and Paul and Pearson (1957 a, 1957 b).

For all analysis it was necessary to have a protein free solution. 2ml. of medium was added to 10 ml. 6% (w/v) meta-phosphoric acid, the solution was mixed and stood in ice for half an hour to allow the precipitate to flocculate. It was then centrifuged and the protein-free extract used for the analysis of carbohydrate, lactic acid and pyruvic acid. All reagents employed were of a recognised analytical grade.

Estimation of Carbohydrate. (Anthrone method).

Reagents.

Anthrone reagent; 200 mg. anthrone were dissolved in 100 ml. 25 N sulphuric acid.

Method.

This estimation was according to the method of Trevelyan and Harrison (1952)

1 ml. of a $\frac{1}{5}$ dilution of the protein free extract was

carefully layered on to the top of 5 ml. anthrone reagent in a pyrex tube. A series of tubes was prepared in this fashion including two reagent blanks and three standards (70 µg. glucose). The contents of the tubes were then mixed with a glass plunger and placed in a boiling water bath for exactly ten minutes, the tubes being handled one at a time and in strict rotation. On removal from the bath the tubes were placed in an ice-bath and, within half an hour, the optical density of the green colour estimated in a spectrophotometer (Unicam, SP 400) set at 620 mµ. The results are expressed in terms of glucose and the range is 10-150 µg. glucose.

Estimation of reducing sugar. (Folin and Wu method).

Reagents.

Solution A; 1.3% (w/v) copper sulphate.

Solution B; To 12.5 g. sodium bicarbonate dissolved in 150 ml. distilled water, 10 g. anhydrous sodium carbonate were added with stirring. To this solution were added 9.2 g. potassium oxalate in 30 ml. warmed water and 6 g. sodium potassium tartarate in 25 ml. water. The solution was made up to 250 ml.

Alkaline copper reagent: This was a mixture of equal volumes of Solutions A and B.

Phosphomolybdic acid reagent: 35 g. molybdic acid and 5 g. sodium tungstate were dissolved in 250 ml. 2N sodium hydroxide and the solution was boiled for 30 minutes. Water was added to 350 ml. then

125 ml. 89% (w/w) phosphoric acid were added. The solution was made up to 500 ml.

Diluted Phosphomolybdic acid Reagent: This contained 150 ml. of the phosphomolybdic acid reagent, 25 ml. phosphoric acid and 325 ml. water.

Sodium sulphate - copper sulphate solution: This was a mixture of 320 ml. 3% (w/v) sodium sulphate and 30 ml. 7% (w/v) cooper sulphate.

Sodium Tungstate solution 10% (w/v).

Method.

This was according to a modification by Dische (1955) of the Folin and Wu method of estimating reducing sugar.

As meta-phosphoric acid was found to interfere with the reaction it was not used to precipitate the proteins in the medium. For this purpose 0.75 ml. 10% sodium tungstate and 6 ml. sodium sulphate - copper sulphate solution were added to 0.75 ml. medium. The precicitated protein was removed by centrifugation and the supernatant liquid used for the estimation.

1 ml. of a $\frac{1}{5}$ dilution of the protein-free extract was added to 1 ml. of alkaline copper reagent in a test tube which was then heated in a boiling water bath for 6 minutes. After removing the tube from the bath and allowing it to cool at room temperature for 2-3 minutes, 5 ml. diluted phosphomolybdic acid reagent were added. The solutions were mixed and the resulting blue colour was measured immediately at 620 mμ. Reagent blanks and standerd glucose solutions were included in each set of estimations.

The range of the method is up to 75 μg. glucose.

Estimation of Lactic Acid.

Reagents.

12% (w/v) copper sulphate.

20% (w/v) copper sulphate

p-hydroxydiphenyl reagent: 1.5 g. recrystallised p-hydroxydiphenyl were dissolved in 10 ml. 5% sodium hydroxide and diluted to 100 ml. with water.

Solid calcium hydroxide

Concentrated sulphuric acid. (ordinary grade)

Method.

This estimation was according to the method of Hullin and Noble (1953). 1 ml. of extract was added to a centrifuge tube containing 1 ml. of 20% copper sulphate solution and the volume made up to 10 ml. with water. To this was added 1 g. solid calcium hydroxide: the mixture was well shaken and it stood for half an hour before centrifugation. This copper-lime treatment breaks down α-Keto acids and prevents their estimation along with the lactic acid. 1 ml. of the supernatant liquid was transferred to a glass-stoppered test tube containing 0.05 ml. 12% copper sulphate solution. A number of such tubes were shaken by an attachment to a microid flask shaker in an ice-bath, and 6 ml. of concentrated sulphuric acid were added dropwise to each. The tubes were quickly stoppered and placed in a water bath at $60^{\circ}C \pm 1^{\circ}C$ for 30 minutes. After cooling to room temperature, 0.1 ml. of p-hydroxydiphenyl reagent was added and the characteristic purple colour developed at $28^{\circ}C \pm 2^{\circ}C$ for twenty

minutes. The excess reagent was then destroyed by 90 seconds immersion in a boiling water bath. A calibration standard was prepared from lithium lactate, equivalent to 100 µg. lactic acid per 1 ml. and it was taken through the entire procedure, as was a reagent blank. Readings of the optical density were made on the Unicam SP 400 spectrophotometer at 560 mµ. and the colour was found to be stable for some hours. Where no HS or BE was present in the original medium, it was found necessary to use larger volumes of protein-free solution. It was of interest that some batches of Analar sulphuric acid failed to give colour development with standard solutions of lactate treated as above. For this reason ordinary grades of sulphuric acid were employed.

Estimation of α-Keto acids.

Reagents.

Dinitrophenylhydrazine reagent: 100 mg. 2.4 dinitrophenylhydrazine were dissolved in 100 ml. 2N hydrochloric acid.

10% (w/v) trisodium phosphate

1.5 N sodium hydroxide

Chloroform

Method.

This estimation was according to the method of Leslie and Paul (1954).

1 ml. of dinitrophenylhydrazine reagent was added to 4 ml. deproteinized medium and the mixture was allowed to react for 10

minutes at 25°C. The hydrazones so formed were extracted in three sucessive volumes of 3 ml. chloroform and the extracts transferred to another tube. The combined chloroform extract was shaken with 5 ml. trisodium phosphate which, in turn, extracted the hydrazones from the chloroform. 4 ml. of the trisodium phosphate layer were pipetted into a third tube and a characteristic reddish colour was developed by the addition of 2 ml. 1.5 N sodium hydroxide. Optical densities were measured in the Unicam SP 400 spectrophotometer at 445 mμ. Standards (30 μg. pyruvic acid) and reagent blanks were included. The range of the method is 5-50 μg. keto-acids expressed as pyruvic acid.

Radioactivity.

In some experiments it was necessary to estimate the residual radioactivity in the medium some time after the addition of radioactive phosphorus (^{32}P). This was effected by diluting the medium to give a suitable count of not more than 3000 per minute. The diluted medium was counted in a liquid counter (20th Century Electronics, type M6M) connected to a conventional scaling unit.

Analysis of the tissue.

Many experiments were designed to study the incorporation of ^{32}P into various tissue fractions, and for this reason the length of time of exposure to the isotope was strictly controlled. The cultures were handled in rotation as the ^{32}P was added, and at the end of the measured interval, again in the same order, the medium was decanted and the cells were quickly washed twice with phosphorus free saline. Alcohol/ether mixture was then added to end all enzymic reactions. Tissue not immediately fractionated was stored in the deep freeze prior to the addition of the alcohol/ether mixture.

The methods of fractionation and subsequent analysis of these small amounts of tissue have recently been collected and published. (Paul, 1958).

Reagents for Tissue Analysis.

Ethanol - ether mixture (3 : 1 v/v).

Ether - carbon tetrachloride (3 : 1 v/v).

Chloroform (B. P.).

Sulphuric acid N and 10N.

Hydrochloric acid concentrated and 2.5 N.

Perchloric acid N and 4N.

Trichloroacetic acid N.

Sodium bicarbonate solution 1% (w/v).

Sodium hydroxide solution 2N and 0.5 N.

Ammonium molybdate solution 2.5% (w/v).

Aminonaphtholsulphonic acid reagent:

1 g. 1-amino-2naphthol-4-sulphonic acid

59.5 g. sodium bisulphite

2 g. anhydrous sodium sulphite

These components were dissolved in 1 litre distilled water.

Nicloux reagent.

12.5 g. silver dichromate were dissolved in 500 ml. conc. sulphuric /
/acid.

Anthrone reagent.

200 mg. anthrone were dissolved in 100 ml. 25N sulphuric acid.

Indole solution.

0.04 g. indole (purified by vacuum distillation) was dissolved in 100 ml. distilled water.

Selenium dioxide solution.

1 g. selenium dioxide was dissolved in 100 ml. 50% (v/v) sulphuric acid.

Nessler Reagent.

3.5 g. gum acacia in 750 ml. water were added to a solution of 4 g. potassium iodide and 4 g. mercuric iodide in 25 ml. water. The solution was made up to 1 litre.

Fractionation prodedure.

The tissue to be analysed was removed from the surface of the culture vessel, by physical means, and transferred, with the alcohol/ether mixture, to a centrifuge tube. This stood at room temperature with occasional stirring for 10 minutes: it was then spun down and the supernatant liquid collected. This procedure was repeated with 2 ml. of the ether- carbontetrachloride mixture and then 2 ml. of ethanol ether. The combined supernatant liquids formed the LIPID FRACTION.

The residue could then be treated in one of two ways. If it were required to obtain a fraction for subsequent chromatography, then the tissue was extracted three times with 2 ml. N trichloro-acetic acid at 0°C, care being taken to hold this temperature during centrifugation. The combined supernatants were ether extracted and stored in the deep freeze as TRICHLOROACETIC ACID (TCA) FRACTION, for chromatography.

Otherwise the residue was extracted with three portions of 2 ml. ice cold N sulphuric acid and the supernatant acid collected

by refrigerated centrifugation. The combined supernatant liquids formed the ACID SOLUBLE 1 (AS1) FRACTION.

2 ml. of N perchloric acid were added to the material remaining in the tube. The tube was then placed in a water bath at 70°C for twenty minutes and the contents were stirred occasionally. The tissue was separated from the acid by centrifugation. This procedure was repeated with a further volume of perchloric acid and the combined supernatant liquids formed the ACID SOLUBLE 2 (AS2) FRACTION. There was left the RESIDUAL FRACTION. This separation is based on Schneiders procedure. (1945).

When ^{32}P had been incorporated into the tissue, however, a different technique based on the Schmidt and Thannhauser (1948) fractionation scheme was employed after the removal of the AS1 fraction. This permitted the separation of various phosphorus fractions. 1 ml. of 0.5 N sodium hydroxide was added to the material in the tube after acid extraction. The tube was stoppered and incubated for 18 hours at 38°C. The digest was cooled to 0°C and 0.2 ml. 2.5N hydrochloric acid and 0.3 ml. 4N perchloric acid were added. The deoxyribonucleic acid (DNA) precipitate which was formed was alloved to flocculate at 0°C for 10 minutes: it was then separated by centrifugation and washed twice with 0.5 ml. N perchloric acid. The combined washings and supernatant liquid contained the RIBONUCLEIC ACID (RNA) FRACTION, while the washed precipitate formed the DNA FRACTION.

Analysis of these fractions.

LIPID FRACTION.

This fraction was shaken with 2 ml. 1% sodium bicarbonate. The aqueous layer which separated was removed to a second tube where it was washed twice with 2 ml. ether. The ether washings were combined with the washed lipid fraction and the total volume was noted. Suitable protions were pipetted into a number of tubes and the solvents were evaporated on a hot air bath. Estimations of total lipid phosphorus were made as follows.

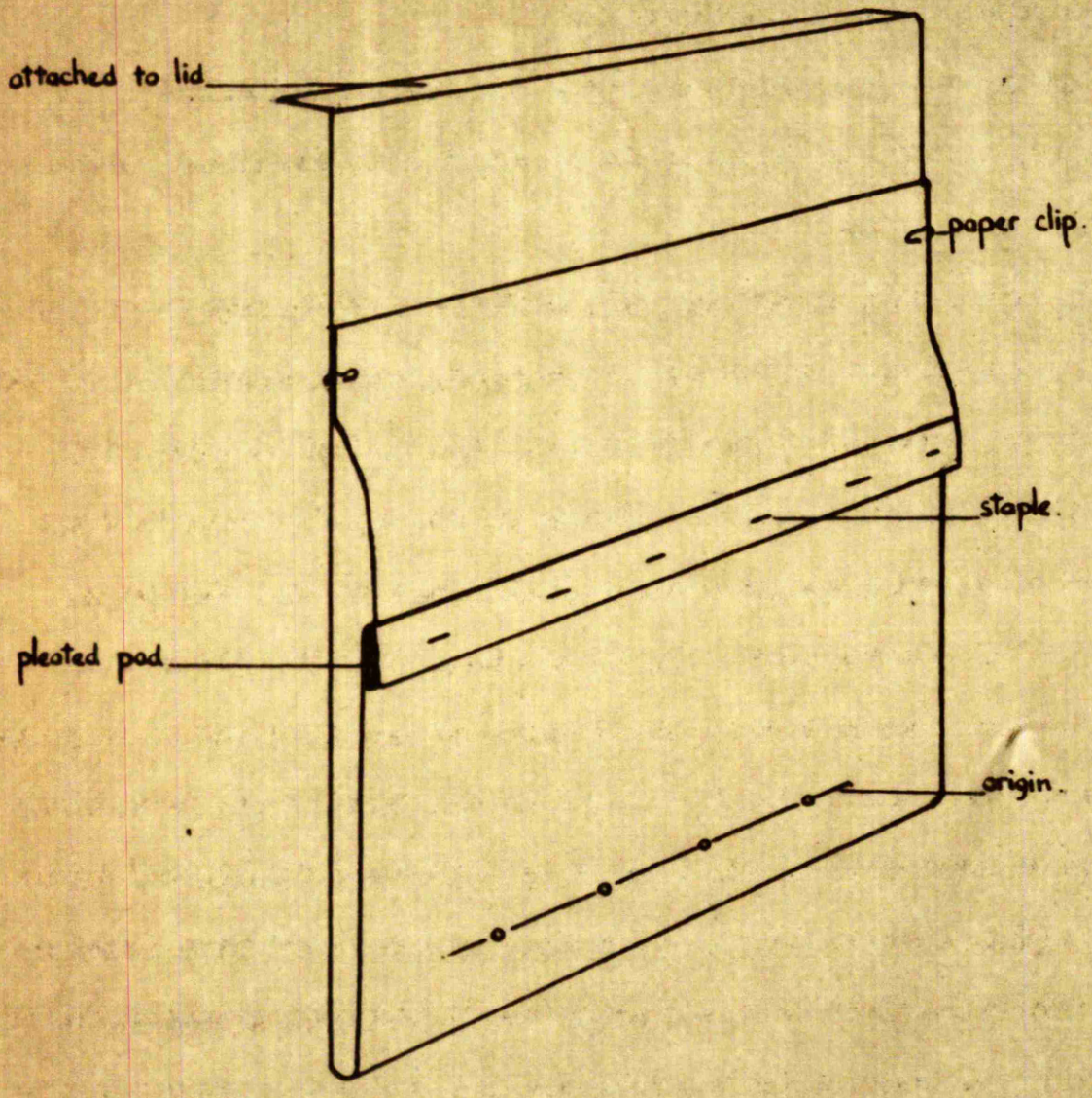
Lipid Phosphorus.

To the dry residue 0.5 ml. 1ON sulphuric acid and 0.5 ml. 4N perchloric acid were added. The contents were digested on a sandbath at 160° C and finally on an electric micro-digestion rack. The phosphorus content of the sample was determined by the method of Griswold, Mumoller and McIntyre (1951). 0.5 ml. of 2.5% ammonium molybdate solution, and 0.5 ml. aminonaptholsulphonic acid reagent were added to the tube and the volume was made up to 5 ml. with water. After heating in a boiling water bath for ten minutes, the blue colour in the cooled solution was measured in a Unicam SP 600 at 820 mμ. The range is from 0.2 to 2μg. phosphorus. Radioactivity was measured on a portion of the blue solution as described previously.

Total Lipid.

This was assayed by Bloor's method (Bloor, 1947).

3 ml. of Nicloux reagent were added to the dry residue and to similarly treated solvent blanks and standards. The tubes were



Folding of paper for chromatography.

heated in a boiling water bath for 15 minutes, cooled and 3 ml. distilled water added to each. The colour was measured in a Unicam SP 400 at 620 mμ. A solution of pure lard was used as a standard and the range found to be 50-300 μg. fat.

TCA FRACTION. (for chromatography)

Part of this fraction was digested with sulphuric and perchloric acids and an estimate of total phosphorus and radio-activity made on it.

The remainder, which had been ether extracted, was lyophylized in the conventional was prior to chromatographic separation of the constituent radioactive nucleotides by the method of Krebs and Hems (1953). Taken up in the minimum of water, this fraction was applied to versene washed Whatman No.1 paper cut and folded as shown in the diagram opposite. It was hung from the lid of the chromatography tank so that the folded end was immersed to a depth of about 2 cm. in the first solvent in the foot of the tank. This solvent consisted of 90 ml. iso-propyl ether and 60 ml. 90% (w/v) formic acid. The separation was continued for about five hours or until the solvent front had reached the folded pad of paper. In this way the highly active inorganic phosphate was washed into the pleated paper, while the nucleotides moved only slightly from the origin.

The air-dried paper was monitored with a Geiger-Muller end-window type counter to locate the two discrete centres of activity. That portion of the paper containing the inorganic phosphorus was cut off, and the remainder was subjected to descending chromatography

in the second solvent which consisted of 100 ml. iso-butyric acid, 60 ml. N ammonia and 1.6 ml. 0.1 M versene. After 16-18 hours the paper was dried in a cool oven and the nucleotides were visualized in ultra-violet light. In addition, an X-ray film was exposed to the chromatogram overnight, and by developing this and replacing it on the chromatogram the exact location of the nucleotides was determined. Marker compounds were run in conjunction with the unknown nucleotides and hence the nucleotides could be indentified. These were cut out as standard size circles with corresponding pieces of blank chromatogram for each nucleotide. The paper circles were dry-ashed individually in porcelain crucibles over a bunsen, and the ash was transferred completely to separate test tubes. The phosphorus content and radioactivity were determined on the sulphuric acid digests of these ashes as described previously. The average value for the blanks was subtracted from the corresponding test estimation to give a true indication of the nucleotide phosphorus present.

ASI FRACTION.

0.5 ml. 4N perchloric acid was added to the fraction and it was digested as described previously. Aliquots of the digest were used for the determination of phosphorus, carbohydrate content and radioactivity. This fraction was also called the acid soluble phosphorus (ASP) fraction.

AS2 FRACTION.

Total nucleic acids were estimated by measuring the ultra-violet adsorption of this fraction at 268 mμ. in a Beckmann ultra-

violet spectrophotometer. (Ceriotti, 1955). The contribution of DNA to this total was determined by the method of Ceriotti (1952). For this purpose 2 ml. of the fraction were pipetted into a glass-stoppered test tube. 1 ml. of indole solution and 1 ml. concentrated hydrochloric acid were added. The tube was placed in a boiling water bath for ten minutes, then it was removed and cooled. A pink interfering colour was washed out of the reaction mixture by three extractions with chloroform, which were discarded. The optical density of the remaining yellow aqueous solution was determined in a spectrophotometer at 490 mμ. The range of the method is from 5 to 30 μg. DNA. Each set of estimations included blanks and standards. Knowing the amount of DNA (μg) in the fraction it is possible to calculate the amount of ultra-violet adsorption caused by it. When this figure is subtracted from the total amount of adsorption at 268 mμ. a value is obtained which corresponds to the amount of RNA in the fraction. A standard figure converts this to μg. RNA.

RESIDUAL FRACTION.

This consisted mainly of protein. To it was added 0.5 ml. selenium dioxide solution and the mixture was digested as before. Total nitrogen in the fraction was determined using Nessler reagent.

The digested mixture was diluted to contain not more than 15 ug. nitrogen per ml. To 2 ml. of this solution, 2 ml. Nessler reagent and 3 ml. 2N sodium hydroxide were added. After 15 minutes the yellow colour was measured at 490 mμ.

RNA FRACTION.

As very small amounts of tissue were involved, it was not possible to purify this fraction further. The estimations of phosphorus carried out on this digested fraction were therefore, only approximate indications of the total RNA phosphorus present. Radioactivity was also determined.

DNA FRACTION.

Aliquots of this fraction were digested with sulphuric and perchloric acids then they were assayed for phosphorus and radioactivity.

RESULTS.

Adaptation Experiments.

Shortly after explantation of chick-embryonic material a rapid outgrowth of new cells is observed from the primary explant. Under certain conditions, and over some weeks, the structure of the original explant is lost and the cell-type becomes uniform. These cells are thought to be fibroblasts, characterised by their rapid unorganized growth. It would seem that a change has occured from the controlled and organized growth observed *in vivo*. These studies were undertaken in an attempt to find a reflection of this change in the pattern of metabolism during the early stages of growth *in vitro*. In a series of experiments, both tissue and medium were analysed at various times after explantation. The incorporation of radioactive phosphorus into several phosphorus fractions was determined after a measurement of the oxygen consumption of the tissue had been made. The experiments were carried out on chick embryonic heart and liver.

Medium analysis.

Two types of conditions were employed in these experiments. In the first group (1C) a high ratio of medium to cells was maintained. Kolle flasks containing 15 ml. medium were used. Samples of medium were withdrawn every day and the experiment was terminated after six days without renewal of the medium. In the second group (1G) a low ratio of medium/cells was maintained: the same initial weight of explants (15-30 mg.) was grown in Warburg flasks containing 2 ml. medium. The medium was changed completely every 48 hours. Cultures were terminated at

various time intervals, and the medium was collected for analysis. In this latter group culture vessels were agitated for the last four hours of each period to permit respiratory measurements. The results of the analyses of the medium are shown in tables 1, 2, 3 and 4. They are also presented diagramatically in figures 1, 2, 3 and 4.

Explanted heart.

The results shown in table 1 confirm previous findings that cultured chick embryonic heart tissue utilises glucose with the production of lactic and pyruvic acid. (Leslie and Paul, 1954. Lipmann, 1932. Willmer, 1942). Figure 1 illustrates that when the glucose concentration has fallen to a low level there is utilisation of the lactic and pyruvic acids in the medium. This has also been observed by Wilson et al., 1942. The diagram also illustrates that the amounts of lactic and pyruvic acids produced in the first few hours are in excess of the glucose utilised. This can be accounted for only in part by the release of endogenous carbohydrate.

In the experiments in which heart tissue was grown in a limited amount of medium (Table 2) a similar pattern of metabolism to the above was found in the first 48 hrs. However, at no time was there evidence of a utilisation of pyruvic or lactic acids. After renewal of the medium there was a continued high rate of glucose utilisation accompanied by a lactic and pyruvic acid production which accounted for over 80% of the glucose utilised. These results suggest that the previously observed utilisation of lactate and pyruvate was caused by

exhaustion of the supplies of available glucose.

Explanted liver.

The most notable feature of the results shown in Table 3 (a high ratio of medium to tissue) was the apparent utilisation of lactic acid in the first few hours of culture. This was accompanied by a rise in the hexose level of the medium. At this time pyruvate was being produced by the tissue. Figure 3 represents the balance between glucose and its products in these experiments. It emphasizes that not all the hexose and pyruvate produced after five hours could be accounted for in terms of lactate utilisation. By 24 hours there was evidence of some hexose utilisation. Later still this was supplemented by an uptake of pyruvate from the medium with the production of lactic acid. Towards the end of the experiment the glucose utilisation tailed off and there was very little lactate production. By 24 hrs. there was evidence of some hexose utilisation. Later still this was supplemented by an uptake of pyruvate from the medium with the production of lactic acid. Towards the end of the experiment the glucose utilisation tailed off and there was very little lactate production.

Table 4 defines the changes which occurred when the tissue was grown in a limited amount of medium. In these conditions there was again an early utilisation of lactate accompanied by a production of hexose. After renewing the medium at 48 hrs. this pattern was not repeated and there was rapid utilisation of glucose with production of lactic and pyruvic acids. The production of hexose in the first few

hours could not be accounted for completely by release of endogenous carbohydrate and by utilisation of lactic acid. The nature of the carbohydrate material synthesized was not established but figure 5 shows that the increase in hexose, as determined by the anthrone method for total carbohydrate, was paralleled by an increase in reducing sugar (method of Folin and Wu). The constant difference between these two estimations may be explained by the existence of some non-reducing hexose in the medium.

TABLE 1.

Changes in the composition of the medium during the first six days of growth of chick embryonic heart explants *in vitro*. A high ratio of medium to tissue was employed. Samples of medium were analysed at the times shown. Results are expressed as the mean ± S.D.

Estimation & Experiment No.	No. of Observations.	Hours of Incubation. 0	5	24	48	72	96	144
Hexose. mg./ml.								
IC1	1	1.354	-	0.664	0.500	0.348	0.188	0.136
IC2	1	1.576	-	1.585	1.508	1.419	1.124	1.040
IC4-9	6	1.575	1.518 ±0.092	1.217 ±0.108	0.904 ±0.046	0.776 ±0.071	0.672 ±0.061	0.583 ±0.053
Lactate. mg./ml.								
IC1	1	0.197	-	0.571	0.614	0.532	0.554	0.562
IC2	1	0.917	-	0.316	0.336	0.495	0.696	0.882
IC4-9	6	0.040	0.533 ±0.028	0.752 ±0.063	0.917 ±0.097	0.030 ±0.073	1.040 ±0.057	0.918 ±0.124
Pyruvate ug./ml.								
IC1	1	3.44	-	33.9	36.8	41.8	31.9	24.1
IC2	1	3.00	-	15.7	31.4	33.2	28.6	36.0
IC4-9	6	9.60	13.5 ± 1.37	36.0 ± 2.67	48.1 ± 4.04	50.0 ± 2.55	49.2 ± 2.79	38.5 ± 2.18

TABLE 2.

Changes in the composition of the medium during the first three days of growth of chick embryonic heart explants in vitro. A low ratio of medium to tissue was employed and the medium was renewed after 48 hrs. Results are expressed as mean ± S.D.

Estimation & Experiment No.	No. of Observations	Hours of Incubation. 0	4	28	48	52	76
Hexose mg./2ml. IGI	3	2.67	2.505 ±0.2.	0.908 ±0.322	0.430 ±0.06	2.16 ±0.068	0.965 ±0.128
IG2	3	2.558	2.455 ±0.14	1.331 ±0.359	0.758 ±0.07	2.337 ±0.051	1.231 ±0.055
IG3	3	2.758	2.587 ±0.11	1.191 ±0.033	0.697 ±0.17	2.307 ±0.12	1.308 ±0.004
Lactate mg./2ml. IGI	3	1.156	1.062 ±0.166	3.078 ±0.459	5.165 ±0.243	1.521 ±0.047	2.165 ±0.219
IG2	3	0.820	1.228 ±0.24	2.471 ±0.09	2.807 ±0.232	1.229 ±0.062	2.227 ±0.084
IG3	3	0.938	1.576 ±0.105	2.800 ±0.3	3.435 ±0.172	1.049 ±0.014	2.085 ±0.05
Pyruvate µg./2ml. IGI	3	14.4	80.9 ±18.12	98.9 ±10.72	12.8 ±10.55	36.6 ±0.082	73 ±10.31
IG2	3	15.43	67.4 ± 5.17	100.3 ± 11.3	105.2 ± 11.9	26.24 ±4.7	78.7 ±12.64
IG3	3	13.32	70.9 ± 4.5	91.97 ±10.0	89 ±17.3	35.3 ± 1.3	68*

* Single observation.

TABLE 3.

Changes in the composition of the medium during the first six days of growth of chick embryonic liver explants *in vitro*. A high ratio of medium to tissue was employed. Samples of medium were analysed at the times shown.

Estimation & Experiment No.	No. of Observations	Hours of Incubation						
		0	5	24	48	72	96	144
Hexose.mg./ml.								
IC1	1	1.354	-	0.832	0.664	0.248	0.072	0.082
IC2	1	1.576	-	1.524	1.484	0.990	0.708	0.560
IC4-9	6	1.451	1.576 ±0.046	1.524 ±0.071	1.199 ±0.077	0.958 ±0.032	0.770 ±0.082	0.633 ±0.017
Lactate.mg./ml.								
IC1	1	0.197	-	0.406	0.508	0.440	0.489	0.354
IC2	1	0.197	-	0.089	0.824	0.643	0.866	0.954
IC4-9	6	0.525	0.409 ±0.021	0.360 ±0.056	0.652 ±0.069	0.779 ±0.052	0.837 ±0.039	0.823 ±0.078
Pyruvate.µg./ml.								
IC1	1	3.44	-	28.0	32.9	22.2	16.2	17.2
IC2	1	3.00	-	8.52	18.42	21.9	15.7	16.6
IC4-9	6	9.6	10.6 ± 1.9	22.1 ±1.9	20.9 ± 2.15	19.4 ±2.26	18.4 ±1.2	13.9 ±4.25

TABLE 4.

Changes in the composition of the medium during the first three days of growth of chick embryonic liver explants in vitro. A low ratio of medium to tissue was employed and the medium was renewed after 48 hrs.

Estimation & Experiment No.	No. of Obs.	Hours of Incubation					
		0	4	28	48	52	76
Hexose mg./2ml. IG1	3	2.67	3.24 ±0.4	2.205 ±0.282	1.462 ±0.177	2.345 ±0.145	1.095 ±0.130
IG2	3	2.588	4.47 ±0.524	2.928 ±0.334	2.503 ±0.206	2.445 ±0.099	1.373 ±0.108
IG3	3	2.758	3.96 ±0.242	2.276 ±0.016	1.583 ±0.13	2.508 ±0.095	1.144 ±0.004
Lactate mg./2ml. IG1	3	1.156	0.507 ±0.034	1.757 ±0.479	2.478 ±0.126	1.352 ±0.13	2.313 ±0.019
IG2	3	0.820	0.156 ±0.005	1.999 ±0.216	1.96 ±0.281	1.251 ±0.222	1.923 ±0.099
IG3	3	0.938	0.112 ±0.069	1.684 ±0.116	2.39 ±0.05	1.142 ±0.41	2.034 ±0.166
Pyruvate µg/2ml. IG1	3	14.4	70.6 ± 4.08	65.0 ±9.499	68.8 ± 1.79	54.5 ± 4.47	73.5 ±10.79
IG2	3	15.43	20.33 ±3.107	61.7*	68.3 ±21.86	31.25 ± 2.70	55.56 ± 7.08
IG3	3	13.32	32.56 ± 7.0	45.7 ± 0.5	56.3 ± 2.04	43.4 ± 5.4	53.7 ± 2.2

* Single Observation.

Fig.1. Balance between the utilisation and production of glucose and its products measured in the medium during the culture of chick embryonic heart tissue. The amount of each substance utilised or produced per hour was calculated from the change in concentration since the previous observation. A high ratio of medium to tissue was employed and the medium was not renewed.

Fig.2. Balance between the utilisation and production of glucose and its products measured in the medium during the culture of chick embryonic heart tissue. A low ratio of medium to tissue was employed and the medium was completely renewed after 48 hr. *in vitro*. The amount of each substance utilised or produced per hour was calculated as above.

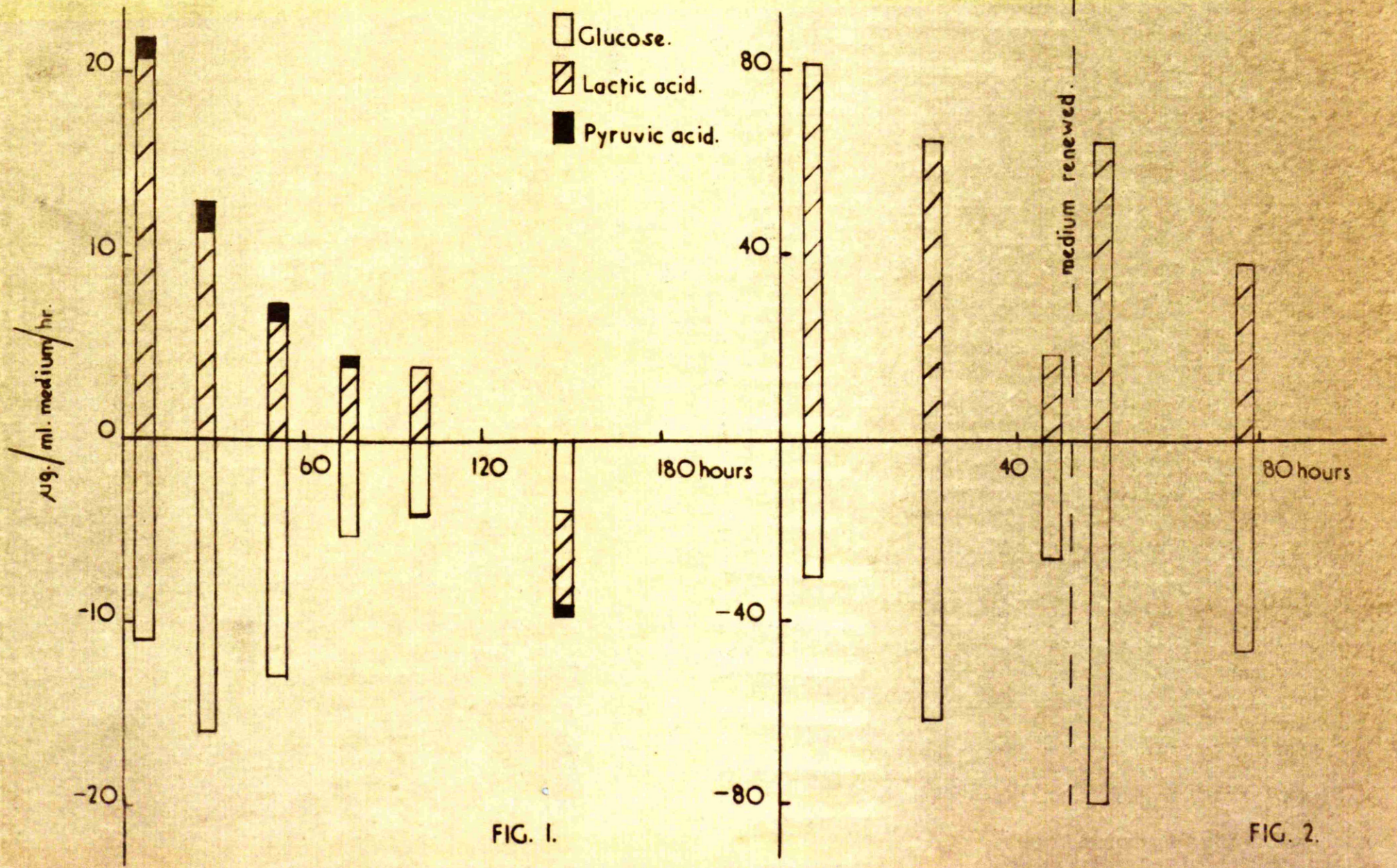

Glucose.
Lactic acid.
Pyruvic acid.
20
10
0
-10
-20
µg./ml. medium/hr.
60
120
180 hours

FIG. 1.

80
40
-40
-80
medium renewed.
40
80 hours

FIG. 2.

Fig.3. Balance between the utilisation and production of glucose and its products measured in the medium during the culture of chick embryonic liver tissue. The amount of each substance utilised or produced per hour was calculated from the change in concentration since the previous observation. A high ratio of medium to tissue was employed and the medium was not renewed.

Fig. 4. Balance between the utilisation and production of glucose and its products measured in the medium during the culture of chick embryonic liver tissue. A low ratio of medium to tissue was employed and the medium was completely renewed after 48 hours *in vitro*. The amount of each substance utilised or produced was calculated as above.

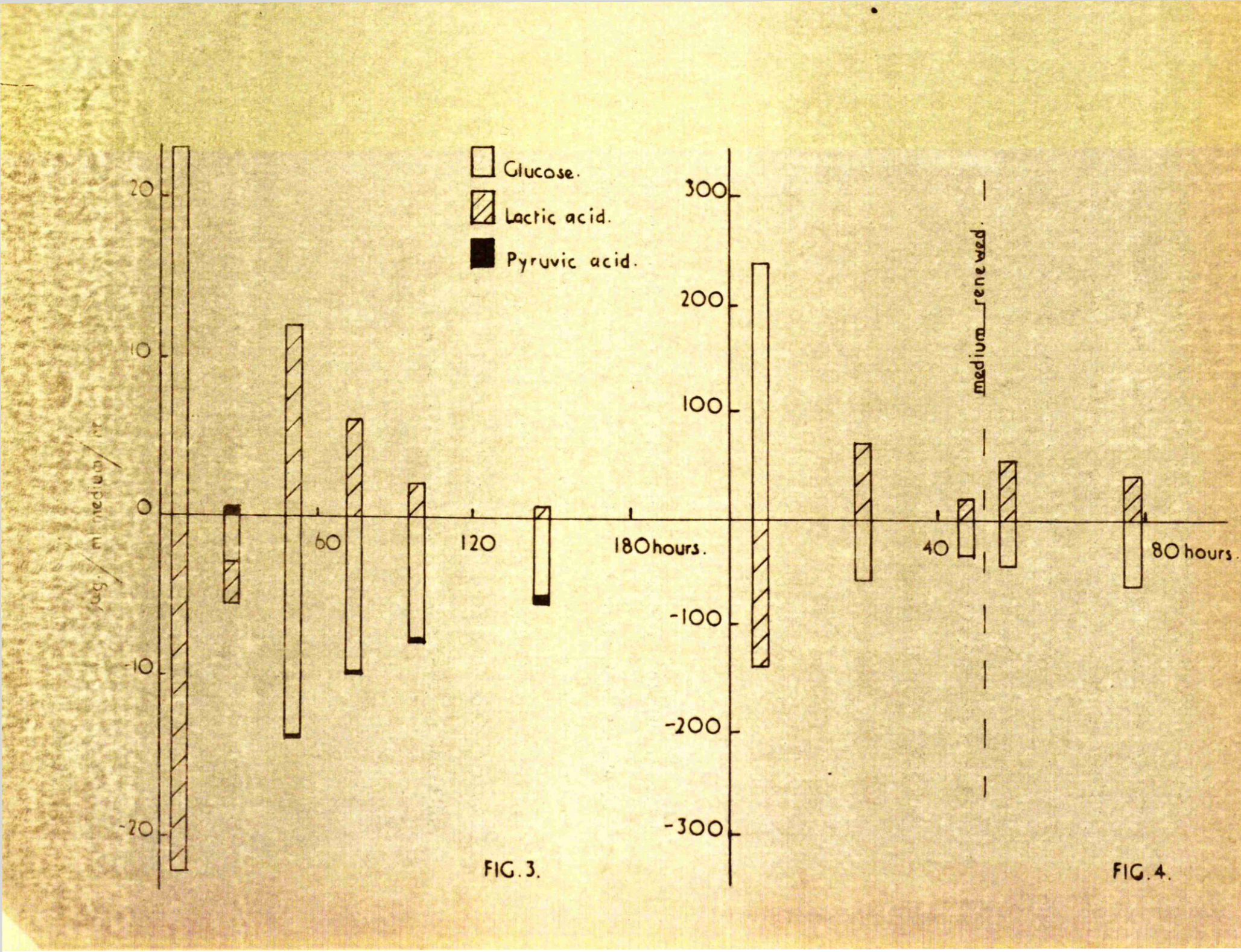

Glucose.
Lactic acid.
Pyruvic acid.
20
10
0
-10
-20
mg. / ml medium / ml.
60
120
180 hours.
300
200
100
-100
-200
-300
medium renewed.
40
80 hours.

FIG. 3.

FIG. 4.

Fig.5. Changes in the concentration of carbohydrate in the medium, as estimated by the Anthrone method and the Folin and Wu method, when chick embryonic liver explants were cultured *in vitro*. A low ratio of medium to tissue was employed and the medium was renewed after 48 hours.

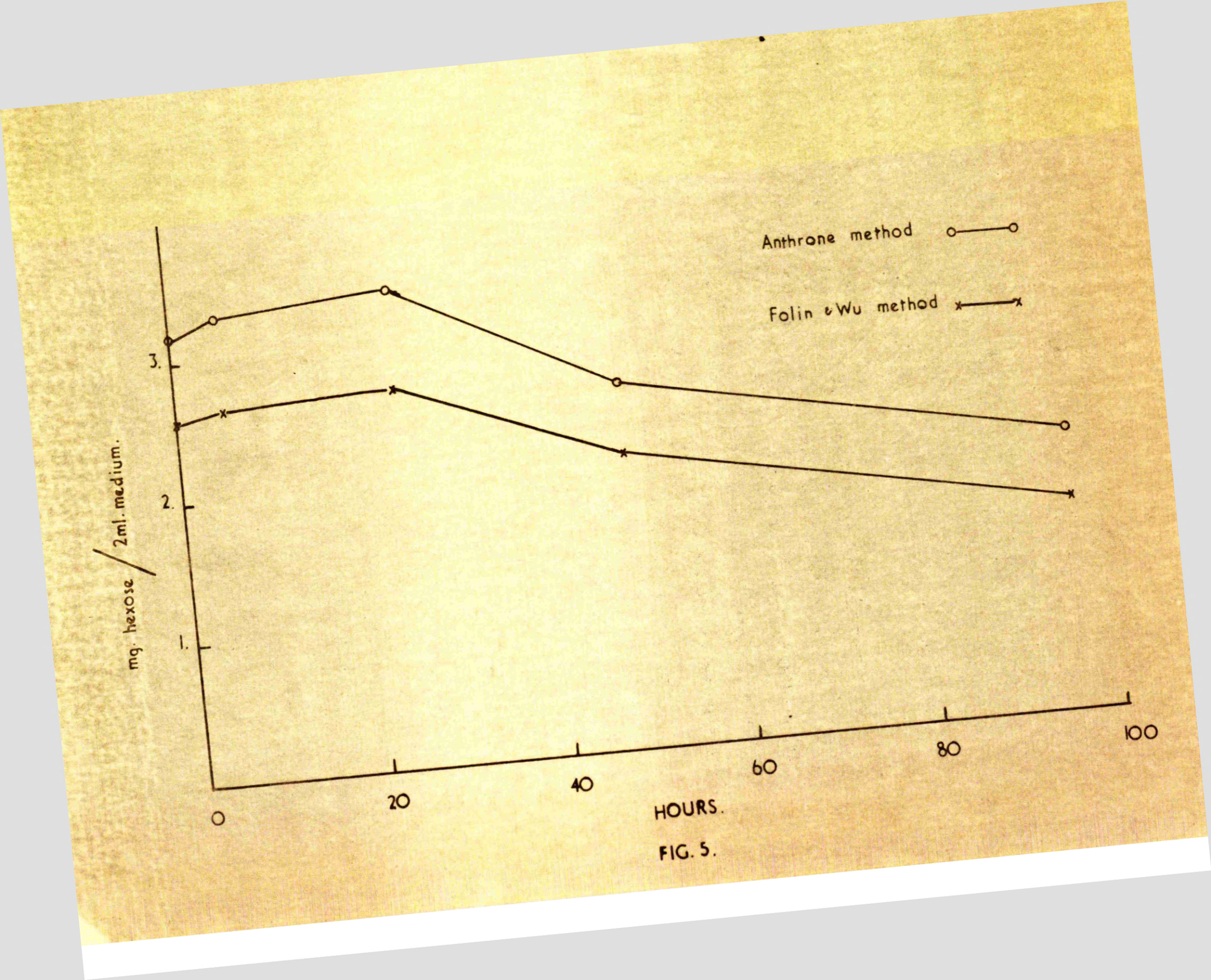

Anthrone method
Folin & Wu method
mg. hexose / 2ml. medium.
3.
2.
1.
0
20
40
60
80
100
HOURS.

FIG. 5.

Tissue Analysis.

In this series of experiments (IH) the conditions were similar to those employed in the IG type of experiment, in which a low ratio of medium to tissue was employed. 15-30 mg. of explants were grown in sterile Warburg flasks containing 2 ml. of medium. The cultures were fed every 48 hours. Respiration measurements were made in the last four hours of each period but no radioactive phosphorus was included in the medium.

Heart. (Table 5).

Analysis of the DNAP content of the tissue at various times after explantation, showed a progressive fall from the initial value. Histological examination revealed necrosis of many muscle cells at the centre of the explant but vigorous growth of fibroblasts at the periphery. This decrease in DNAP content was paralleled by an immediate and continued fall in the total hexose content of the explants.

In contrast to this, there was an accumulation of lipid in the explanted tissue. The amount of protein nitrogen in each culture remained fairly constant throughout the six days of the experiment.

Liver (Table 6).

These results showed a rapid and prolonged fall in the DNAP content of the embryonic liver tissue after explantation. Histological observations showed a good survival of all tissue elements but a

central necrosis of hepatocytes. There was, however, a good outgrowth of fibroblasts from the original explant over the floor of the culture vessel.

The hexose content of the liver explants was maintained at a steady level until the fourth day when a slight fall was noted. There was no immediate drop in the tissue carbohydrate, as was observed in the chick heart cultures. The analysis of lipid content showed a gradual decline until the fourth day when there was evidence of a slight increase in the amount present. The protein nitrogen of the cultures fell progressively throughout the experiment.

TABLE 5.

Changes in the carbohydrate, lipid, protein and DNA content of 15-day chick embryonic heart explants during the first 148 hours in vitro

Expt. No. Fraction.	No. Obs.	Hours in vitro. 0	4	28	52	100	148
µg.hexose per flask. 1H5	4	221.2 ± 48.5	131.5 ± 29.2	103.5 ± 10.96	88 ± 11.43	82.35 ± 12.2	77.3 ± 9.2
1H6	4	207.2 ± 40.7	109.2 ± 11.2	119.8 ± 11.5	92.5 ± 18.2	75.9 ± 11.4	77.5 ± 7.6
µg.lipid per flask. 1H5	4	371 ± 73.1	486 ± 138	509.3 ± 65.8	554.8 ± 83.6	497 ± 71.5	454 ± 70.7
1H6	4	269.8 ± 42.0	391 ± 56	484 ± 52.8	434 ± 14.2	393 ± 13.7	514.8 ± 19.4
µg.protein per flask 1H5	4	306.5 ± 36.7	352 ± 67.4	288 ± 32.8	318 ± 10	287 ± 28.6	274.8 ± 32.2
1H6	4	301.5 ± 41.0	257 ± 29.5	325.8 ± 21.0	313.5 ± 28.4	317.7 ± 26.0	338.5 ± 33.6
µg.DNAP per flask 1H5	4	4.29 ± 0.57	4.13 ± 0.65	2.06 ± 0.55	2.02 ± 0.19	1.57 ± 0.11	1.07 ± 0.204
1H6	4	4.01 ± 0.36	3.18 ± 0.34	3.11 ± 0.78	2.57 ± 0.09	2.22* ± 0.11	1.95 ± 0.34

* 3 Observations.

TABLE 6.

Changes in the carbohydrate, lipid, protein and DNA content of 15-day chick embryonic liver explants during the first 148 hours in vitro.

Expt.144.	No. Obs.	Hours in vitro. 0	4	28	52	100	148
µg.hexose per flask.	4	77.4 ±10.3	80.4 ±22.9	72.9 ± 8.8	76.35 ± 8.54	59.2 ± 5.8	61.3 ± 4.3
µg.lipid per flask.	4	1255 ± 166	957 ±200.1	911 ±228	894 ±228.7	969 ± 71	* 988 ±277.6
µg.protein per flask.	4	630.3 ± 71.5	576 ± 71.8	522 ± 64.3	481 ±112.4	490.5 ± 39.4	304.3 ±29.96
µg.DNAP per flask.	4	10.46 ± 0.83	7.32 ± 0.68	4.52 ± 0.40	3.54 ± 0.44	2.75 ± 0.27	1.82 ± 0.14

* 3 Observations.

Incorporation of Radioactive Phosphorus.

In these experiments the explants were grown in 2 ml. medium in sterile Warburg flasks. The respiration of the first group was measured immediately over a 4 hour period and ^{32}P added from the side-arm for the last two hours. The medium was then collected and the tissu washed and fractionated by the modified Schmidt and Thannhauser procedure. These measurements were repeated on further samples at 24, 48 and 72 hours. As before the medium was renewed at 48 hours. The results are expressed as specific activity (counts per min. per 100 µg. P).

Heart.

From Table 7 it is seen that the majority of values recorded are accompanied by a fairly large standard deviation. This has to be taken into consideration when evaluating the graphs of mean activities.

Acid Soluble Phosphorus Fraction (ASP).

Activity in this fraction fell off after 24 hours, suggesting a general lowering of metabolic activity. After renewal of the medium the rate of incorporation rose sharply to reach a value higher than the initial one. During the next 24 hours a further fall in activity occurred. This is illustrated in figure 6.

Lipid Phosphorus Fraction. (LP).

In common with the acid soluble fraction, this fraction showed a fall in activity in the first twenty-four hours and a rise after feeding

at forty-eight hours. This rise was not so pronounced as in the previous fraction. The last 24 hrs. showed a fall off in activity. (figure 7).

Ribonucleic Acid Phosphorus Fraction (RNAP).

This fraction is the most likely to be contaminated with highly radioactive materials. It was not possible to purify it further as only small amounts of material were available. The resultant variation is clearly seen in figure 8: experiments 1G2 and 1G3 give diametrically opposed results to 1G1. Where one group shows a continuous fall in activity from the initial level, the other shows a progressive rise. It is not possible to draw any general conclusions from these results.

Deoxyribonucleic Acid Phosphorus Fraction (DNAP).

In the DNAP fraction there was again some contradiction between the first experiment and the subsequent two. (figure 9). All showed a rise in activity in the first 24 hours, but 4 hours after feeding, 1G1 showed a steep increase in activity while the activity of the other two fell. It is possible, however, that their activity subsequently rose before falling to the lower level at 76 hours.

Liver.

Table 8 contains a complete record of these studies. Again the average values are associated with a fairly large standard deviation. However, each of the three experiments showed the same general pattern of phosphorus incorporation within the different fractions.

Acid Soluble Phosphorus Fraction.

The initial level of activity was maintained at least until 24 hours (figure 10). After feeding, the characteristic rise to values in excess of the initial level was observed. This was typical of both heart and liver cultures.

Lipid Phosphorus Fractions.

The most notable feature of figure 11 is the constantly observed fall in activity of about 80% during the first 24 hrs. in culture. As in all the liver fractions, there was evidence of increased activity after feeding. In this fraction the level of activity remained below the initial level throughout the experiment.

RNAP and DNAP Fractions.

Both these fractions showed decreased incorporation during the first 24 hours in vitro. The activity was stimulated to varying degrees on renewal of the medium. (Figure 12 and 13).

TABLE 7.

The incorporation of radioactive phosphorus into fractions of chick embryonic heart after various times *in vitro*. Incorporation was for two hours: each value is the average of three and is expressed as specific activity (counts per min. per 100 µg.P.)

Expt. No.	Fraction.	Hours in vitro 4	28	52	76
IG1	ASP.	144,600 ± 48,720	96,700 ±44,480	398,330 ± 55,760	190,000 ± 21,950
IG2		682,667 ± 6,899	415,330 ± 44,060	1,288,330 ± 212,600	440,330 ± 51,510
IG3		528,667 ± 49,810	294,500 ± 29,250	1,310,000 ± 437,000	* 498,000
IG1	LP	7,727 ± 2,416	4,070 ± 2,805	15,967 ± 2,544	3,630 ± 667
IG2		25,167 ± 4,537	11,937 ± 1,356	17,517 ± 2,101	15,827 ± 6,893
IG3		17,860 ± 791	13,440 ± 1,001	13,180 ± 7,472	* 5,635
IG1	RNAP	13,780 ± 8,110	22,687 ± 8,667	30,500 ± 7,895	27,300 ± 8,671
IG2		172,130 ± 28,720	86,850 ± 20,930	63,750 ± 26,460	71,860 ±11,810
IG3		57,770 ± 14,010	50,130 ± 6,914	31,810 ± 8,395	* 36,200
IG1	DNAP	* 3,490	9,218 ± 7,707	* 23,900	3,386 ± 1,578
IG2		8,320 ± 1,288	* 14,685	8,900 ± 3,560	6,463 ± 987
IG3		5,967 ± 2,152	8,220 ± 4,434	8,010 ± 6,067	* 4,990

* 2 estimations.

TABLE 8.

The incorporation of radioactive phosphorus into fractions of chick embryonic liver after various times *in vitro*. Incorporation was for two hours: each value is the average of three and is expressed as specific activity (counts per min. per 100 µg. P.)

Expt. No.	Fraction.	Hours in vitro. 4	28	52	76
IG1	ASP	115,833 ±10,750	123,200 ± 64,830	408,330 ±170,900	126,570 ± 34,620
IG2		269,000 ±15,870	253,800 ± 31,410	1,406,670 ± 143,900	472,330 ±138,500
IG3		348,233 ±44,240	160,900 *	943,000 ± 101,400	* 429,750
IG1	LP	12,990 ± 1,933	2,827 ± 126	8,087 ± 1,597	3,650 ± 627
IG2		14,680 ± 621	2,753 ± 665	8,730 ± 3,616	* 5,962
IG3		19,293 ± 4,861	4,030 ± 1,000	7,123 ± 2,226	* 3,885
IG1	RNAP	20,557 ± 3,513	9,670 ± 5,365	22,700 ± 2,100	29,067 ± 3,554
IG2		45,830 ± 7,199	17,280 ± 3,459	34,430 ± 28,370	31,125 ± 8,375
IG3		37,170 ±12,550	14,430 ± 8,209	16,540 ± 6,599	* 15,635
IG1	DNAP	7,333 ± 2,133	2,595 ± 955	21,270 ± 2,874	18,190 ± 10,890
IG2		4,737 ± 708	1,497 ± 182	3,903 ± 1,636	3,895 ± 555
IG3		8,503 ± 2,306	3,450 (one only)	5,287 ± 2,792	* 7,780

* 2 estimations.

Fig.6. The incorporation of radioactive phosphorus into the acid soluble phosphorus (ASP) fraction of chick embryonic heart tissue cultured for the stated time *in vitro*. Incorporation was allowed to proceed for the last two hours in culture. Each point is the average of three measurements.

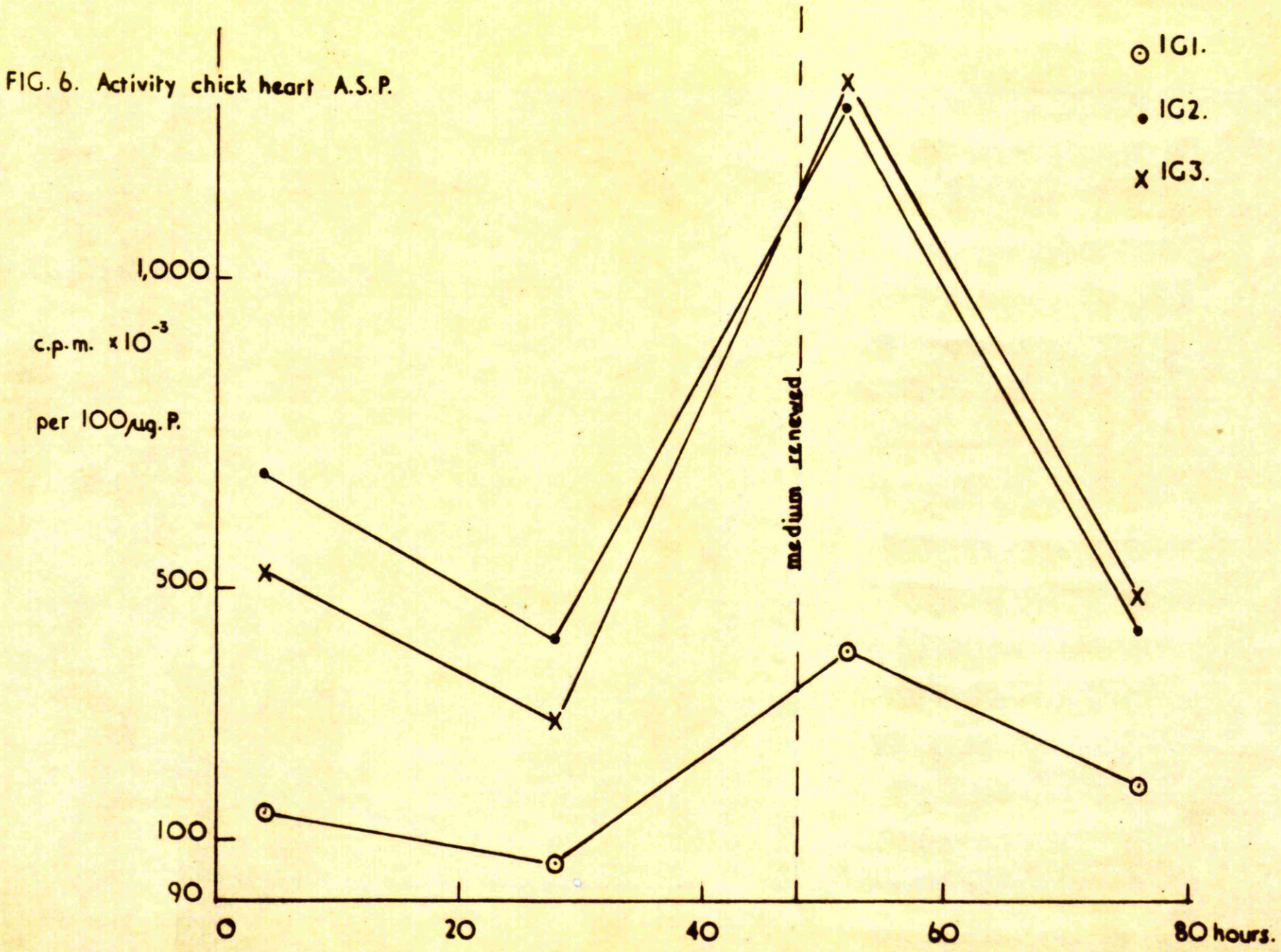


FIG. 6. Activity chick heart A.S.P.
IG1.
IG2.
IG3.
1,000
c.p.m. x 10^-3
per 100µg. P.
500
100
90
medium renewed
0
20
40
60
80 hours.

Fig.7. The incorporation of radioactive phosphorus into the lipid phosphorus (LP) fraction of chick embryonic heart tissue cultured for the stated time *in vitro*. Incorporation was allowed to proceed for the last two hours in culture. Each point is the average of three measurements.

FIG. 7. Activity chick heart L.P.

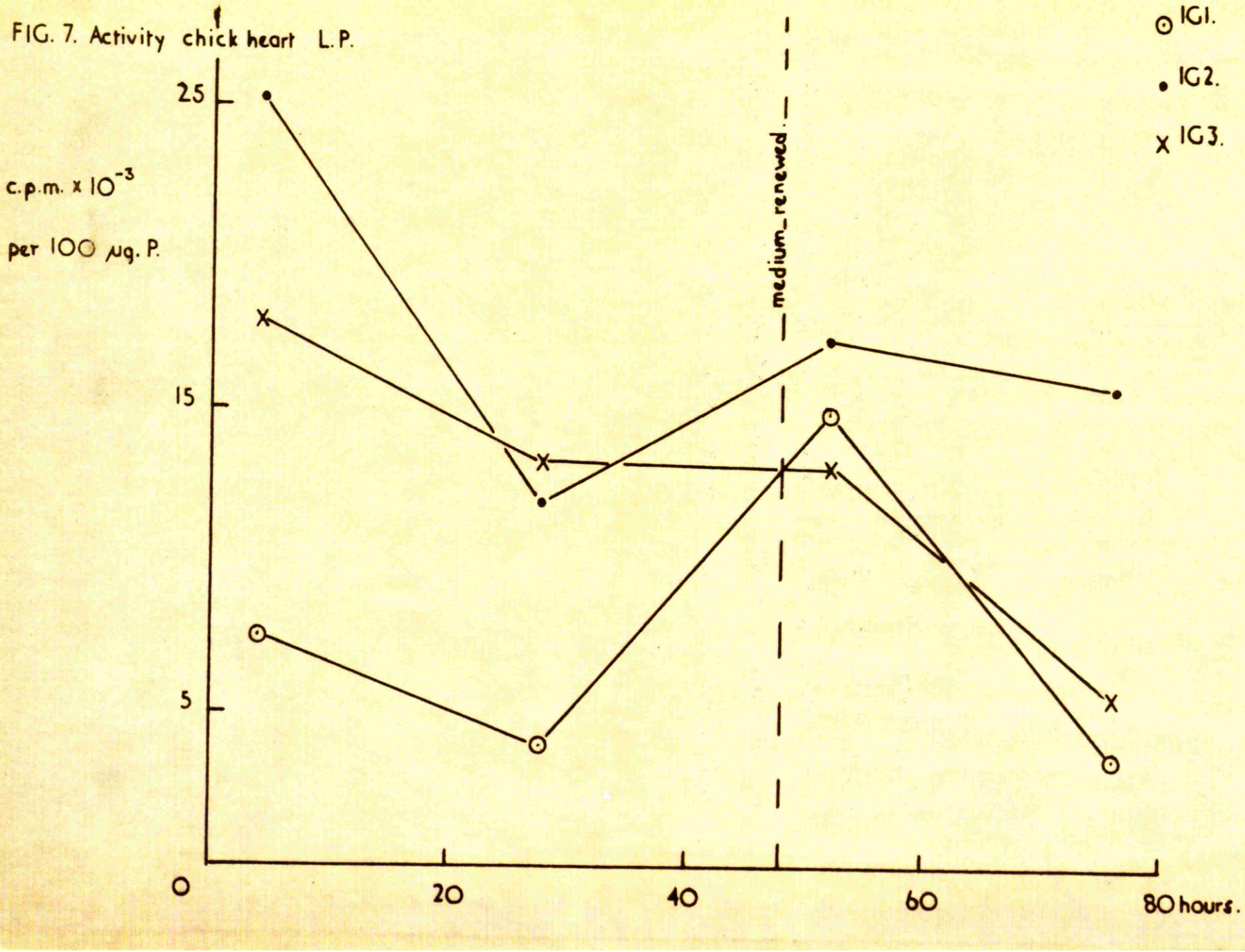


Fig.8. The incorporation of radioactive phosphorus into the ribonucleic acid phosphorus (RNAP) fraction of chick embryonic heart tissue cultured for the stated time *in vitro*. Incorporation was allowed to proceed for the last two hours in culture. Each point is the average of three measurements.

FIG. 8. Activity chick heart R.N.A.P.

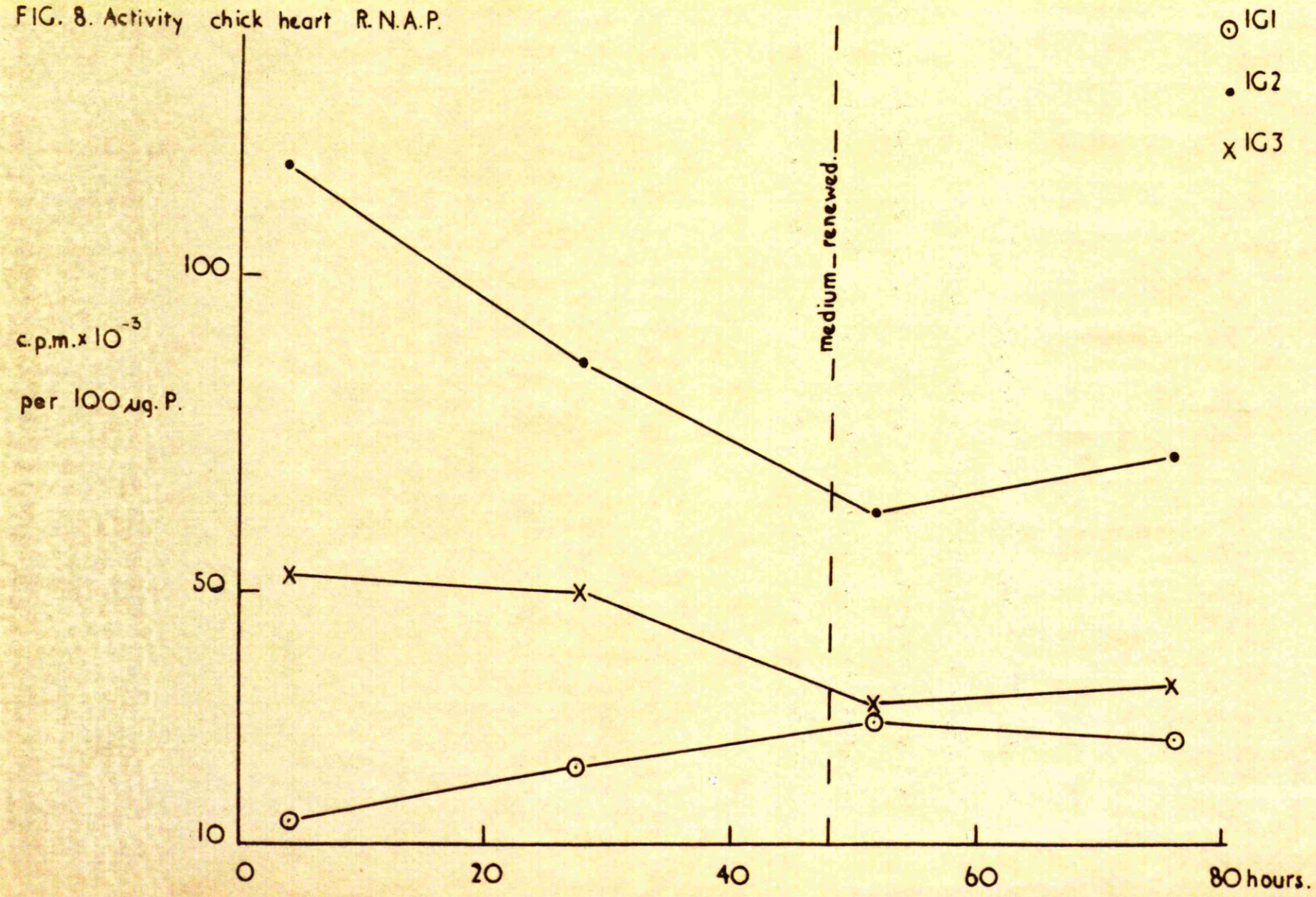


Fig.9. The incorporation of radioactive phosphorus into the deoxyribonucleic acid phosphorus (DNAP) fraction of chick embryonic heart tissue cultured for the stated time *in vitro*. Incorporation was allowed to proceed for the last two hours in culture. Each point is the average of three measurements.

FIG. 9. Activity chick heart D.N.A.P.

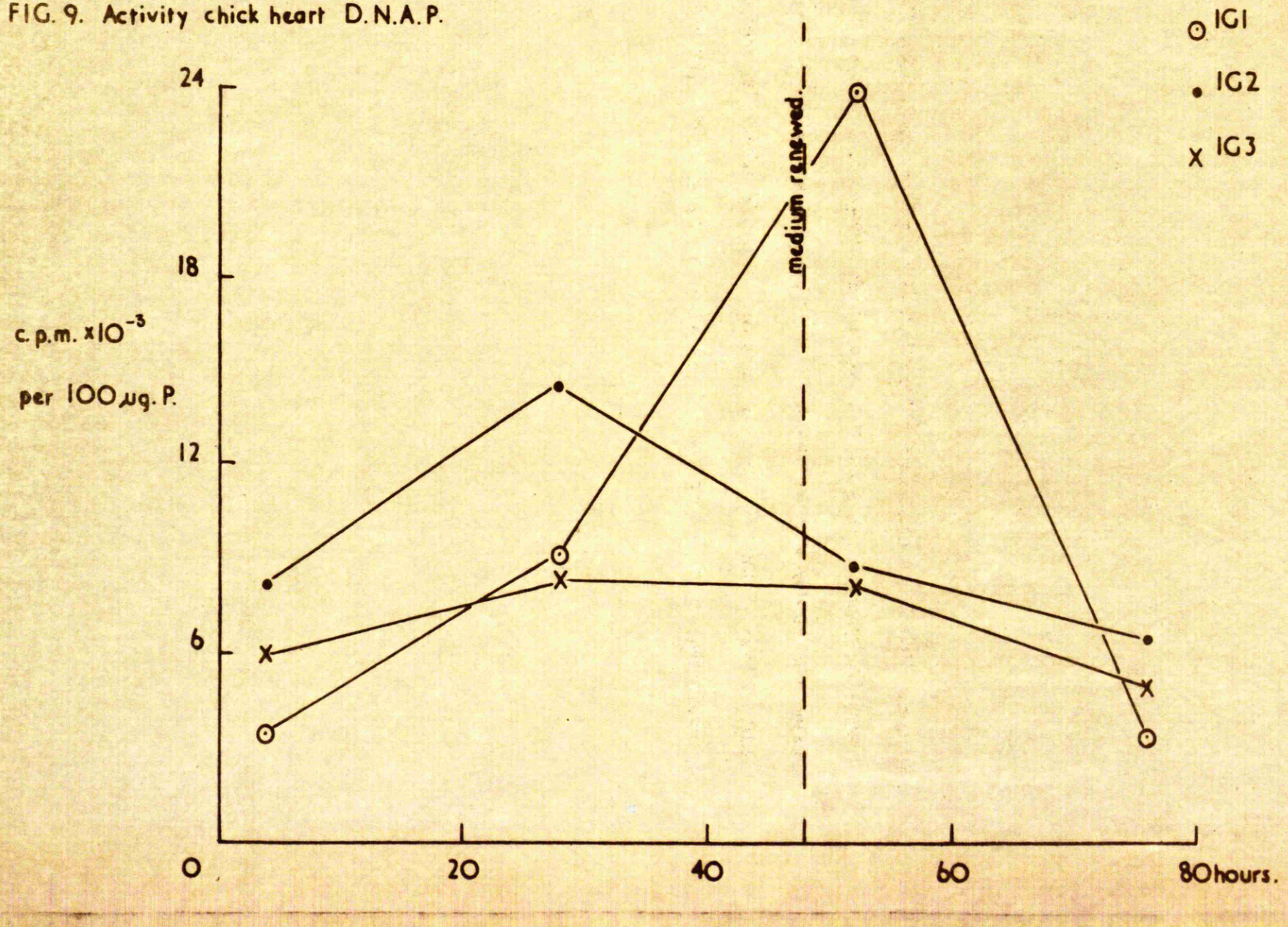


Fig.10. The incorporation of radioactive phosphorus into the acid soluble phosphorus fraction of chick embryonic liver tissue cultured for the stated times *in vitro*. Incorporation was allowed to proceed for the last two hours in culture. Each point is the average of three measurements.

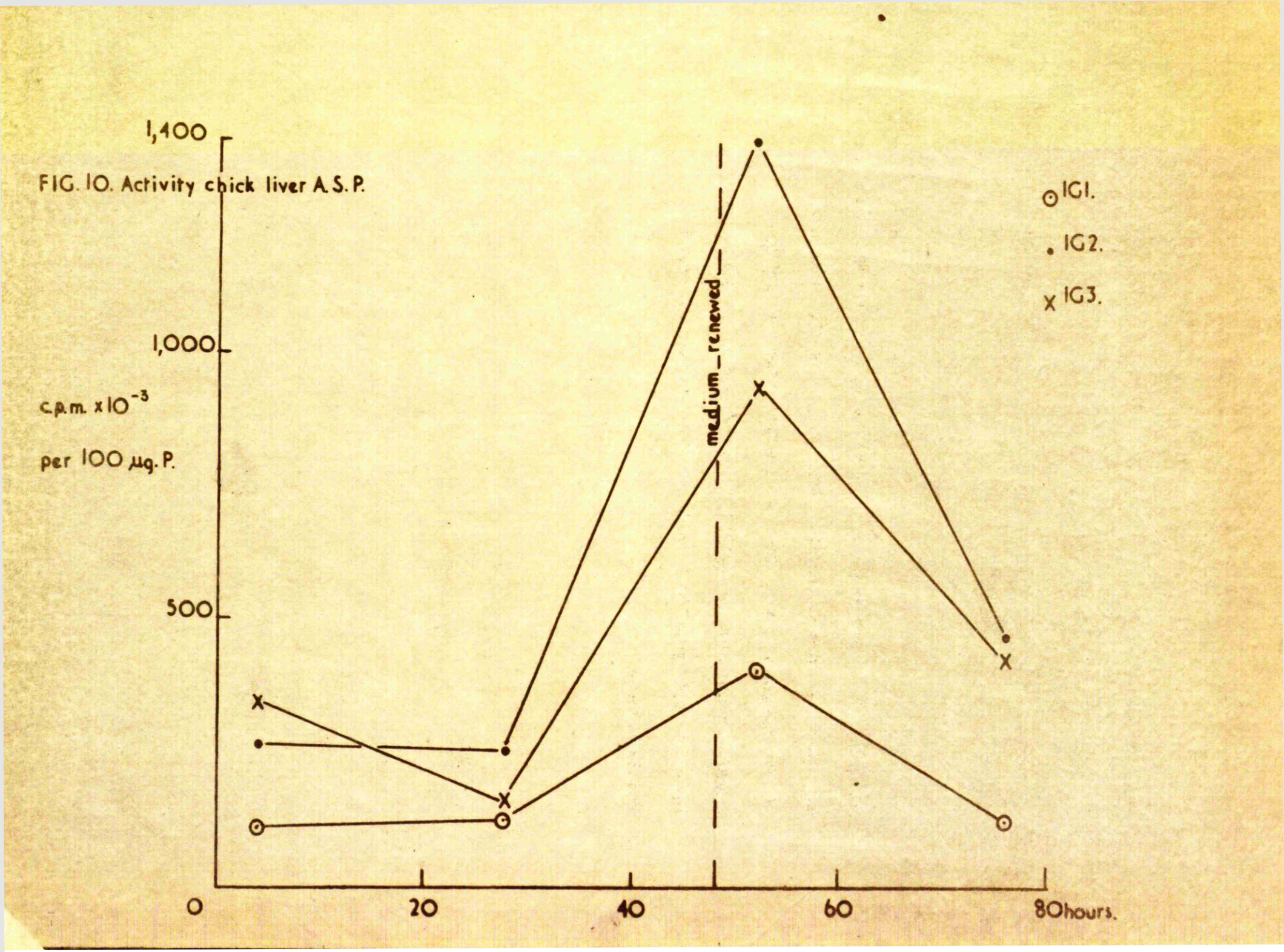


FIG. 10. Activity chick liver A.S.P.

Fig.11. The incorporation of radioactive phosphorus into the lipid phosphorus fraction of chick embryonic liver tissue cultured for the stated time *in vitro*. Incorporation was allowed to proceed for the last two hours in culture. Each point is the average of three measurements.

FIG. 11. Activity chick liver L.P.

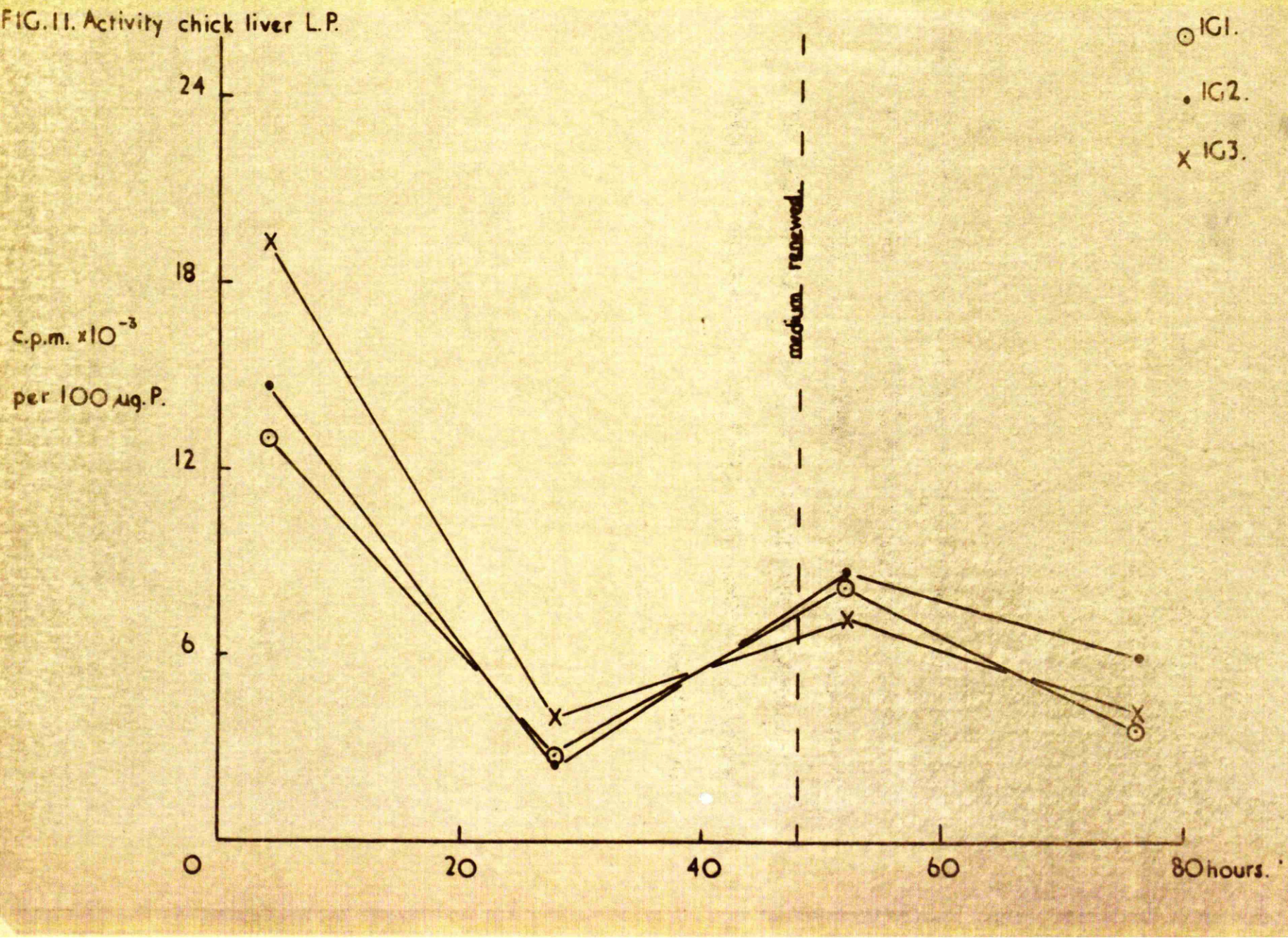


Fig.12. The incorporation of radioactive phosphorus into the ribonucleic acid phosphorus fraction of chick embryonic liver tissue cultured for the stated times *in vitro*. Incorporation was allowed to proceed for the last two hours in culture. Each point is the average of three measurements.

FIG. 12. Activity chick liver R.N.A.P.

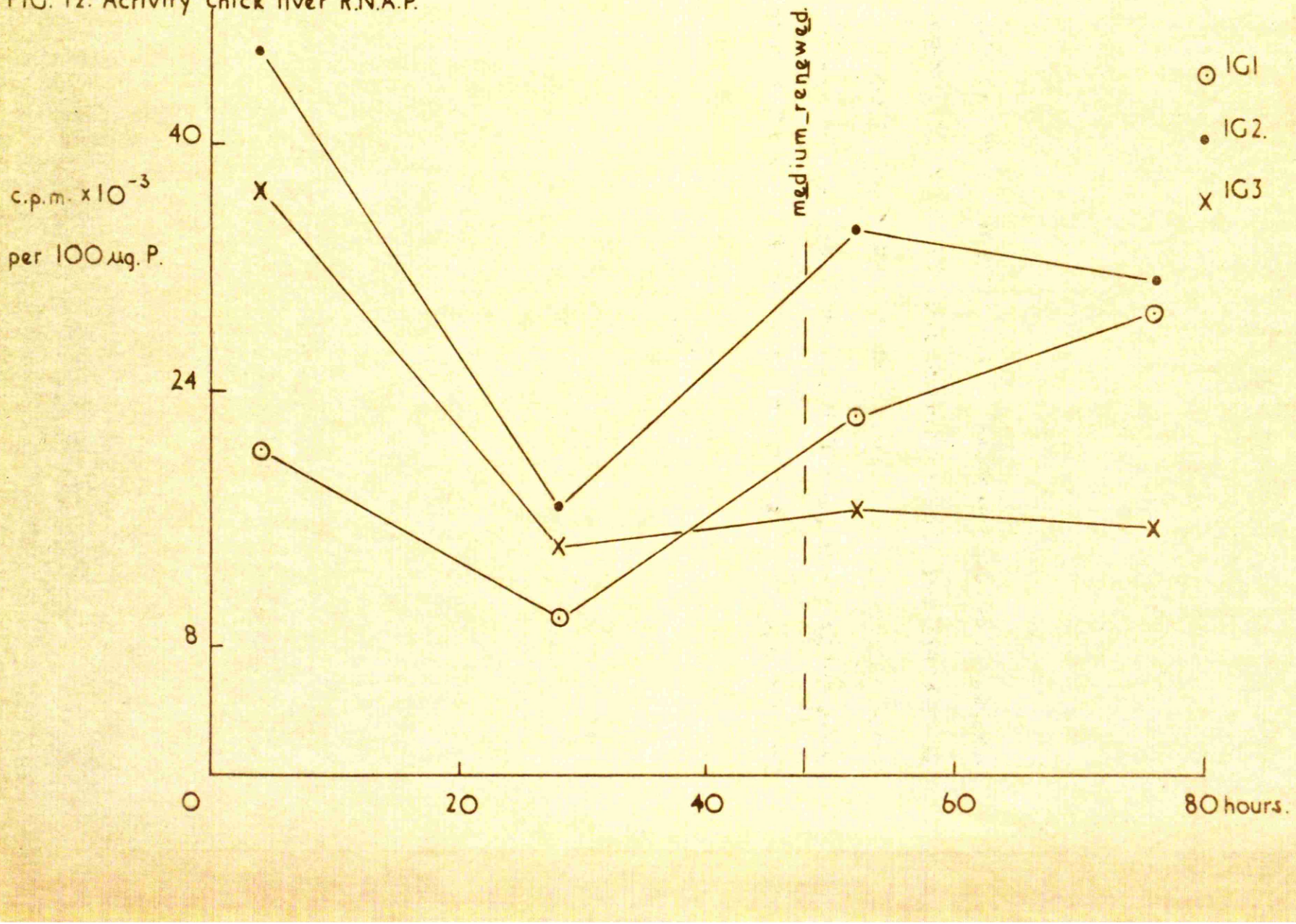
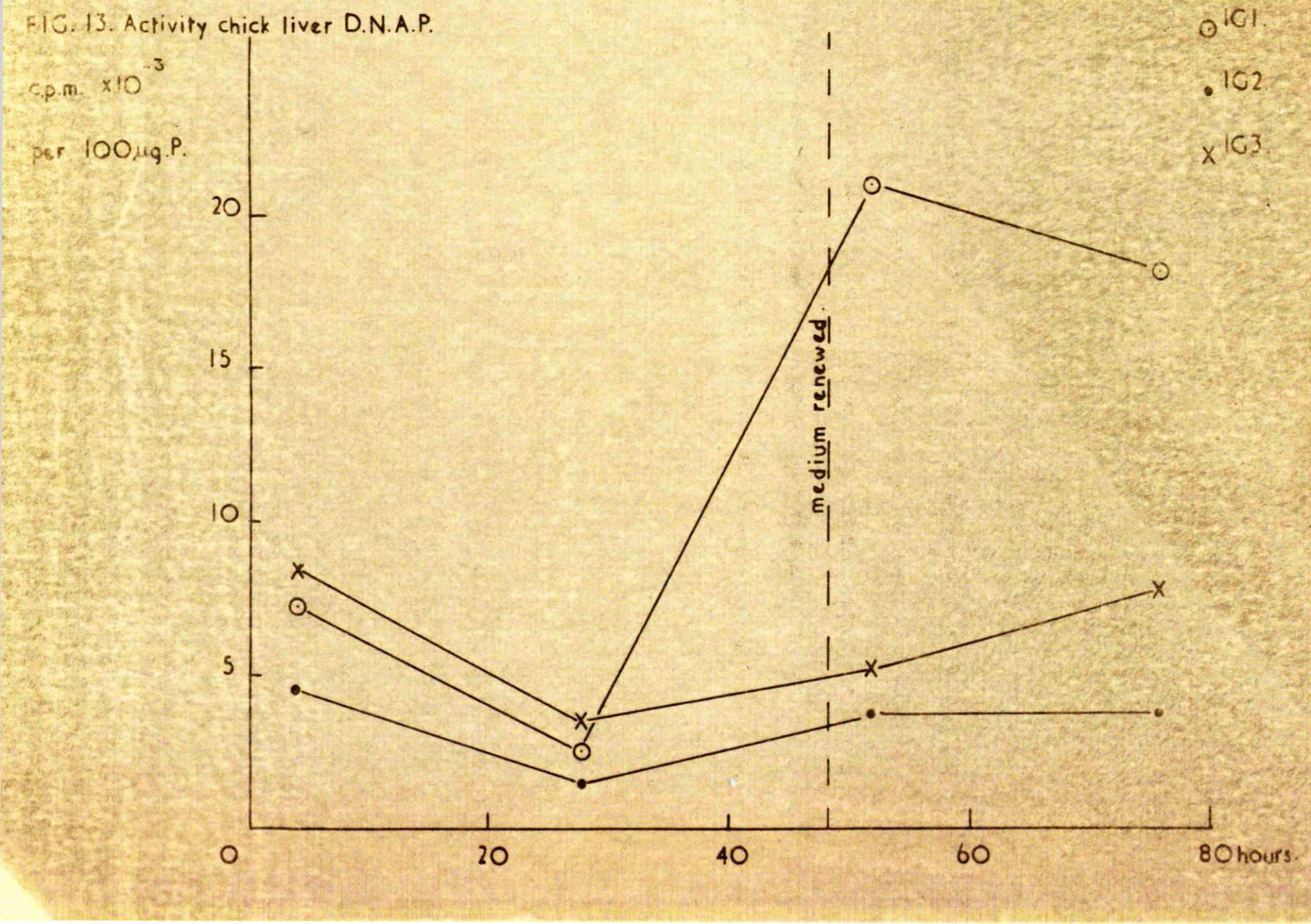


Fig.13. The incorporation of radioactive phosphorus into the deoxyribonucleic acid phosphorus fraction of chick embryonic liver tissue cultured for the stated times *in vitro*. Incorporation was allowed to proceed for the last two hours in culture. Each point is the average of three measurements.

FIG. 13. Activity chick liver D.N.A.P.



Respiration Studies.

Measurements of oxygen uptake were made in experiments 1H and 1G in which fragments of tissue were grown in sterile Warburg flasks. The experiments in group 1J employed the same technique. Alkali and a filter paper tube were added to the centre cup immediately before measuring respiration. It was necessary to grease the lip of this cup to prevent creeping of alkali, as storage for some time in the incubator produced a film of moisture on the cup. Respiration was usually measured over a four hour period at the end of which, tissue and medium were harvested. Results are expressed as ul oxygen consumed per hour, per mg. DNAP. This is represented by the symbol $Q_{O_2}^{air}$ (DNAP). The results of experiments 1H and 1J have been grouped together while 1A and 1G, radioactive experiments, have been presented separately. In all experiments the medium was renewed at 48 and 96 hours.

Heart.

In tables 9 and 10 are summarised the measurements of oxygen uptake made on explanted chick embryonic heart. The measurements are presented graphically in figure 14. It is seen that immediately after removal from the embryo, the respiration of the explants was very low and the average uptake of oxygen approximated to zero. During the next 48 hours it increased progressively, but on renewal of the medium there was a marked drop in the rate of respiration. It continued to fall gradually throughout the rest of the experiment although it never returned to the low level found originally.

Liver.

In tables 11 and 12 are summarised the measurements of oxygen uptake made on explanted chick embryonic liver. The measurements are presented graphically in figure 15. There is some disagreement between the two sets of results, especially in the first four hour period: the radioactive experiments showed a $Q_{O_2}^{air}$ of 23 x 10^3 while in the other experiments the average value was 6 x 10^3. However, in relation to the newly explanted heart tissue, the initial respiration was high. This declined during the next 48 hours and no significant effect was noted on renewal of the medium. The respiration measured at the later time intervals showed that results for the two types of tissue were quite similar.

TABLE 9.

The respiration of chick embryonic heart tissue after various times *in vitro*. Respiration was measured over a 4 hr. period and is expressed as $10^3 \times$ µl. oxygen consumed per hour, per mg. DNAP.

Experiment No.	Hours in vitro. 4	28	52	100	148
IH5	* 0.373	* 0.16	2.47	1.93	2.26
	* 0.212	5.80	1.38	5.27	5.94
	* 3.27	4.50	2.13	4.28	6.75
	3.68	1.75	3.57	1.34	4.56
IH6	* 0.38	1.07	7.00	1.78	2.22
	* 1.21	4.07	3.27	5.45	8.08
	* 0.46	3.09	3.83	5.55	8.82
	0.80	3.10	4.33	4.95	4.76
IJ1	* 5.52	* 8.5	8.86	17.18	5.79
	* 9.39	* 2.25	7.37		3.97
	* 4.94	4.06	13.80		*7.31
		5.69	12.10	9.9	2.82
IJ2	12.60	4.78	6.45		4.61
	* 0.63	9.04	13.88		3.51
	3.13	* 2.09	7.44		3.26
	* 1.44	* 0.96	4.96		
Production	27.82	13.96			7.31
Uptake	20.21	46.95	102.84	57.6	67.35
Total Uptake	* 7.61	32.99	102.84	57.6	60.04
No. observations	15	16	16	10	15
Average value	* 0.51	2.06	6.40	5.76	4.00

* apparent production of gas.

TABLE 10.

The respiration of chick embryonic heart tissue after various times in vitro. Respiration was measured over a 4 hr. period and is expressed as 10^{3}_{K} µl oxygen consumed per hour, per mg DNAP. Radioactive phosphorus was present in the medium.

Experiment No.	Hours in vitro.				
	4	28	48	52	76
IG1	1.03	4.06	13.7	8.5	2.12
	1.53	2.97	6.0	5.85	1.59
	0.36	7.76		4.63	2.78
IG2	0.87	4.36	6.5	3.5	1.45
	0.82	2.16	3.08	1.18	2.85
	0.69	3.44	0.82	0.54	3.48
IA2	2.27	8.28	16.85	10.28	9.11
	2.73	5.46	17.33	10.45	4.33
		11.13	13.92	8.50	6.38
Total uptake	10.30	49.62	78.20	53.43	34.09
No. observations	8	9	8	9	9
Average value	1.29	5.51	9.78	5.94	3.79

TABLE 11.

The respiration of chick embryonic liver tissue after various times *in vitro*. Respiration was measured over a 4 hr. period and is expressed as 10^3_K µl oxygen consumed per hour, per mg. DNAP.

Experiment No.	Hours in vitro.				
	4	28	52	100	148
IH3	5.72	3.30	1.75	2.44	2.29
	7.96	2.84	2.56	2.75	2.29
	4.96	2.33	1.94	3.86	2.58
			1.06		5.82
IH4	5.21	2.56	7.65	4.45	5.96
	7.73	3.18	4.80	6.22	14.80
	8.45	3.24	3.87	7.89	6.65
	9.51	4.77	3.40	10.45	9.01
IJ3	6.30	8.20	19.10	14.21	19.86
	4.20	6.78	11.38	16.0	15.40
	8.31	6.22	16.90	9.07	17.20
	4.40	8.23	20.10	4.32	20.15
IJ4	3.55	5.37	10.24	6.73	3.95
	6.84	9.95	9.45	7.17	7.45
	5.77	5.66	4.00	4.22	18.16
	2.28	5.96	4.19	2.94	2.24
Total uptake	91.20	78.59	122.39	102.72	153.91
No. observations	15	15	16	15	16
Average value.	6.08	5.24	7.65	6.85	9.62

TABLE 12.

The respiration of chick embryonic liver tissue after various times *in vitro*. Respiration was measured over a 4 hr. period and is expressed as 10^{3}_{x} µl oxygen consumed per hour, per mg. DNAP. Radioactive phosphorus was present in the medium.

Experiment No.	Hours in vitro.				
	4	28	48	52	76
IG1	25.60	8.24	9.21	7.90	3.43
	33.50	8.28	1.57	7.21	7.01
	20.30	17.80	11.88		4.96
IG2	6.72	1.68	1.82	1.43	1.13
	5.81	2.67	1.15	1.05	1.93
	7.85	1.19	1.04	2.61	0.64
IA2	38.30	6.87	13.68	15.00	10.81
	39.50	5.06	9.76	6.94	9.71
	29.20	5.60	7.38	11.57	9.78
Total uptake	206.78	57.39	57.49	55.81	49.40
No. observations	9	9	9	8	9
Average value.	22.98	6.38	6.39	6.98	5.49

Fig.14. The respiration of chick embryonic heart tissue at the stated times *in vitro*. Measurements were made over the last four hours in culture, and are expressed as µl. oxygen consumed per hour, 'per mg. DNAP. Q_{O2}^{air} (DNAP).' Each point is the average of at least eight measurements.

FIG. 14. Respiration of chick heart.

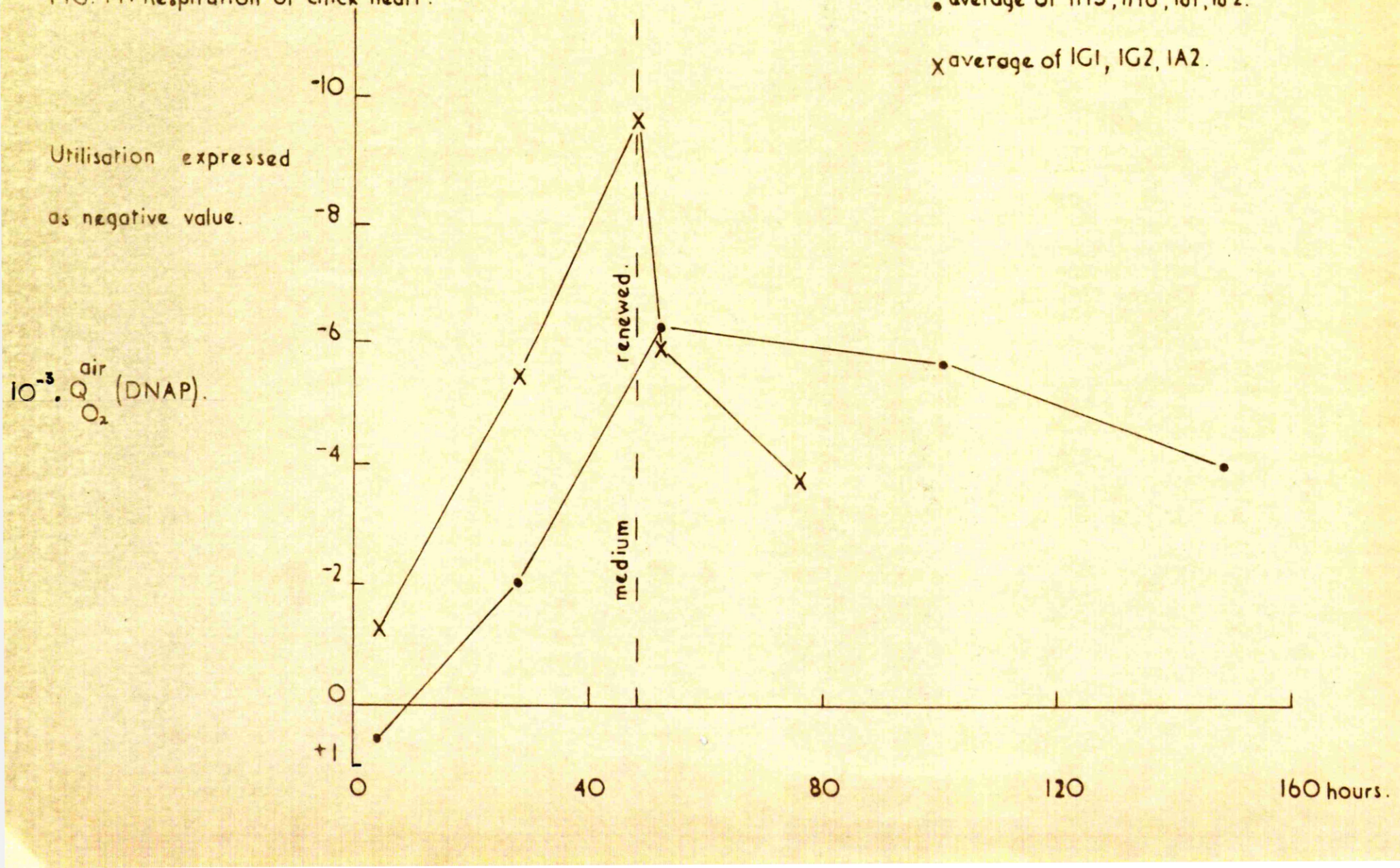
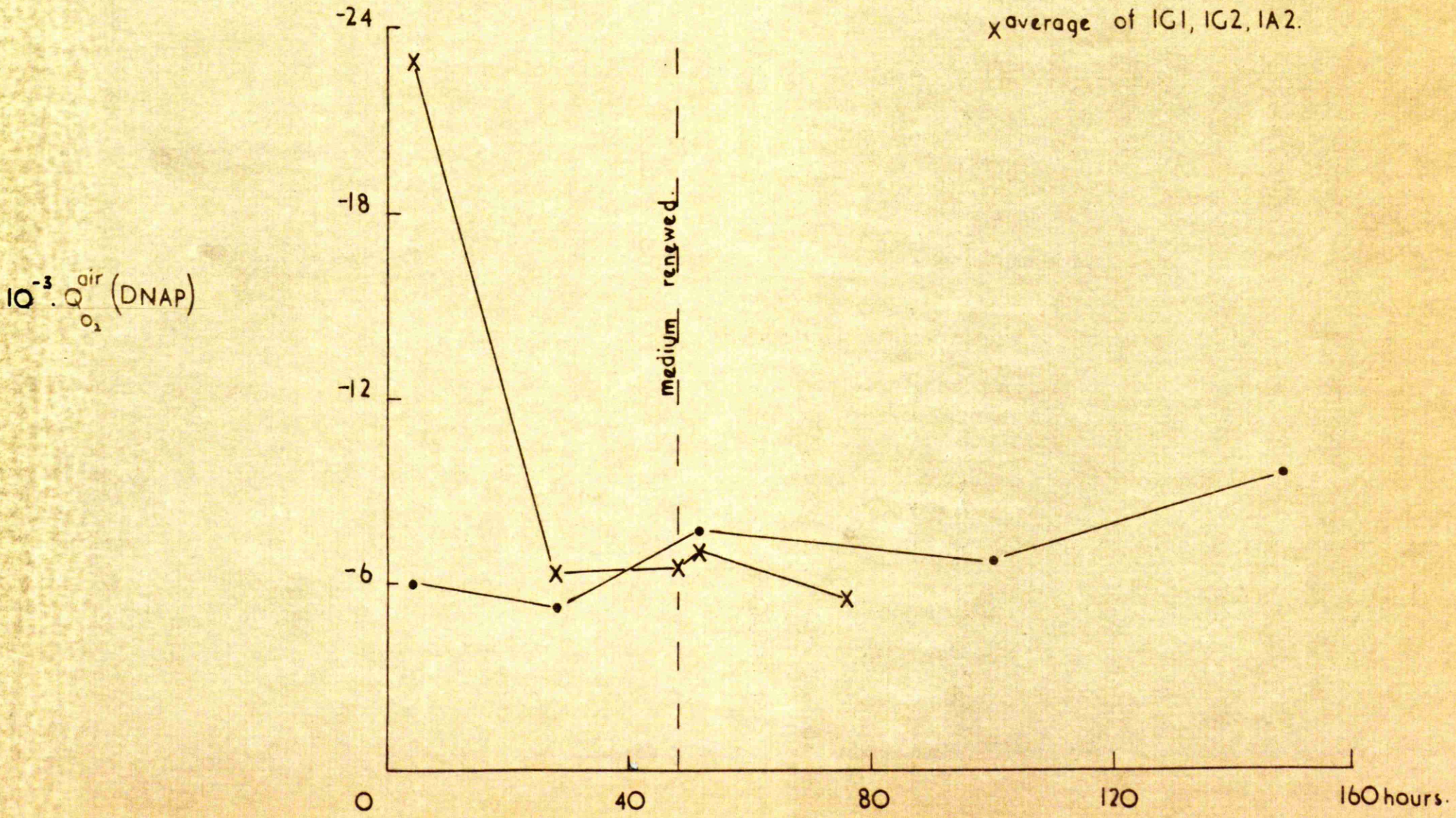


FIG.15. The respiration of chick embryonic liver tissue at the stated times *in vitro*. Measurements were made over the last four hours in culture and are expressed as µl. oxygen consumed per hour, 'per mg. DNAP. Q_{O2}^{air} (DNAP).' Each point is the average of at least eight measurements.

FIG. 15. Respiration of chick liver.

•average of 1H3, 1H4, 1J3, 1J4.

Xaverage of 1G1, 1G2, 1A2.



Studies with Insulin.

From the foregoing results it becomes clear that these tissues undergo a change in the pattern of metabolism on transfer from an *in vivo* to an *in vitro* system. One of the important differences between these two environments is the complete absence of hormones in the latter, and it was thought that this might in part explain the changes that occur. For this reason it was decided to study the effects produced when insulin was added to tissue culture media.

The preliminary studies with insulin were carried out with Earle's strain L-cells and the uptake of glucose was used as the criterion of an effect. Both Eagle's medium (p.110), supplemented with 10% HS, and embryo extract medium were used in the experiment. 50 ml. conical flasks were inoculated with 1.6×10^6 cells and medium was added to give a final volume of 3ml. in each flask. With both types of medium three test flasks, containing 2 units insulin/ml., and three control flasks were set up. Samples of the initial medium were retained and further samples withdrawn at 5 hrs. and 24 hrs. when the experiment was terminated. The medium was analysed for glucose and from the results the uptake was calculated. Table 13 shows that a significant effect of insulin on glucose uptake was not observed at all times.

Early work of Gey and Thalhimer (1924) showed an effect of insulin on cultures of chick fibroblasts, which was confirmed and extended some years later by Leslie and Paul (1954). In view of this, and taking into account the disappointing results with strain L-cells,

TABLE 13.

The uptake of glucose by cultures of strain L-cells grown in insulin-containing or control medium. The results are expressed as µg. glucose utilized per ml. of medium.

Description of medium.	No. Obs.	Hours after start of exp.	
		5	24
CONTROL E.E. medium	3	65 ± 2	229 ± 11.1
INSULIN E.E. medium	3	50 ± 9.2	261 ± 10.1
CONTROL Eagle's medium	3 (2 at 5 hr.)	12	146 ± 24.9
INSULIN Eagle's medium	3	65 ± 3.6	162 ± 9.5

it was decided to use chick embryonic material for a more complete investigation into the nature of the insulin effect. It proved difficult to produce enough replicate cultures of chopped liver and heart and the material finally chosen was trypsinized chick embryo carcass. The preparation of this is described on page 26.

In the first experiment of this type (3HI) seven test and seven control cultures were set up. Medium was renewed and samples retained for analysis every day up to five days when the cells were also harvested and stored for analysis. Eagle's medium, supplemented with 20% HS, was used in this experiment. A second experiment using embryo extract medium (3H2) was also performed in the same manner but it was terminated after three days. The results of the medium analysis from these experiments are presented in Tables 14 and 15. They show a possibly increased utilisation of glucose by the cultures grown with insulin, resulting in an increased production of lactic acid. This was accompanied by a fall in pyruvic acid production. The results of the tissue analysis are summarised in Tables 16 and 17. It appears that the presence of insulin had no effect on the nett synthesis of protein or lipid over a period of days: there is no significant difference between the values obtained for control and insulin containing cultures. On the other hand there is evidence that insulin had a depressing effect on the synthesis of RNA and DNA. These results are in contrast to the findings of Leslie and Davidson (1951) who, working with chick heart explants, demonstrated an increased synthesis of lipid phosphorus, RNA phosphorus and protein nitrogen in explants grown with insulin.

TABLE 14. (Expt. 3H1).

The utilisation of glucose and the production of lactic and pyruvic acid by cultures of embryonic chick carcass measured at 24 hour intervals, in insulin-containing and control medium (Eagle). The medium was renewed daily.

Analysis	No. Obs.	Conditions	Hours after start of experiment. 22	47	72	96	119
Glucose (µg) utilisation per ml. medium	7	CONTROL	278 ±302	170 ± 63	286 ± 63	361 ± 98	186 ±106
	7	INSULIN	319 ±216	278 ±135	454 ± 40	279 ±105	272 ± 61
Lactic Acid (µg.) production per ml. medium	7	CONTROL	86.3 ± 3.4	156 ± 23.9	277 ± 22.8	346 ± 16.9	353 ± 5.9
	7	INSULIN	115.3 ± 7.8	236 ± 19.1	326 ± 12.4	358 ± 15.5	363 ± 19.1
Pyruvic Acid (µg) production per ml. medium.	7	CONTROL	20.3 ± 0.99	21.6 ± 1.30	17.9 ± 1.77	11.4 ± 3.25	3.13 ± 1.01
	7	INSULIN	21.3 ± 0.98	15.9 ± 0.93	6.6 ± 2.85	4.2 ± 2.11	3.6 ± 1.6

TABLE 15. (Expt. 3H2).

The utilisation of glucose and the production of lactic and pyruvic acid by cultures of embryonic chick carcass, measured at 24 hour intervals. The cultures were growing in insulin-containing and control embryo extract medium, which was renewed daily.

Analysis	No. Obs.	Conditions.	Hours after start of experiment		
			23	47	71
Glucose (µg.).	6	CONTROL	231 ± 24.58	164.5 ± 22.9	147.3 ± 27.8
Utilisation per ml. medium	6	INSULIN	256 ± 68.1	203.7 ± 23.3	162 ± 25.35
Lactic acid (µg.)	6	CONTROL	125.5 ± 3.92	177 ± 18.54	178.5 ± 21.02
production per ml. medium	6	INSULIN	179 ± 7.46	233.2 ± 24.26	232.8 ± 23.92
Pyruvic acid	6	CONTROL	24.9 ± 1.99	22.85 ± 0.45	19.9 ± 2.52
(µg.)production per ml. medium	6	INSULIN	21.35 ± 0.43	16.25 ± 2.20	16.15 ± 3.23

TABLE 16. (Expt. 3H1).

Changes in the composition of embryonic chick carcass cultures during 5 days growth in Eagle's medium with and without insulin.

No. Obs.	CONDITIONS	Total Protein Nitrogen (µg.)	Total lipid (µg.)	Total RNAP (µg.)	Total DNAP (µg.)
7	CONTROL	75.1 ± 3.2	585 ± 92.5	6.11 ±0.45	0.82 ±0.05
7	INSULIN	70.0 ± 3.1	561 ± 39.7	4.35 ±0.87	0.56 ±0.13
3	Initial content	56.9	414	3.57	1.02

TABLE 17. (Expt. 3H2).

Changes in the composition of embryonic chick carcass cultures during 3 days growth in embryo-extract medium, with and without insulin.

No. Obs.	Conditions.	Total protein nitrogen (µg.)	Total lipid (µg.)	Total RNAP (µg.)	Total DNAP (µg.)
6	CONTROL	82.2 ± 1.54	573 ±114	10.42 ± 1.38	2.17 ±0.30
6	INSULIN	86.3 ± 1.27	590 ± 26.6	8.04 ± 0.98	1.57 ±0.17
3	Initial content.	52.95	198	3.59	0.70

In the preliminary experiments with L-cells (Table 13) attention was focussed on a possible effect of insulin on glucose uptake from the medium and because this was not consistently observed the material was considered unsuitable. However this inconsistency was also apparent in the trypsinized carcass cultures and there was therefore no advantage to be gained by using this material for the more elaborate experiments which were planned. It was decided first of all to repeat the more complete analyses on a series of cultures of L-cells (Exp.3I) and also on a strain of human buffy-coat cells (Exp.3J). For this purpose approximately 2.5 x 10^6 cells were dispensed to a series of 50 ml. conical flasks to which was then added either insulin-containing or control medium to 3 ml. The medium was renewed daily and retained for analysis. On the final day of the experiment the cells were also harvested and stored for analysis. An exception to this general procedure was experiment 3I2 in which L-cells were grown in roller tubes and which were rotated throughout the experiment.

The results of the analysis of the medium from these experiments are shown in Tables 18, 19 and 20. They show a decreased production of pyruvic acid coincident with an increased lactic acid production and glucose utilisation when strain L-cells were grown in the presence of insulin. These changes were not statistically significant at all time intervals, but they confirm earlier findings with cultures of chick embryonic carcass cells. The analysis of medium obtained from cultures of human buffy-coat cells (Table 20) failed to demonstrate, in a convincing manner, an effect of insulin on their carbohydrate

TABLE 18. (expt. 311).

The utilisation of glucose and the production of lactic and pyruvic acid by cultures of strain-L cells grown in embryo extract medium, which was renewed daily, with and without insulin.

Analysis	No. Obs.	Conditions.	Hours after start of experiment			
			24	48	72	96
Glucose (µg.) utilisation	6	CONTROL	164.7 ± 33.04	449.3 ± 39.0	610.7 ± 44.01	592 ± 31.5
per ml. medium	6	INSULIN	213.3 ± 30.2	479.3 ± 32.64	640.7 ± 40.67	720.7 ± 31.7
Lactic Acid (µg.) production	6	CONTROL	264 ± 16.32	556 ± 27	628 ± 16	645 ± 10.3
PER ML. MEDIUM	6	INSULIN	302.8 ± 33.4	584 ± 6.6	664.5 ± 6.05	683.5 ± 8.21
Pyruvic Acid (µg.) production	6	CONTROL	23.4 ± 0.67	12.2 ± 0.43	8.02 ± 0.56	7.36 ± 1.28
per ml. medium	6	INSULIN	20.9 ± 1.40	7.45 ± 0.34	3.36 ± 0.69	3.57 ± 0.69

TABLE 19. (Expt. 312).

The utilisation of glucose and the production of lactic acid by cultures of strain L-cells in embryo-extract medium, which was renewed daily, with and without insulin. The cells were grown in roller tubes, rotated on the roller - drum.

Analysis	No. Obs.	Conditions.	Hours after start of the experiment. 19	42	63	87	109
Glucose (µg.) utilisation	7	CONTROL	256 ± 16.9	392 ± 48.4	1091 ± 32.7	1325 ± 61.1	1350 ± 66.4
per ml. medium	7	INSULIN	275 ± 33.6	308 ± 133	1252 ± 72.5	1446 ± 61.4	1333 ± 47.9
Lactic Acid (µg.) production	7	CONTROL	168.2 ± 26.1	217.4 ± 21.7	297.7 ± 21.1	340.6 ± 49.6	424.3 ± 12.7
per ml. medium	7	INSULIN	175.8 ± 17.3	253.4 ± 52.9	382.7 ± 31.73	417.2 ± 8.3	440.3 ± 20.9

The pyruvic acid analyses were technically unsatisfactory.

TABLE 20. (Expt. 3J1).

The utilisation of glucose and the production of lactic and pyruvic acid by cultures of a strain of human buffy-coat cells grown in Eagle's medium which was renewed daily, with and without insulin.

Analysis	No. Obs.	Conditions	Hours after start of experiment 18	47	68
Glucose (µg) utilisation per ml. medium	6	CONTROL	181.5 ± 34.3	282.7 ± 27.4	321.2 ± 32.3
	6	INSULIN	242.5 ± 23.4	379.2 ±135.0	366.5 ± 34.8
Lactic Acid (µg) production per ml. medium	6	CONTROL	261 ± 19.4	287 ± 10.7	299 ± 36.0
	6	INSULIN	277 ± 5.3	303 ± 11.7	349 ± 21.0
Pyruvic Acid (µg) production per ml. medium	6	CONTROL	54.1 ± 2.1	29.5 ± 1.2	23.9 ± 2.13
	6	INSULIN	53.6 ± 3.0	27.2 ± 1.97	19.2 ± 0.99

metabolism. Only at the earliest time interval was there evidence of an increased uptake of glucose by the cells in the presence of insulin. A depression of pyruvic acid production was noted only at 68 hr. and this was to a marginal degree.

Analysis of strain L-cells and buffy-coat cells showed that there was no differnec between cultures grown in the presence of insulin and control cultures. The cells were analysed for protein nitrogen, lipid, DNAP and RNAP: in none of these fractions was any real difference detected between test and control cultures.

TABLE 21. (Expt. 311).

Changes in the nucleic acid composition of cultures of strain L-cells during 4 days growth in embryo extract medium, with and without insulin.

No. Obs.	Conditions.	Total RNAP (µg.)	Total DNAP (µg.)
6	CONTROL	32.31 ± 6.07	6.64 ±0.56
6	INSULIN	35.44 ± 2.28	6.26 ±0.49
3	Initial content	10.7	2.42

TABLE 22. (expt. 312).

Changes in the composition of cultures of strain L-cells during 5 days growth in embryo extract medium, with and without insulin. The cells were grown in roller tubes rotated on the roller drum.

No. Obs.	Conditions	Total protein nitrogen (µg.)	Total lipid (µg.)	Total RNAP (µg.)	Total DNAP (µg.)
7	CONTROL	81.9 ± 2.48	289 ± 66.7	4.08 ±2.95	2.12 ±0.63
7	INSULIN	77.8 ± 3.76	221 ± 13	3.38 ±1.37	2.03 ±0.44
3	Initial content	35.3	160	0.59	0.50

TABLE 23. (Expt. 3J1).

Changes in the composition of cultures of a strain of human buffy-coat cells during 3 days growth in Eagle's medium, with and without insulin.

No. Obs.	Conditions.	Total protein nitrogen (µg.)	Total lipid (µg.)	Total RNAP (µg.)	Total DNAP (µg.)
6	CONTROL	99 ± 4.2	928 ±132	11.08 ± 1.79	5.94 ±0.54
6	INSULIN	100.7 ± 7.3	778 ± 51	12.27 ± 0.76	6.48 ±0.36
3	Initial content	39.7	287	8.19	2.52

The earlier work on the adaptation of primary explanted tissue to *in vitro* conditions, included studies on the incorporation of radioactive phosphorus into various phosphorus fractions. In the majority of fractions it was noted that newly explanted material showed a fall in activity in the first twenty-four hours. It was thought likely that the presence of insulin might have some effect on this aspect of metabolism. This idea was strengthened by previous reports in the literature. Kaplan and Greenberg (1944,a) injected insulin and radioactive sodium hydrogen phosphate into normal rats and studied the effect on the acid soluble phosphates of the liver. They found that insulin administration caused a marked increase in the ^{32}P content of the total acid soluble phosphate, the inorganic phosphate and the adenosine triphosphate (ATP) of the liver. Further evidence (Kaplan and Greenberg, 1944,b) led to the conclusion that insulin action is concerned with the formation of energy rich phosphate bonds. About the same time Sacks (1945) reported similar work on striated muscle of cats. Using tracer doses of ^{32}P he found that insulin increased the turnover rate of phosphocreatine and the two labile phosphate groups of ATP. This conclusion is in accord with the findings of Haugaard, Marsh and Stadie (1951) who, working with rat diaphragms, deduced a greatly increased turnover of high energy phosphate to have occurred in the presence of insulin. They did not employ radioactive phosphorus in their studies.

It was decided, for these reasons, to extend the experiments with insulin to include a study of its effect on the phosphorus metabolism of cell cultures. The initial experiments were made with trypsinized chick

embryonic material and the subsequent studies were with cultures of established cell strains. For this purpose an aliquot of cells was pipetted accurately into each of a number of flasks. To these was added some growth medium and the cells were allowed to settle on the glass overnight. Before beginning the experiment, the cultures were examined microscopically and any unhealthy flasks were rejected. The growth medium was replaced completely with experimental medium which contained either insulin in acid solution or an equivalent amount of hydrochloric acid. The cultures were then stored in the hot room for 4 - 5 hours prior to the addition of radioactive phosphorus. Working in the hot room, 0.1 ml. of a solution of radioactive phosphorus, calculated to give 4 μ. curies per ml. of medium, was added to each flask at one minute intervals. After exactly one hour the medium was quickly removed from each flask in turn. The cells were washed twice with phosphorus free saline (appendix) and were either fractionated immediately or stored in the deep freeze. The method of fractionation is described on p.37 et seq.

The results in the following section are expressed as the mean of a group of observations plus or minus the standard deviation. The probability (p) of the difference between the control and the test cultures being due to chance alone is quoted except where, by inspection the value is seen to be very high. A probability greater than 0.05 is regarded as an indication that no real difference exists between the control and test cultures. The term 'significant' difference is therefore applied to findings which would occur with a probability of 0.05 or less under control conditions.

Using the criterion established above, it can be concluded from the results quoted in Tables 24 and 25 that insulin caused an increased rate of incorporation of ^{32}P into the lipid phosphorus fraction of chick carcass cultures. This was observed in all three types of medium. Increased activity in the presence of insulin was also observed in the acid soluble phosphorus fraction: this increase could not be proved significant when cells were grown in BSS. In none of the experiments was there evidence of increased incorporation into the RNA or DNA fractions.

The experiments were repeated on strain L-cells and, where indicated, the acid soluble fraction was subjected to chromatography to obtain separation of the acid soluble nucleotides. The first experiments in this series contained only two flasks in each group, as it was hoped the material would provide reproducible results. Experiment 302 was performed in Kolle flasks each containing 10 $x10^6$ cells in 15 ml. Eagle's medium: experiments 303 and 304 were performed in large conical flasks containing 50 x 10^6 cells in 40 ml. medium. Table 26 gives an indication that insulin caused an increased incorporation of ^{32}P into the acid soluble and lipid phosphorus fractions of strain L-cells. Table 27 indicates an increased activity in the IMP, ATP and ADP under these conditions. However, the agreement between duplicate cultures was not exact enough to allow definite conclusions to be reached. It was decided to repeat the experiment on larger samples and to subject the findings to statistical analysis.

TABLE 24. (Expt. 3E1)

The incorporation of radioactive phosphorus into fractions of trypsinized chick carcass cultures during one hour. The cultures were maintained for the duration of the experiment, in embryo-extract medium, Eagle's medium or balanced salt solution (Hank's) with and without insulin. The results are expressed as specific activity (counts per minute per 100 µg. P.)

Medium	No. Obs.	Conditions	ASP Activity	P	LP Activity	P	RNAP Activity	P	DNAP Activity	P
Embryo Extract	3	CONTROL	2,125,000 ± 87,890	0.01	70,300 ± 6,052	0.01	124,300 ± 24,630		32,200 ± 6,480	0.
Medium	3	INSULIN	2,705,000 ± 86,750		91,500 ± 1,950		133,630 ± 18,970		43,200 ± 7,690	
Eagle's medium	3	CONTROL	2,740,000 ± 346,000	0.1	63,030 ± 8,210	0.05	133,700 ± 5,017	0.1	50,370 ± 8,340	02
	3	INSULIN	3,687,000 ± 632,700		111,670 ± 31,500		180,670 ± 37,980		129,400 ± 78,470	
B S S (Hank's)	3	CONTROL	2,915,000 ± 277,800	0.2	114,300 ± 35,440	0.05	197,730 ± 22,690		91,570 ± 11,760	0.2
	3	INSULIN	3,503,000 ± 548,700		200,800 ± 28,450		199,700 ± 60,510		± 136,900 ± 21,940	

TABLE 25. (Expt. 3E2 and 3E3).

The incorporation of radioactive phosphorus into fractions of trypsinized chick carcass cultures during one hour. The cultures were grown in Eagle's medium or embryo-extract medium with and without insulin. The results are expressed as specific activity (counts per minute per 100 µg. P.)

Medium	No. Obs.	Conditions.	ASP Activity	ASP P.	LP Activity	LP P.	RNAP Activity	RNAP P.	DNAP Activity	DNAP P.
3E2. Eagle's Medium	6	CONTROL	* 660,680 ± 84,520	0.02	141,966 ± 22,100	0.001				
	6	INSULIN	819,200 ± 92,220		* 208,640 ± 22,230					
3E3. Embryo extract medium	4	CONTROL	690,500 ±182,700				44,640 ±12,590		±57,875 -38,580	
	4	INSULIN	582,500 ± 44,190				24,180 ± 4,350		52,975 ± 9,602	

* 5 Observations.

TABLE 26. (Expt. 3G2, 3G3, 3G4).

The incorporation of radioactive phosphorus into fractions of cultures of strain L-cells during one hour. The cultures were grown in Eagle's medium or embryo-extract with and without insulin. The results are expressed as specific activity (counts per min. per 100 μg. P.)

Conditions	Flask No.	ASP	LP	RNAP	DNAP
Expt. 3G2 Eagle's medium					
CONTROL	1	529,500	285,000	99,430	20,120
	2	841,000	224,000	97,100	24,630
INSULIN	3	925,000	327,300	133,430	21,900
	4	995,000	320,000	82,570	18,680
Expt. 3G3 Eagle's medium					
CONTROL	1	239,530	50,070	15,210	3,560
	2	267,500	54,630	22,420	2,930
INSULIN	3	330,670	57,600	23,280	3,460
	4	287,670	41,200	38,390	4,650
Expt. 3G4 Embryo-extract medium.					
CONTROL	1	685,830	14,780	33,300	7,430
	2	649,830	18,430	35,990	7,660
INSULIN	3	808,330	20,050	41,630	9,410
	4	671,830	14,110	44,670	7,175

TABLE 27. (Expt. 334)

The incorporation of radioactive phosphorus into the acid soluble nucleotides of cultures of strain L-cells. The cells were grown in embryo-extract medium with and without insulin. Incorporation was allowed to proceed for one hour. The results are expressed as specific activity (counts per min. per 100 µg. P.)

Conditions	Flask No.	Inorganic Phosphorus.	ITP and IDP	IMP	ATP	ADP
CONTROL	1	2,001,000	991,500	490,000	720,000	546,000
	2	1,769,000	779,000	376,000	803,500	446,000
INSULIN	3	2,502,000	969,000	615,000	935,000	661,500
	4	1,894,000	973,500	505,000	846,500	584,500

Inosine monophosphate (IMP)

Inosine diphosphate (IDP)

Inosine triphosphate (ITP)

Adenosine diphosphate (ADP)

Adenosine triphosphate (ATP)

The results of these six experiments in which statistical analysis was possible contribute a little to the elucidation of the action of insulin. In experiments 3G1 (Table 28) and 3G6 and 3G7 (Table 30) there is convincing evidence that the presence of insulin brings about an increased incorporation of ^{32}P into the acid soluble fraction of strain L-cells. In experiment 3G7 the acid soluble fraction was further fractionated to show an increased activity in the IMP and ATP of the cells grown in insulin-containing medium. (Table 31). However, once again, it was difficult to obtain consistent results. Experiments 3G5 and 3G13 (Table 29) and experiment 3G10 (Table 30) failed to show a significant increase in activity in the ASP fraction in the presence of insulin. The ratio of cell population to volume of medium was constant throughout the series of experiments; in the majority of these the glucose concentration was the same, the medium was prepared to the same formula, the same preparation of insulin was employed and the experimental routine was not varied. A systematic search was therefore undertaken in an attempt to pin point the factor causing the variation in results, which was observed in all the fractions analysed. The following factors were investigated: preparatio of insulin, glucose concentration, potassium concentration, phosphorus concentration and pH of the medium. The effect of varying the growth phase of the cells under test was studied.

TABLE 28. (Expt. 361)

The incorporation of radioactive phosphorus into fractions of cultures of strain L-cells grown in Eagle's medium, with and without insulin. Incorporation was allowed to proceed for one hour. The results are expressed as specific activity (counts per min. per 100 µg. P.)

Description	No. Obs.	Conditions	ASB		LP		RNAP		DNAP	
			Activity	P.	Activity	P.	Activity	P.	Activity	P.
3×10^6 cells in 50 ml. conical flasks with 3ml. medium	9	CONTROL	811,500 ±75,600	0.02	274,480 ±29,500	0.02	209,800 ±49,800		34,890 ± 9,780	0.02
	9	INSULIN	974,400 ±163,300		333,000 ±61,390		221,500 ±147,000		57,300 ±23,960	

TABLE 29. (Expt. 3G5 and 3G13)

The incorporation of radioactive phosphorus into fractions of cultures of strain L-cells grown with and without insulin in large conical flasks (50 x 10^6 cells per flask). Incorporation was allowed to proceed for one hour. The results are expressed as specific activity (counts per min. per 100 µg. P.)

Medium.	No. Obs.	Conditions.	ASP	LP	RNAP	DNAP
3G5 Embryo extract	3	CONTROL	1,998,700 ± 116,000	519,000 ± 31,240	638,570 ± 93,660	* 69,920
medium	3	INSULIN	2,057,000 ± 166,700	534,300 ±170,700	597,000 ± 31,590	85,870 ±13,040
3G13 Eagle's medium	3	CONTROL	339,300 ± 58,400			
without HS.	3	INSULIN	350,300 ± 35,070			

* 2 observations.

TABLE 30. (Expt. 3G6, 3G7, 3G10).

The incorporation of radioactive phosphorus into fractions of strain L-cells grown with and without insulin in Kolle flasks (20 x 10^6 cells per flask). Incorporation was allowed to proceed for one hour. The results are expressed as specific activity (counts per min. per 100 µg. P.)

Medium	No. Obs.	Conditions.	ASP		LP		RNAP		DNAP	
			Activity	P.	Activity	P.	Activity	P.	Activity	P.
3G6 Eagle's medium (without HS)	3	CONTROL	752,300 ± 48,410	0.02	155,300 ± 23,660	0.3	59,470 ± 3,971	0.02	8,160 ± 650	0.2
	3	INSULIN	916,000 ± 48,540		170,530 ± 4,966		69,370 ± 2,554		9,310 ± 912	
3G7 Eagle's medium (without HS)	3	CONTROL	582,500 ± 17,510	0.001	157,500 ± 9,984	0.01	43,150 ± 7,859	0.05	4,257 ± 336	0.1
	3	INSULIN	709,800 ± 32,510		196,800 ± 11,100		54,630 ± 381		5,693 ± 1,114	
3G10. Eagle's medium (with dialysed) HS)	4	CONTROL	1,325,250 ± 193,400	0.2						
	4	INSULIN	1,528,000 ± 96,620							

TABLE 31. (Expt. 367).

The incorporation of radioactive phosphorus into the acid soluble nucleotides of cultures of strain L-cells. The cells were grown in Eagle's medium with and without insulin. Incorporation was allowed to proceed for one hour. The results are expressed as specific activity (counts per min. per 100 µg. P.)

No. Obs.	Conditions	Inorganic P. Activity	P.	ITP & IDP Activity	P.	IMP Activity	P.	ATP Activity	P.	ADP Activity	P.
3	CONTROL	644,700 ±157,800		674,000 ± 32,790	0.2	406,170 ±168,600	0.05	642,300 ± 49,100	0.02	425,300 ± 72,110	0.1
3	INSULIN	706,300 ±271,300		861,667 ±183,100		715,700 ± 31,970		877,000 ±117,000		569,000 ± 74,910	

TABLE 32. (Expt. 3G5 and 3G7).

The utilisation of glucose and production of lactic and pyruvic acid in cultures of strain L-cells grown with and without insulin over a 5 hour period.

Conditions	Flask No.	Glucose µg/ml. utilised	Lactic Acid produced µg/ml.	Pyruvic Acid produced µg/ml.	Total Acid production (µg)	% glucose not appearing as acid.
CONTROL	1	119	29	16.5	45.5	62%
3G7	2	82	30	15.1	45.1	45
(Eagle)	3	106	24	15.6	39.6	59.4
INSULIN	4	140	49	16.0	65.0	46.5
3G7	5	90	33	14.0	47.0	48
	6	90	44	17.3	61.3	68
CONTROL	1	202	43	8.7	51.7	74
3G5	2	342	30	8.2	38.2	89
(Embryo Extract)	3	272	31	7.7	38.7	86
INSULIN	4	232	30	7.7	37.7	84
3G5	5	222	21	9.2	30.2	87
	6	192	24	8.7	32.7	83

Preparation of insulin.

Two preparations of insulin were compared in this experiment; the sterile solution of Boots' insulin normally used, and a solution prepared from a crystalline sample of Novo-insulin prepared in the Novo - Terapeutisk Laboratorium, Copenhagen. The experiment was on Strain L-cells, 5×10^6 cells per 50 ml. conical flask containing 5 ml. Eagle's medium. Four flasks were included in both insulin-treated groups. The results from these two groups were compared with each other and with a similar control group. Table 33 shows that both preparations of insulin had the same quantitative effect in increasing the incorporation of radioactive phosphorus into the acid soluble and lipid phosphorus fractions of the cells. These findings indicate that the differences in the previous results cannot be explained by changes occurring in the stored insulin solution.

Glucose concentration.

The normal concentration of glucose present in the medium was 200 mg. per 100 ml. Occasionally it was 400 mg./100 ml. (Expt. 306 and 305) but this could not be correlated with a consistent effect of insulin: in experiment 306 (Table 30) an increased activity was observed in the ASP and RNAP fractions in the presence of insulin while these effects were absent in experiment 305 (Table 29). Table 34 compares the effect of insulin on cells grown in 50 mg. glucose per 100 ml. with the effect on cells grown in the normal concentration of glucose. In neither group was there any statistical difference between

TABLE 33. (Expt. 3017).

The incorporation of radioactive phosphorus into fractions of strain L-cells grown in Eagle's medium in 50 ml. conical flasks (5×10^6 per flask). Incorporation was allowed to proceed for one hour. The effects of adding either Boots' insulin or Novo insulin to control medium were compared. The results are expressed as specific activity (counts per min. per 100 µg. P.)

Conditions	No. Obs.	ASP.	LP.
CONTROL	4	777,700 ± 33,100	14,965 ± 1,591
INSULIN (Boots')	4	842,500 ± 34,700	17,580 ± 490
INSULIN (Novo-Terapeutisk)	4	834,500 ± 11,400	18,360 ± 1,204

TABLE 34. (Expt. 3G16).

The incorporation of radioactive phosphorus into fractions of strain L-cells grown in embryo extract medium with and without insulin. 5×10^6 cells were grown in 50 ml. conical flasks containing 5 ml. medium. Incorporation was allowed to proceed for one hour. The effects of varying the glucose concentration of the medium were studied. The results are expressed as specific activity (counts per min. per 100 µg. P).

Glucose Concentration	No. Obs.	Conditions	ASP	LP
200 mg. per 100 ml. medium	3	CONTROL	753,700 ± 93,620	9,490 ± 462
	3	INSULIN	729,300 ± 68,080	9,650 ± 745
50 mg. per 100 ml. medium	3	CONTROL	766,300 ± 109,200	9,883 ± 1,562
	3	INSULIN	672,700 ± 45,010	8,437 ± 1,198

The control and insulin containing cultures. This suggests that the anomaly in the previous results was not connected with a variation in the glucose level.

Potassium concentration.

Previous work by Leslie and Paul (1954), in which a reproducible increase in DNAP of chick heart explants was observed in the presence of insulin, was carried out in a medium containing Tyrode's solution. This solution contains 0.2 g. KCl per litre whereas Hanks' BSS contains 0.4 g. KCl per litre. A further indication that the potassium level might be of importance in modifying the effect of insulin was found in the work of Hastings, Renold and Teng, (1955) who studied the effects of ions and hormones on carbohydrate metabolism. An experiment was therefore set up in which cells were grown in medium containing varying amounts of potassium. HS was omitted from the medium as the concentration of potassium in it is very variable. The effect of insulin on the incorporation of radioactive phosphorus was studied at the various levels of potassium concentration. The results are shown in Table 35. This series of experiments failed to demonstrate any effect of insulin on the incorporation of radioactive phosphorus into the fractions under investigation. There was no evidence that the potassium concentration modified the effect of insulin.

TABLE 35. (Expt. 353 and 3018).

The incorporation of radioactive phosphorus into tissue culture cells grown in Eagle's medium with and without insulin. Incorporation was allowed to proceed for one hour. The effect of varying the potassium concentration in the medium was studied. The results are expressed as specific activity (counts per min. per 100 µg. P.) Each 50 ml. flask contained 4.5×10^6 cells per 5 ml. medium.

Potassium concentration in BSS.	No. Obs.	Conditions.	ASP Activity	ASP P.	LP Activity	LP P.
353 buffy coat cells. no potassium	3	CONTROL	900,000 ± 73,700	0.2	20,120 ± 5,110	>0.2
	3	INSULIN	987,000 ± 43,060		21,070 ± 1,880	
0.2 g. KCl/litre	3	CONTROL	1,005,000 ± 38,580	>0.2	19,510 ± 783	0.2
	3	INSULIN	1,017,000 ± 50,270		17,880 ± 1,170	
0.4 g. KCl/litre	3	CONTROL	847,000 ± 115,600	0.2	19,043 ± 225	
(conc. in Hanks')	3	INSULIN	954,000 ± 48,410		15,790 ± 1,480	

TABLE 35 (Contd.) (Expt. 383 and 3G18)

The incorporation of radioactive phosphorus into tissue culture cells grown in Eagle's medium with and without insulin. Incorporation was allowed to proceed for one hour. The effect of varying the potassium concentration in the medium was studied. The results are expressed as specific activity (counts per min. per 100 µg.P.) Each 50 ml. flask contained 4.5×10^6 cells per 5 ml. medium.

Potassium concentration in BSS.	No. Obs.	Conditions.	ASP Activity	ASP P.	LP Activity	LP P.
3G18 strain L-cells. 0.4 g. KCl/litre	3	CONTROL	373,000 ± 59,600	0.2	43,740 ± 6,560	0.2
	3	INSULIN	432,000 ± 32,620		54,370 ± 8,520	
0.8 g. KCl/litre	3	CONTROL	334,700 ± 3,082	0.3	35,370 ± 6,990	0.3
	3	INSULIN	354,700 ± 31,570		43,600 ±10,460	
1.6 g. KCl/litre	3	CONTROL	296,000 ± 13,530	>0.2	25,970 ± 5,830	0.2
	3	INSULIN	306,300 ± 38,630		42,800 ±18,820	

Phosphorus concentration.

If it is assumed, for the moment, that one of the primary effects of insulin is on phosphate metabolism and the transport of phosphorus into the cell, then the occasional absence of an insulin effect may be explained by postulating that the phosphorus content of the medium has become a limiting factor. The main source of phosphorus in the medium was usually Hanks' BSS which contains 2.4 mg. P per 100 ml. An experiment was set up in which strain L-cells were grown in medium prepared from BSS containing only 0.6 mg. P per 100 ml. and the effect of insulin on these cells was compared with its effect on cells grown in normal medium. 5×10^6 cells were grown in 5 ml. medium in a 50 ml. conical flask: 3 flasks were included in each group. The results (Table 36) show that the only significant ($p = 0.08$) effect of insulin was on the cells grown in the lower concentration of phosphorus. This makes unlikely the suggestion that the phosphorus concentration became a limiting factor for insulin action in some of the previous experiments.

pH of the Medium.

This is a factor which was not strictly controlled from experiment to experiment. The pH was normally adjusted by the dropwise addition of sodium bicarbonate to the medium until the colour of the phenol red indicated the pH to be approximately 7.4. It was not possible to make an accurate adjustment by this method. However in experiment 335 Eagle's medium was prepared with Earle's BSS and measured amounts of 6.6% (w/v) sodium bicarbonate added to aliquots of the medium to give a range of pH values from 7.0 - 8.2. Cultures of buffy-coat cells (5×10^6 cells

per 50 ml. flask) were set up in 5 ml. of this medium which was then equilibrated with 5% carbon dioxide / air mixture. Insulin was include in the medium of half the cultures and the experiment was carried out in the usual way.

The results are contained in Table 37. They show that no significant difference in incorporation was found between any of the pairs of results. In as far as it went, the experiment failed to provide evidence of a pH related insulin effect. However, it is seen that at higher pH values there was more rapid incorporation of radioactive phosphorus into fractions of both control and insulin containing cultures. If insulin cultures were maintained at a more alkaline pH than the corresponding control cultures, then there would appear to be more rapid incorporation of ^{32}P in the presence of insuli It is unlikely that this situation existed for the insulin was made up in acid solution, which would weight the results in the opposite direction. The pH of the test and control media was adjusted until, by visual comparison, the solutions were matched.

TABLE 36. (Expt. 3G15).

The incorporation of radioactive phosphorus into cultures of strain L-cells grown in Eagle's medium with and without insulin. Incorporation was allowed to proceed for one hour. The effect of varying the phosphate concentration in the medium was studied. The results are expressed as specific activity (counts per min. per 100 µg. P).

Phosphorus concentration in BSS.	No. Obs.	Conditions.	ASP		LP	
			Activity	P.	Activity	P.
2.4 mg. P	3	CONTROL	499,700 ±161,700		28,470 ± 3,360	0.3
per 100 ml.	3	INSULIN	430,000 ± 32,650		37,570 ±14,720	
0.6 mg. P	3	CONTROL	764,000 ± 39,880	0.08	55,770 ± 8,940	
per 100 ml.	3	INSULIN	880,700 ± 71,840		* 53,400	

* 2 observations.

TABLE 37.

The incorporation of radioactive phosphorus into cultures of a strain of buffy coat cells grown in Eagle's medium, with and without insulin. Incorporation was allowed to proceed for one hour. The effect of altering the pH of the medium was studied. The results are expressed as specific activity. (counts per min. per 100 μg. P).

pH of medium	No. Obs.	Conditions	ASP		LP	
			Activity	P.	Activity	P.
7.0	3	CONTROL	117,200 ± 13,890	0.1	10,363 ± 3,962	
	3	INSULIN	137,400 ± 7,385		7,577 ± 473	
7.4	3	CONTROL	161,800 ± 23,130	>0.2	8,453 ± 1,148	>0.2
	3	INSULIN	160,100 ± 5,292		8,253 ± 1,101	
7.8	3	CONTROL	179,500 ± 3,122	0.2	8,790 ± 1,243	0.2
	3	INSULIN	193,700 ± 15,320		10,430 ± 1,448	
8.2	3	CONTROL	184,000 ± 18,560	0.2	10,093 ± 1,440	0.2
	3	INSULIN	203,700 ± 9,000		11,580 ± 1,042	

Growth Phase of the Cells.

It was decided to investigate this problem with a simpler experimental technique in which the total activity of the medium was measured. The possibility of this method was suggested by recent work of Goodman and Rothstein (1957) with yeast cells. In the first experiment (3P5) cells were labelled by growing them overnight in radioactive medium. They were then washed and transferred to non-radioactive medium with and without insulin. Samples of the medium were removed at various time intervals and the activity of them was assayed. It was thought that if insulin acted by controlling the release of phosphorus from the cell a difference would be detected between the level of activity in the control and test medium. The details of the experiment and the results are given in Table 38. By inspection it is seen that there was no difference in the rate of release of radioactive phosphorus by the control and insulin containing cultures.

The second experiment (3P9) used this technique to study the effect of insulin on cells which had been growing for some time (18 hr.) in the experimental medium before being exposed to the hormone. Normally the cells grew only for four hours in the experimental medium before incorporation was studied. In this short time it might not have been possible for the cells to synthesize the full complement of metabolites necessary for insulin reaction.

The experimental procedure was as follows. Thirty, 50 mm. Petri dishes were inoculated with 5×10^6 strain L-cells. The dishes were

divided at random into three groups of ten, A, B and C: to groups A and B were added 5 ml. Eagle's medium containing 1 μ.curie ^{32}P/ml. while to group C were added 5 ml. non-active medium. All the cells were incubated overnight. The following day the media were removed and collected. The radioactive cells of group A were quickly washed with BSS and 1.5 ml. of non-active medium, from group C, added to each of the ten dishes in the group. To half of these cultures insulin was then added. Group A was a study of the release of radioactive phosphorus into non-active medium, with and without insulin. To groups B and C were added 1.5 ml. of the pooled radioactive medium. To half the cultures in each group insulin was added. The cultures in groups B and C provided a study of the effect of insulin on the incorporation of radioactive phosphorus from the medium. After four hours the medium from each cultures was removed. It was later diluted and counted. This experimental procedure is summarised and the results are given in Table 39. It is seen that there is no significant difference between the results in any one group. The release of phosphate by the cells is not enhanced by insulin, nor is the uptake of phosphorus from the medium increased under these circumstances in which the cells were grown overnight in the experimental medium.

TABLE 38. (Expt. 3P5)

The activity of the medium in which radioactive strain L-cells had been growing for various times after their removal from medium containing 0.8 μc. ^{32}P per ml. The cells were washed with BSS before transfer to the non-active medium prepared with and without insulin. The results are expressed as counts per minute per 1 ml. medium.

Hrs. after transfer to non-active medium.	CONTROL	INSULIN	No. obs. in each group.
0	864	863	3
3	2517	2561	3
5	3240	3172	3
21	4857	4880	3
24	5163	5330	3

TABLE 39. (Expt. 3P9).

The experimental procedure of expt. 3P9.
The level of activity in the medium from groups A, B and C strain L-cells at the completion of the experiment. Activity is expressed as counts per minute per 1 ml. medium.

GROUP A	GROUP B	GROUP C.
10 cultures L-cells grown overnight in medium with 1 μc. ^{32}P/ml.	10 cultures L-cells grown overnight in medium with 1 μc. ^{32}P/ml.	10 cultures L-cells grown overnight in non-active medium
Following day:- All medium removed and retained.		
Medium replaced with 15ml. non-active medium from group C. Insulin added to half the cultures.		Medium replaced with 1.5 ml. pooled radioactive medium. Insulin added to half the cultures in each group.
After 4 hours all the medium removed for counting		

No. obs.	CONTROL	INSULIN	CONTROL	INSULIN	CONTROL	INSULIN
5	230,300 ± 64,120	210,840 ± 32,300	1,267,000 ± 91,530	1,250,000 ± 53,370	1,053,000 ± 32,420	1,033,000 ± 37,910
	GROUP A		GROUP B		GROUP C.	

Different cell types.

A consideration of previous results and those shown in Table 40 reveals that in none of the experiments with the strain of human-buffy coat cells was a significant effect produced on the incorporation of radioactive phosphorus into various phosphorus fractions by insulin. Only one experiment was carried out with HeLa cells and it also was negative. A confirmation of the results obtained with strain L-cells and chick embryonic material was sought with material known to be insulin responsive. For this purpose rat diaphragm was chosen.

Rat diaphragm.

Sacks and Sinex (1953) worked with rat diaphragm in vitro to establish the relation between phosphate transfer and glucose metabolism in the presence of insulin. Using a Warburg type of flask, radioactive tracer and insulin were tipped in from the side arm after the diaphragm and medium had been equilibrated for 15 minutes. 1 - 2 hours later the experiment was terminated and various phosphorus fractions were isolated from the diaphragm. An increased oxidation of glucose was observed accompanied by a 50% increase in phosphate transfer from the medium to the diaphragm in the presence of insulin. This increase was noted in the following fractions: phosphocreatine phosphorus, labile phosphorus of ATP and hexose monophosphate phosphorus. No accompanying change in the rate of oxygen consumption was detected. These results suggested that rat diaphragm would be a suitable material for the proposed studies.

TABLE 40. (Expt. 381 and 382).

The incorporation of radioactive phosphorus into cultures of a strain of buffy coat cells grown in Eagle's medium, with and without insulin and into cultures of HeLa cells grown in embryo extract medium. Incorporation was allowed to proceed for one hour. The results are expressed as specific activity (counts per min. per 100 µg. P.)

No. obs.	Conditions.	ASP Activity	P.	LP Activity	P.	RNAP Activity	P.	DNAP Activity	P.
Expt. 381 buffy-coat									
6	CONTROL	* 866,400 ± 53,530	0.1	136,130 ± 46,160					
6	INSULIN	978,000 ± 124,000		120,050 ± 49,780					
Expt. 382 buffy-coat									
6	CONTROL	522,500 ± 22,640	0.1	20,800 ± 1,330		40,917 ± 8,015		7708 ± 930	
6	INSULIN	552,500 ± 30,190		18,170 ± 1,317		35,670 ± 2,045		7,735 ± 913	
HeLa cells.									
3	CONTROL	265,000 ± 86,650		12,970 ± 4,535					
3	INSULIN	317,000 ± 42,330		12,290 ± 2,331					

* 5 observations.

Six rats were decapitated and the diaphragms removed quickly into a beaker of ice-cold BSS. These were trimmed free of adhering tissue, halved and the two hemidiaphragms put into paired 25 ml. conical flasks. One flask of each pair contained 3 ml. control medium and the second 3 ml. insulin-containing medium. The medium was Eagle's medium without horse serum. 12 μ. curies radioactive phosphorus were added to each flask in turn at one minute intervals. The flasks were gassed with 5% CO_2/air mixture and shaken in a 37°C water-bath for two hours. At the end of the incubation period the hemidiaphragms were removed from the flasks one at a time. They were blotted and washed three times in cold isotonic salt solution before being dropped into 2 ml. alcohol-ether mixture cooled in a bath of alcohol/solid carbon dioxide. The hemidiaphragms were chopped finely with scissors and fractionated in the usual way to obtain a lipid fraction, AS1 for chromatography, AS2 for DNA estimation and a residue containing protein.

The results are expressed relative to the nitrogen content of the tissue, except for the acid soluble nucleotides where the total amount present was not accurately known. (Tables 41 and 41 a). It was not possible to obtain sufficient material to analyse the inosine phosphates. Students t- test was applied to the results in each group and the probability associated with this value is quoted.

No definite pattern of results was obtained in these three experiments. Only in experiment 3N1 was there evidence of an enhanced rate of incorporation of ^{32}P into the AS1 fraction in the presence of insulin. Later experiments 3N2 and 3N3 did not confirm this observation

and values of activities for the paired hemi diaphragms were in close agreement with each other. However analysis of the carbohydrate content of the AS2 fraction in these experiments showed insulin had significantly increased the amount of glucose present. This would seem to separate the effect of insulin on glucose uptake from its effect on phosphate metabolism. There was no evidence of increased activity in any of the acid soluble nucleotides examined.

TABLE 41 a. (Expt. 3M1 and 3M3).

The analysis of fractions obtained from rat hemidiaphragms previously incubated in medium containing radioactive phosphorus for two hours. One hemidiaphragm of each pair was in insulin-containing medium and the other in control medium.

3M1 Pair No.	Conditions	ASI Total count/mg.N.	LP Total count /mg.N.	ATP sp. act.	ADP. sp. act.
1	CONTROL	23,600	282		
	INSULIN	32,700	342		
2	CONTROL	23,700	308		
	INSULIN	33,900	-		
3	CONTROL	25,800	234	116,200	64,500
	INSULIN	37,400	571	138,200	29,200
4	CONTROL	30,800	319	53,800	28,600
	INSULIN	37,600	406	73,000	42,900
5	CONTROL	34,200	502	89,200	74,000
	INSULIN	36,100	340	79,900	34,200
6	CONTROL	25,600	320	106,900	51,100
	INSULIN	30,700	510	127,600	98,600
P.		0.01	0.3	0.2	0.5

TABLE 41 a. (Contd.) (expt. 3N1 and 3N3).

The analysis of fractions obtained from rat hemidiaphragm previously incubated in medium containing radioactive phosphorus for two hours. One hemidiaphragm of each pair was in insulin-containing medium and the other in control medium.

Pair No.	Conditions	AS1 Total count/mg.N.	LP Total count/mg.N.	ATP sp.act.	ADP sp.act.	AS2 fraction. mg. Glucose / mg. N.
Expt. 3N3.						
1	CONTROL	19,900	115	180,500	-	0.0396
	INSULIN	22,900	131	115,500	-	0.0481
2	CONTROL	23,400	143	153,500	26,300	0.0415
	INSULIN	22,500	126	269,000	33,700	0.0438
3	CONTROL	23,900	99	466,000	23,800	0.0353
	INSULIN	21,200	80	100,800	18,450	0.0491
4	CONTROL	26,400	170	142,800	23,500	0.0393
	INSULIN	23,900	112	312,000	29,400	0.0487
5	CONTROL	28,000	213	187,500	51,000	0.0384
	INSULIN	26,500	188	290,500	41,100	0.0575
6	CONTROL	-	115	592,000	19,200	0.0396
	INSULIN	35,700	209	98,800	23,700	0.0893
P.		0.4	>0.5	0.5	>0.5	0.05

TABLE 41 b. (Expt. 3N2).

Expt. 3N2.

Pair No.	Conditions.	AS1 Total count/mg. N.	LP Total count/ mg. N.	AS2 fraction mg. glucose/mg. N.
1	CONTROL	34,300	333	0.066
	INSULIN	32,800	306.1	0.0976
2	CONTROL	34,200	363	0.0797
	INSULIN	34,300	765	0.1050
3	CONTROL	32,700	344	0.0691
	INSULIN	33,600	645	0.0580
4	CONTROL	27,000	288	0.0594
	INSULIN	33,100	347	0.0860
5	CONTROL	46,400	337	0.0617
	INSULIN	30,300	555	0.0880
P.		>0.5	0.08	0.07

'Brown Fat' from rat.

This material was used in a further attempt to investigate whether insulin increases the amounts of lipid phosphorus, ribonucleic acid phosphorus and protein nitrogen in a culture of explants.

The tissue has been the object of much speculation for more than half a century. Formerly the view was held that 'brown' adipose tissue developed from special pre-adipose structures of mesenchymal cells. 'White' adipose tissue was thought to arise from branched connective tissue cells which gradually filled with fat. It was emphasized that the two were not interchangeable and that they developed by different means. Later workers, however, claimed that brown fat can develop into white fat. The origin of brown adipose tissue has been thoroughly investigated by Sidman, using the organ culture technique (1956 a). He concluded that it does not differ fundamentally from white adipose tissue. Further work on this tissue showed a direct effect of insulin on cultures of brown fat (Sidman, 1956 b). Visual inspection of stained sections showed that insulin in concentrations of 0.04 - 4.0 µg./ml. increased glycogen synthesis and lipid deposition.

The present experiment employed the 'floating raft technique' (p.25). 48 pieces of chopped brown fat were individually positioned on each nylon raft which was floated on 3 ml. Eagle's medium. After 48 hrs. the medium and tissue were collected for analysis. The medium contained graded amounts of insulin.

The chemical analysis of the tissue (Table 42) shows little

increase in the synthesis of carbohydrate, lipid or protein in the presence of insulin. However the analyses were on very small amounts of material and the methods may not have been sensitive enough to detect a change. The analysis of the medium (Table 43) showed the expected increase in glucose utilisation by tissue grown in insulin-containing medium. There was some evidence of a lowered lactic acid production by this tissue. On the whole, this system proved less satisfactory than had been anticipated and it was therefore abandoned.

TABLE 42.

The composition of explants of brown adipose tissue from a rat after two days growth in Eagle's medium which contained graded amount of insulin. The results are expressed relative to the protein N. content of the tissue, except for the total ultra violet absorption.

6

Conditions Insulin conc.	Total carbohydrate (µg.glucose/µg.pro.N)	Total lipid (µg.) per µg. pro. N.	Total ultra violet absorp.at 268 mu.	Total protein nitrogen (µg.)
CONTROL	0.613	27.6	0.564 0.524	- 66.8
1 unit/ml.	0.600 0.671	32.5 33.6	0.688 0.548	63.4 64.1
10^{-2} unit/ml.	0.905 0.810	33.6 30.3	0.548 0.656	62.9 66.0
10^{-4} unit/ml.	0.445 0.578	30.0 34.2	0.504 0.844	64.9 67.9
10^{-6} unit/ml.	0.590 0.523	32.0 39.8	0.536 0.580	64.4 63.6

66

pro - protein
absorp - absorption

TABLE 43.

The utilisation of glucose and the production of lactic and pyruvic acid by cultures of rat brown adipose tissue grown in Eagle's medium containing graded amounts of insulin for two days. All results are expressed relative to the protein nitrogen content of the tissue.

Conditions Insulin conc.	Glucose utilisation (µg) per ml. medium.	Lactic acid production (µg) per ml. medium.	Pyruvic acid production (µg.) per ml. medium.
	All results expressed relative to protein N of tissue.		
CONTROL	0.63	0.478	0.0096
1 unit/ml.	2.85	0.268	0.0125
	4.41	0.093	0.0098
10^{-2} unit/ml.	3.27	0.222	0.0076
	3.90	0.424	0.0080
10^{-4} unit/ml.	2.30	0.416	0.0096
	4.06	0.398	0.0061
10^{-6} unit/ml.	2.22	0.295	0.0085
	2.75	0.472	0.0079

D I S C U S S I O N .

DISCUSSION.

Results obtained from the study of adaption of tissue cells from *in vivo* to *in vitro* conditions show a change in the pattern of metabolism during the first three days in culture. Initially the heart explants exhibited a minimal uptake of oxygen with the utilisation of glucose and production of lactic acid. As heart tissue in the adult mammal is usually regarded as highly aerobic these findings were rather unexpected. Willmer's observations (1942) with fresh heart cultures give support to the idea that glycolysis is an important energy producing pathway in these newly explanted tissues. Warburg found it possible to maintain chick embryonic heart under conditions of strict anaerobiosis (Warburg and Kubowitz, 1927). However Danes (1955) studying the metabolism of chick heart fibroblasts by the precise Cartesian diver technique showed that these cells are capable of respiration or, in an atmosphere of nitrogen, anaerobic glycolysis. She was working with a cell culture from a 48 hour explant.

In the 24 hours following explantation the rate of oxygen consumption greatly increased and 48 hrs. after explantation the heart fragments exhibited a fairly high rate of respiration. Analysis of the medium showed that oxidation of lactic acid occurred on depletion of the glucose reserves. This depletion may, to some extent, have necessitated the switch in metabolism, but renewal of the glucose level to the original 200 mg/100 ml. did not induce a return to the low levels of oxygen consumption originally found. It is interesting

to note that renewal of the medium caused an increase in the incorporation of ^{32}P into the acid soluble fraction to a higher level than the initial one. These findings provide evidence for a change in metabolism and suggest that there is an initial lag phase in which the explanted tissue adapts itself to in vitro conditions. This may not be a true lag-phase, in which adaptive enzyme systems are formed but could be explained by the death of one type of cell and its replacement by the more readily cultivated fibroblast. It is possible that by 48 hrs. the majority of surviving cells are fibroblasts and that the 'change' in the pattern of metabolism simply mirrors the emergence of the fibroblast as the dominant type of cell. This theory was not confirmed by histological observation. It is probable that the tissue is damaged during preparation and consequently the original medium may contain inhibitory metabolites from damaged cells. A third possibility is that the metabolism of the chick heart in vivo is controlled by a variety of factors which do not persist in in vitro conditions. The loss of these mechanisms leads ultimately to the rapid, unorganised growth characteristic of most cell strains. Whatever the reason for these alterations may be, they are of importance in themselves. It would seem that the dramatic change in oxygen consumption is of particular interest.

The results of the analysis of chick embryonic liver during the first few days of cultivation also show evidence of a change in the pattern of metabolism. Again there is a profound alteration in the oxygen consumption of the tissue after 24 hours in culture: the very

high initial levels are replaced by more moderate uptakes and after three days in culture the rate of oxygen utilisation is similar to that found in heart cultures of the same age. The initially high oxygen consumptions are associated with a utilisation of lactid acid from the medium and a production of hexose. An apparent contradiction of these results was found in the work of Roffo who also studied changes in the medium during cultivation of chick embryonic liver. However these explants had been in culture for some time. (Roffo,1929). Since a reversal to the normal pattern of glucose utilisation and lactate production was established by 24 hours in the present study, there is no disparity between the two groups of results. A study of the incorporation of ^{32}P into the acid soluble fraction of the explanted liver showed, as in the comparable heart studies, that renewal of the medium caused a substantial rise in the rate of incorporation. This was to a level higher than the initial one. From these findings it can be concluded that after two days in culture the liver tissue had a reduced oxygen consumption, an altered pattern of carbohydrate metabolism and an increased rate of incorporation of ^{32}P to various phosphorus fractions.

Again it is possible that these changes might coincide with the death of some liver cells and their replacement by fibroblasts. However, histological examination of the explants at 24 hr. showed that although there were often areas of central necrosis there were also large numbers of surviving hepatocytes. An alternative explanation is that exhaustion of the lactate in the medium, or an inadequate

supply of oxygen played a part in the switch of metabolism. To test this hypothesis a culture of liver cells was grown in roller tubes, to ensure efficient oxygenation, and additional lactic acid was added to the medium. The characteristic alteration in the pattern of metabolism persisted. This seems to point to the existence of some controlling factor, present under normal conditions, but absent when the tissue is cultured in vitro. It is interesting that the metabolic pattern which is established after a few days in culture is similar to that which develops in chick embryonic heart cells. This lends support to the idea that in the absence of certain specific factors cells in vitro adopt a simpler standard pattern of metabolism. Jones and Bonting (1956) working with embryonic chick lung and intestine, showed that two days after explantation these cultures were able to adapt themselves to the substrate provided and could alter the balance between aerobic and anaerobic glycolysis according to the gas and energy substrates available. This does not appear to be true for newly explanted tissue.

Having decided that these changes in explanted tissues might be explained by the loss of controlling factors present in vivo, it was natural to turn to a consideration of the effects of a hormone on cell cultures. The effects of insulin on metabolic reactions have been widely studied in a great variety of animals and tissues and it is possible to compare the findings presented here with previously published observations. Perhaps the most reproducible effect of insulin is on glucose utilisation by isolated muscle. Villee and Hastings (1949,a)

showed that insulin in the medium increased the glucose uptake, the fraction appearing as carbon-dioxide and the fraction synthesized to glycogen in rat diaphragm. An impairment of these abilities was exhibited by diaphragm from a diabetic rat. The experiments with chick embryonic material, L-cells and buffy-coat cells showed, in the majority of analyses, a greater glucose uptake in the presence of insulin. These observations are in agreement with previous evidence that insulin promotes the utilisation of glucose by a variety of tissue culture cells. (Leslie and Paul, 1954).

A more marked phenomenon in these cultures was the depression of the level of pyruvic acid in the medium in the presence of insulin. This was always coupled with an increased level of lactic acid such that the sum total of lactic and pyruvic acids in the insulin-containing medium was larger than that of the control medium. A review of the literature connecting pyruvate metabolism and insulin shows that the majority of observed effects by the hormone are on diabetic tissue only. For example, Villee and Hastings (1949, b) incubated normal rat diaphragm in the presence of isotopic pyruvate and studied the rate of pyruvate utilisation and oxidation. They measured the uptake of oxygen and production of carbon dioxide from the labelled acid. Insulin, added to normal diaphragm, was without effect but returned the impaired pyruvate metabolism of diabetic diaphragms to control levels. Similarly Osborn, Chaikoff and Felts (1951) who concluded that insulin shifts the metabolism of the 2-C fragment derived from an oxidative fate to one involving synthesis, based

their claim on studies with diabetic liver. An earlier report of an effect of insulin on pyruvate utilisation was that of Rice and Evans (1943). These workers found an increased utilisation of pyruvate by pigeon breast muscle following an 80 minute equilibration with the hormone. It is to be remembered that Rice and Evans were using the material with which Krebs and Eggleston (1938) first demonstrated the striking effect of insulin on oxygen uptake. Later workers were unable to reproduce these results with mammalian muscles (Shorr and Baker, 1939). It may be that pigeon breast muscle is unique in its response to insulin. There is, therefore, little evidence of excess insulin having any effect on pyruvate utilisation or oxidation in normal tissue.

Leslie and Paul (1954) who found lowered levels of pyruvic acid when chick heart explants were grown in insulin-containing medium, suggested that the hormone had channelled the 'missing' pyruvate into the citric acid cycle. This theory was derived from the previous reports which have been considered above. Their results, however, do not preclude an alternative explanation which is implied in the results of lactic acid analyses presented here. These show that decreased levels of pyruvic acid in the test medium are accompanied by increased amounts of lactic acid. This suggests that under the experimental conditions one of the effects of insulin is to alter the balance between pyruvic and lactic acids in favour of the latter. A further proof that the oxidation of pyruvic acid is not the determining factor lies in the fact that the total amount of lactic

and pyruvic acid produced was never less than the amount of glucose utilised.

A more detailed consideration of the results reveals one or two exceptions to this theory. In the first place the trypsinized carcass cells, the strain L-cells and the buffy-coat cells, having been grown in the absence of insulin for some time, must be regarded as essentially diabetic material. The results of medium analysis from cultures of L-cells and buffy-coat cells (Tables 18 and 20) reveal that oxidation of pyruvic acid is not only impaired in these cells, it is virtually non-existent. Under these circumstances there may be a considerable lag phase before a possible effect of insulin on the citric acid cycle becomes apparent. The results with the chick carcass cells are rather different for the analyses at 24 hours showed that some oxidation of pyruvic acid had occurred. This was not significantly increased by the presence of insulin.

It is known that the interconversion of lactic acid and pyruvic acid depends on the presence of lactic dehydrogenase and diphosphopyridine nucleotide (DPN). A deficiency of DPN in the oxidized form would lead to an accumulation of lactic acid. It may be that insulin, by stimulating other enzymic reactions, requiring DPN, rapidly reduces the DPN available in the cells. This hypothesis is supported by the work of Glock and McLean (1955) who studied the levels of oxidized and reduced DPN in diaphragm muscle from diabetic rats. Insulin deficiency led to a rise in the concentration of oxidized DPN per g. muscle and to a corresponding fall in the level

of DPNH.

It has been claimed that insulin is an anabolic hormone, stimulating growth and the storage of excess foodstuffs. In addition to its noted effect on carbohydrate metabolism, insulin has been shown to enhance protein and fat synthesis. Early experiments, in which rats were fed deuterium, confirmed that in diabetes there is an impaired synthesis of fatty acids from glucose in the liver and carcass. (Stetten and Boxer, 1944). Bloch and Kramer (1948) showed that insulin had a stimulating effect on the incorporation of labelled acetate into higher fatty acids in rat liver slices. Krahl (1953) studied the incorporation of glycine-1- ^{14}C into protein fractions of liver and diaphragm from normal and diabetic rats. He found in both tissues that this was reduced in severe diabetes and that the addition of insulin, particularly in the presence of glucose, restored synthesis to normal levels. In their paper Leslie and Paul (1954) demonstrated that insulin can act upon chick heart cells in vitro to increase the rate of synthesis of phospholipids and protein. In contrast to these findings the results presented here for the analyses of chick carcass cells, L-cells and human blood cells cultivated for some time in the presence of insulin, showed that these contained no more lipid nor protein than control cultures. Earlier reports of a positive effect of insulin on the growth of fibroblast cultures are summarised in the introduction. It is interesting to note that Latta and Bucholz (1939) failed to establish an effect of insulin on cell growth below levels of 3.3 units/ml. while Friedheim and Roukhelman (1930) reported a

decrease in the growth of fibroblasts supported by medium containing 2 units insulin/ml. as compared to control cultures. The reason for these conflicting results is not apparent.

A previous summary had indicated that a connection exists between insulin action and phosphate metabolism. This was illustrated by the use of radioactive phosphorus which was incorporated to an increased extent into the acid soluble fraction, the tissue inorganic phosphate, the phosphocreatine phosphate and the labile phosphate groups of adenosinetriphosphate (ATP) in the presence of the hormone. A further consideration of the work of Haugaard, Marsh and Stadie (1951) leads to the conclusion that insulin action is concerned with an increased efficiency of oxidative phosphorylation. Rat diaphragms were incubated with glucose in the presence and absence of insulin. Subsequently they were analysed for ester phosphate, ATP and glycogen. Under the influence of insulin there was a large increase in glycogen formation but no significant change in the total acid soluble phosphate content. This increased glycogen synthesis must have necessitated an extra formation of ATP which occurred without an increased oxygen uptake. The conculsion drawn from these results was that insulin promotes the formation of more energy rich phosphate bonds per molecule consumed, than is possible in its absence.

Several studies have been made of reactions which depend on the availability of high energy phosphates and these have been found to be significantly depressed in diabetic animals. Charalampous and Hegsted (1949) studied the acetylation of an aromatic amine following

its injection into intact normal and diabetic rats. In diabetes there was a marked decrease in acetylation which was corrected by injection of insulin. This return to normality could also be brought about by a number of substances which either contained high energy phosphate bonds or stimulated oxidations leading to their formation. A second study has been made by Goranson and Erulkar (1949) using an *in vitro* system. They measured the phosphorylation of creatine by homogenates of heart and brain from normal and diabetic rats. The homogenates contained creatine, inorganic phosphates, cytochrome C, ATP and oxidizable substrates (succinate or malate). The diabetic homogenates showed a greatly impaired ability to synthesize phosphocreatine: this capacity was restored to normal on the addition of insulin. All these results indicate that in diabetic tissue the coupling between oxidation and phosphorylation is impaired. They suggest that in the presence of insulin there is an increased efficiency of oxidative phosphorylation. This would explain the accumulation of results which show that radioactive phosphorus is incorporated to a greater degree in a number of phosphate compounds in the presence of insulin.

These findings again stress the importance of the citric acid cycle in this aspect of insulin action. It will be remembered that early findings (Table 18) showed that all the glucose utilised by a culture of L-cells in a given time could be accounted for as acid produced. However in later experiments in which ^{32}P incorporation was studied, this was not found (Table 32) and only some 50% of the glucose utilised could be accounted for in this way. An explanation of

the difference in results is suggested by a recent paper of Phillips and Feldhaus (1956) who reported the oxygen uptake of strain L-cells to be altered by previous culture conditions. It is reasonable to conclude that in the majority of the incorporation experiments an oxidative pathway of glucose metabolism existed.

The results presented here showed that using L-cells or chick embryonic material it was possible to obtain a highly significant effect of insulin on the incorporation of ^{32}P into the total acid soluble fraction, the acid soluble nucleotides and the lipid phosphorus fraction. This effect could not however be reproduced with certainty. It is interesting to note that in the remaining experiments there was again a positive difference between the ASP activity in the presence and absence of insulin, but the probability of this difference being significant was very low. This suggests that the results are not contradictory as they at first appear, but they indicate varying degrees of response to the presence of insulin. It would be convenient to relate this degree of response of the cells to the extent of operation of the citric acid cycle and to postulate that a negligible increase in ^{32}P incorporation in the presence of insulin, indicated that Kreb's cycle was functioning to only a minor degree. Experimental evidence does not support this theory. Experiment 365 (Table 32), in which insulin was not effective in significantly increasing the incorporation of ^{32}P to any of the phosphorus fractions examined, showed that over 80% of the glucose utilised was completely oxidized. In contrast, experiment 367 (Table 32) which clearly

demonstrated an insulin induced effect, had a lower percentage of oxidized glucose. It seems the cause of the variation must be found elsewhere.

A report by Chen (1954 b) that a greater response to insulin was elicited in the absence of embryo extract prompted a careful scrutiny of the results to see if the inclusion of embryo extract in the experimental medium could be correlated with the absence of an effect of insulin on ^{32}P incorporation. This relationship was not established beyond doubt, and in experiment 3E1 (Table 24) for example a highly significant effect of insulin was noted in embryo extract medium. It was realised that the insulin molecule with a net anionic charge and other reactive groups may be rendered less active by combination with a medium constituent, like embryo extract. Of special interest were the reports of Lowell (1944) and Vallance-Owen, Hurlock and Please (1955) that some serum proteins may block or reduce the effect of the hormone. For this reason, embryo extract and horse serum were omitted from the medium of the later short-term experiments.

A number of experiments were designed in an attempt to locate the factor causing the variation in response to insulin by the cells. These experiments are described and discussed on p.65 et. seq. In summary it can be said that alteration of the preparation of insulin, or of the glucose, potassium or phosphate concentration of the medium were not factors in this variation. It was pointed out that the more alkaline cultures in a series of pH graded controls showed a faster rate of incorporation of ^{32}P into the ASP fraction. This was discussed

as a possible, but unlikely, reason for the appearance of the typical 'insulin' effect, since errors would most probably have weighted the results in the opposite direction. The final investigation into this problem considered the effect of the growth phase upon the response of the cells to the hormone. It was concluded that cells grown overnight in the experimental medium behaved in no way differently from cells subjected to the normal experimental treatment.

A comprehensive survey of the results reveals that the increased incorporation of ^{32}P into various phosphorus fractions in the presence of insulin is an elusive observation which cannot be reproduced with certainty. It was never observed in a culture of buffy coat cells and the single test on HeLa cells was negative. The response of Strain L-cells was variable but, on occasion, highly significant. A detailed investigation failed to show the cause of this variation. Further experiments were carried out with rat diaphragm muscle, a tissue noted for its sensitivity to insulin. Care was taken to exclude all subjective factors and it was not known by the analyst which flasks contained insulin until the completion of the experiment. Again an inconsistency in the results was noted (Tables 41a, 41b). The first experiment provided evidence that insulin had increased the incorporation of ^{32}P into the ASl fraction but the two later experiments failed to verify this conclusion, although the insulin appeared to be active in these experiments for its presence coincided with an increased uptake of glucose by the diaphragm.

From these findings two general conclusions can be reached.

In the first place, in as far as it has been examined, the metabolism of some cell strains is unaffected by the presence of insulin. This was confirmed by the results of Leslie, Fulton and Sinclair (1956) who found that cultures of HeLa cells showed no greater increase in cell numbers in the presence of insulin than did control cultures. Such an increase was observed with human skin fibroblasts. Secondly, in insulin responsive tissue under the stated experimental conditions, there are certain unspecified factors which may inhibit the normal action of the hormone.

While acknowledging the existence of this variation, it is possible to construct a picture of the action of insulin on cells in vitro. Medium analysis has shown that the hormone causes an increased uptake of glucose, an increased output of lactic acid and a decreased release of pyruvic acid by the cells. It has been suggested that the effect of insulin is directly or indirectly on the level of coenzyme I in the cells. There was no evidence of insulin increasing the rate of fat, protein or nucleic acid anabolism. Consideration of the phosphate metabolism of the cells gave support to previous observations that insulin induces an increased incorporation of ^{32}P into the acid soluble phosphorus and lipid phosphorus fractions of the tissue. A more detailed investigation revealed increased activities in some acid soluble nucleotides, most notably in the adenosine-triphosphate of the cells.

It is interesting to consider these findings in relation to the several mechanisms proposed to explain the action of insulin. The

first of these supposes that the hormone has some action on the cell membrane which facilitates the entry of glucose. Park, Bornstein and Post (1955) showed that insulin added to rat diaphragm *in vitro* led to an accumulation of free glucose within the cells. Further experiments by Park and Johnson (1955) demonstrated a similar *in vivo* effect of the hormone: injected insulin increased the glucose content of the diaphragm and other muscles of the rat. This confirms the earlier work of Levine *et.al.* (1950) who studied the rate of disappearance of certain metabolites from the plasma of eviscerated-nephrectomized dogs. On injection of insulin there was a more rapid fall in the level of plasma glucose and plasma galactose. The preparation was unable to metabolise galactose and the increased disappearance of the sugar from the plasma was thought to be brought about by an enhanced transfer of the free sugar into the cell under the influence of insulin. The work was extended to other sugars and it was noted that all sugars responsive to insulin in this way were characterized by having the same configuration in carbon atoms 1, 2 and 3: for example d-xylose and l-arabinose were responsive whereas d-mannose and d-arabinose were not. (Goldstein *et.al.* 1955). Wick and Drury (1951) have published several papers in support of the experiments of Levine and his co-workers. Of particular interest is their report that in the rabbit the volume distribution of sorbitol between the plasma and the intra-cellular compartment was unaffected by insulin. As glucose and sorbitol differ in structure only at carbon atom 1 this would seem to underline the importance of the terminal

chemical group in determining the response of a sugar to insulin. These experiments lead to the conclusion that insulin acts, at least in the peripheral tissues of some species, to facilitate the transfer of free glucose into the cell.

The second postulate, that insulin has a direct effect on the hexokinase system, sprang from the reports of Price, Cori and Colowick (1945). They noted that in cell free systems prepared from normal rats an anterior pituitary extract had an inhibitory effect on the hexokinase reaction. This inhibition could be removed by the addition of insulin. Several workers attempted to repeat these experiments but failed to confirm the previous findings (Stadie and Haugaard, 1949(. However failure to demonstrate effects of insulin on the hexokinase reaction in cell free extracts does not preclude the possibility that these may occur in intact tissue. Cori (1949) injected eviscerated rats with glucose, fructose and mannose and after a suitable time interval analysed the entire carcass for residual sugar. In the case of fructose and mannose 40% was utilized in an hour and this precentage remained unaltered in the presence of insulin. With glucose, the hormone produced a highly significant increase in utilisation. Cori interpreted these experiments to mean that insulin acts upon gluco-hexokinase and at no other point in the glycolytic scheme. This does not take account of the fact that fructose may be metabolised by the alternative hexose monophosphate shunt pathway. Further experiments designed to support the theory are those of Teng et al.(1952). They measured the synthesis of glycogen from glucose or pyruvate in rat liver slices.

The synthesis from glucose involved the hexokinase reaction whereas the production of glycogen from pyruvate did not. Only the first system was shown to be impaired in diabetic liver. While these experiments indicate some action of insulin at the hexokinase level they do not necessarily implicate this enzyme. These data can all be interpreted in terms of an action of insulin on the glucose transfer system.

The relation of insulin to phosphate metabolism has been considered previously (p.61.) and it was concluded that diabetes is associated with an impaired coupling of oxidation and phosphorylation. It remains to be decided whether this is a resultsof a lowered uptake of glucose by diabetic tissue, or whether the results indicate a further site of insulin action. It is even possible that the effect of insulin on ATP formation may be the primary action of the hormone and the effect on glucose uptake a secondary one dependent on the supply of ATP. Certainly, all the known defects in the metabolism of the diabetic animal could be explained on this basis. There is much conflicting evidence on the subject and it is not feasible to reach any definite conclusions. Krahl (1953) studied the incorporation of glycine-1-^{14}C into glutathione and protein fractions of normal and diabetic rat diaphragms *in vitro*. He showed that the rate of incorporation was lowered in diabetic tissue but could be restored to normal by the addition of glucose alone. Under these conditions the metabolic defect was corrected by increasing the uptake of glucose which does not accord with the theory that the primary defect in

diabetes is in oxidative phosphorylation. However, Haft and Miller (1958) reported that enhanced sugar uptake by isolated rat liver failed to simulate the observed insulin effect on fatty acid synthesis. This points to the involvement of insulin at some site besides the entry of glucose to the cell. There is much evidence to support both points of view, and results have varied according to the tissues studied.

These proposed mechanisms for the action of insulin have emphasized that one of the first effects of the hormone is to stimulate the uptake of glucose by various tissues. At the same time it is important to remember that the secretion of the hormone in the intact animal is primed by a rise in blood glucose. Munro (1956), by feeding carbohydrate to fasting rats, has elicited a release of insulin from the pancreas. In this way he has obtained a system in which the action of the hormone may be studied at physiological levels and in intact animals. A study of protein metabolism under these conditions revealed the temporary accumulation of a peptide in muscle tissue, with a consequent fall in blood amino acids. This effect was not observed in rat liver. In view of these results the inferences drawn from studies of the effects of insulin on liver tissue <u>in vitro</u>, are to be accepted with some reservation.

At the conclusion of a comprehensive review of the subject of insulin action Stadie (1954) says, 'it is impossible at present to espouse one theory of insulin action to the complete exclusion of any other'. More recently Krahl (1957), in a speculative article, has

overcome this difficulty by proposing one theory which would seem to embrace them all. He suggests that insulin combines with lipoprotein present at the cellboundary and in this way initiates 'a sequence of inter-molecular rearrangements which are propagated along the boundary and into the cell interior '. In consequence the entry of glucose to the cell is facilitated, but the structural alterations are such that only certain sugars can be admitted. The rearrangements within the cell bring enzymes into more favourable relations with their substrates which explains the enhanced hexokinase activity and protein and fat synthesis. A third effect is to alter the structure of the mitochondria and so enhance the efficiency of substrate oxidation and energy transport in these particles. It is a difficult theory to prove and Krahl concludes, 'The problem of explaining the action of insulin has thus become a problem in solid state physics',

It can be concluded that the effects produced by insulin added to cell cultures are in general agreement with those produced by the hormone on other systems. This emphasizes the fact that insulin has an action independent of other hormones in the body, although its effect may be modified by them. The findings are consistent with the theory that insulin causes an increased entry of glucose to the cell. They also suggest that the hormone has an effect on the phosphate metabolism of the cell, possibly enhancing the regeneration, if not the synthesis of ATP. In view of these results it is surprising that no increased synthesis of fat, protein or nucleic acid was observed in cultures grown in insulin-containing medium for several days. Taken

on an average the changes produced by insulin in these cultures were slight and withdrawal of this hormone alone would not be sufficient to explain the profound alterations in metabolism observed during trahsition of explants from *in vivo* to *in vitro* conditions. It is possible, however, that cells once adapted to the absence of hormones may have a less sensitive response to them.

SUMMARY.

Summary.

1. 15-day chick embryonic heart and liver explants were cultured *in vitro* for periods up to six days. Metabolic measurements were made at various time intervals.

2. Initially heart explants produced more lactate than could be accounted for by carbohydrate available in the medium and tissue. At later time intervals, when the medium was partially depleted of glucose, lactate and pyruvate were utilised by the heart tissue.

3. Immediately after explantation liver tissue utilised lactate with the production of hexose and pyruvate. Within twenty four hours this metabolic pattern was reversed and lactate was then synthesised at the expense of available hexose.

4. Analysis of the heart tissue after six days *in vitro* showed that the lipid content had increased during this period. The amount of protein nitrogen remained fairly constant but there was a decrease in the deoxyribonucleic acid phosphorus and the carbohydrate content of the tissue.

5. Analysis of the liver tissue after six days growth *in vitro* showed that there was a gradual decline in the amounts of all tissue components.

6. The specific activity of the acid soluble phosphorus and the lipid phosphorus fractions of explanted chick heart decreased during the first twenty-four hours *in vitro*. On renewal of the medium there was a sharp rise in activity, often to a level in excess of the initial one. The specific activity of the deoxyribonucleic acid phosphorus fraction increased after explantation but later declined.

7. The specific activity of the acid soluble phosphorus fraction of explanted chick liver remained fairly constant until feeding at 48 hours. All other fractions showed a fall in activity during this period. When the medium was renewed there was evidence of increased activity in all fractions: the specific activity of the acid soluble fraction rose to a value in excess of the initial one.

8. The oxygen consumption of newly explanted chick heart was initially very low. During the following forty-eight hours it increased progressively. On renewal of the medium respiration decreased significantly, but did not return to the original low level

9. Newly explanted liver tissue had a relatively high rate of oxygen consumption which declined during the following forty-eight hours. Renewal of the medium had no effect on oxygen uptake by this tissue.

10. Some possible reasons for these changes in the pattern of metabolism of chick embryonic tissue on transfer from <u>in vivo</u> to <u>in vitro</u> conditions were discussed.

11. Further studies considered the effects of insulin on the metabolism of cell cultures.

12. Insulin, added to the culture medium of chick carcass cells or strain L-cells, increased the uptake of glucose from the medium. This was accompanied by an increased production of lactic acid and a decreased production of pyruvic acid by the cells. The possibility that insulin altered the balance between lactic and pyruvic acids by an effect on the DPN level in the cells was discussed. There

was no increased oxidation of pyruvic acid.

13. Insulin added to the culture medium of chick carcass cells, strain L-cells or a strain of human buffy coat cells was without effect on the rate of synthesis of tissue components during five days growth.

14. Studies with radioactive phosphorus showed that, under certain conditions, insulin increased the rate of incorporation of ^{32}P into the acid soluble phosphorus and lipid phosphorus fractions of strain L-cells and chick carcass cells. The effect could not be reproduced with certainty.

15. An investigation into the variation in results showed that it could not be explained by alterations in the preparation of insulin, the glucose concentration, the potassium concentration, the phosphorus concentration, the pH of the medium or the growth phase of the cells.

16. A similar inconsistency was observed in studies on the effect of insulin on the incorporation of radioactive phosphorus into rat diaphragm in vitro.

17. A short study was made of the effect of varying concentrations of insulin on the metabolism of brown adipose tissue from rats.

18. All these findings were considered in relation to the current theories of insulin action.

ACKNOWLEDGMENTS.

Acknowledgements.

The author wishes to express her appreciation to Dr. J. Paul for his guidance in this work and to Professor J.N. Davidson for making facilities available to her within the Biochemistry Department. She is also greatly indebted to the technical staff of the Tissue Culture Laboratory. The work was made possible by the financial support of the Scottish Hospital Endowments Research Trust.

BIBLIOGRAPHY.

Bibliography.

Berenblum, I., Chain, E., and Heatley, N.G. (1939). Biochem, J. 33, 68.

Bloch, K., and Kramer, W. (1948). J. biol. Chem., 173, 811.

Bloor, W.R. (1947). J. biol. Chem., 170, 671.

Burrows, M.T. (1910). J. Amer. med. Ass., 55, 2057.

Carrel, A., and Burrows, M.T. (1910). J. Amer. med. Ass., 55, 1379.

Carrel, A., (1912) J. exp. Med., 15, 516.

Carrel, A., (1913, a). J. exp. Med., 17, 14.

Carrel, A., (1913, b). J. exp. Med., 18, 287.

Carrel, A., and Baker, H.E. (1926). J. exp. Med., 44, 503.

Carrel, A., (1928). Arch. exp. Zellforsch., 6, 70.

Ceriotti, G., (1952). J. biol. Chem., 198, 297.

Ceriotti, G., (1955). J. biol. Chem., 214, 59.

Charalampous, F.C., and Hegsted, D.M. (1949). J. biol. Chem., 180, 623.

Chen, J.M. (1954, a). Exp. Cell Res., 7, 518.

Chen, J.M. (1954,b). J. Physiol., 125, 148.

Cori, C.F. (1949). First Internat. Congr. Biochem., 9, 25.

Danes, B.S. (1955). Exp. Cell Res., 8, 543.

Davidson, J.N., Leslie, I., and Waymouth, C., (1949). Biochem. J. 44, 5.

Davidson, J.N. and Leslie, I., (1950). Nature, Lond., 165, 49.

DeMars, R., (1958). Biochim. biophys. acta, 27, 435.

Dische, S., (1955). J. clin. Path., 8, 253.

Eagle, H., (1955, a). J. biol. Chem., 214, 839.

Eagle, H., (1955, b). J. exp. Med., 102, 37.

Eagle, H., (1955, c). J. exp. Med., 102, 595.

Eagle, H., (1956, a). Proc. Soc. exp. Biol., N.Y., 91, 358.

Eagle, H., (1956, b). Arch. Biochem. Biophys., 61, 356.

Eagle, H., (1958). J. biol. Chem., 233, 551.

Earle, W.R. (1943). J. nat. Cancer Inst., 4, 165.

Earle, W.R. Evans, V.J., and Schilling, E.L. (1950). J. nat. Cancer Inst., 10, 943.

Earle, W.R., Schilling, E.L., Bryant, J.C., and Evans, V.J., (1954). J. nat. Cancer Inst., 14, 1159.

Ebeling, A.H., (1921). J. exp. Med., 34, 231.

Ebeling, A.H., (1924). C.R. Soc. Biol., Paris, 90, 31.

Ebeling, A.H., (1925). J. exp. Med., 41, 337.

Ehrensvard, G., Fischer, A., and Stjerholm, R., (1949). Acta physiol. scand., 18, 218.

Evans, V.J., and Earle, W.R., (1947). J. nat. Cancer Inst., 8, 103.

Evans, V.J., Bryant, J.C., McQuilken, W.T., Fioramonti, M.C., Sanford, K.K., Westfall, B.B., and Earle, W.R., (1956). Cancer Res., 16, 87.

Fell, H.B., and Robinson, R., (1929). Biochem. J. 23, 767.

Fell, H.B., and Mellanby, E., (1952). J. Physiol. 116, 320.

Fell, H.B., and Mellanby, E., (1953). J. Physiol. 119, 470.

Fischer, A., (1922, a). J. exp. Med., 36, 379.

Fischer, A., (1922, b). J. exp. Med., 35, 367.

Friedheim, A.H., and Roukhelman, N., (1930). C.R. Soc. Biol., Paris, 103, 10.

Gaillard, P.J., (1955). Exp. Cell Res., 3, 154.

Gey, G.O., and Thalhimer, W., (1924). J. Amer. med. Ass., 82, 1609.

Gill, P.M., (1938). Biochem. J. 32, 1792.

Glock, G.E., and McLean, P., (1955). Biochem. J. 61, 381.

Goldstein, M.S., Henry, W.L., Huddlestun, B., and Levine, R., (1953). Amer. J. Physiol. 173, 207.

Goodman, J., and Rothstein, A., (1957). J. gen. Physiol. 40, 915.

Goranson, E.S., and Erulkar, S.D. (1949). Arch. Biochem. 24, 40.

Griswold, B.L., Numoller, F.L., and McIntyre, A.R., (1951). Anal.Chem., 23, 192.

Grobstein, C., (1953). J. exp. Zool., 124,383.

Grobstein, C., (1958). J. nat. Cancer Inst., 20, 107.

von Haam, E., and Cappel, L., (1940, a). Amer. J. Cancer, 39, 350.

von Haam, E., and Cappel, L., (1940, b). Amer. J. Cancer, 39, 354.

Haft, D.E., and Miller, L.L., (1958). Amer. J. Physiol., 193, 469.

Hanks, J.H., (1948). J. cell. comp. Physiol., 31, 235.

Harris, M., and Kutsky, P.B., (1953). J. cell. comp. Physiol., 42, 449.

Harris, M., (1954). J. exp. Zool., 125, 85.

Harris, M., and Kutsky, R.J., (1954). Exp. Cell Res., 6, 327.

Harrison, R.G., (1907). Proc. Soc. exp. Biol., N.Y., 4, 140.

Harrison, R.G., (1928). Arch. exp. Zellforsch., 6, 4.

Hastings, A.B., Renold, A.E., and Teng, C.T., (1955). Rec. Prog. Hormone Res., 11, 381.

Haugaard, N., Marsh, J.B., and Stadie, N.C., (1951). J. biol. Chem., 189, 59.

Healy, G.M., Fisher, D.C., and Parker, R.C., (1954). Canad. J. Biochem. Physiol, 32, 319.

Hull, W., and Kirk, P.L., (1950). J. gen. Physiol., 33, 327.

Hullin, R.P., and Noble, R.L., (1953). Biochem., J. 55, 289.

Jones, M., and Bonting, S.L., (1956). Exp. Cell Res., 10, 631.

Jones, M., Featherstone, R.M., and Bonting, S.L., (1956). J. Pharmacol., 116, 114.

Kaplan, N.O., and Greenberg, D.M., (1944, a). J. biol. Chem., 156, 525.

Kaplan, N.O., and Greenberg, D.M., (1944,b). J. biol. Chem., 156, 553.

Krahl, M.E., (1953). J. biol. Chem., 200, 99.

Krahl, M.E., (1957). Perspectives Biol. Med., 1, 69.

Krebs, H.A., and Eggleston, L.V., (1938). Biochem. J., 32, 913.

Krebs, H.A., and Hems, R., (1953). Biochim. biophys. acta, 12, 172.

Lasnitski, I., (1954). Cancer Res., 14, 632.

Lasnitski, I., (1955,a). Exp. Cell Res., 8, 121.

Lasnitski, I., (1955,b). J. Endocr., 12, 236.

Latta, J.S., and Bucholz, D.J., (1939). Arch. exp. Zell forsch., 23,146.

Leighton, J., (1951). J. nat. Cancer Inst., 12, 545.

Leighton, J., (1954). J. nat. Cancer Inst., 15, 275.

Leslie, I., and Davidson, J.N., (1951). Biochem. J., 49, xli.

Leslie, I., and Paul, J., (1954). J. Endocr., 11, 110.

Leslie, I., Fulton, W.C., and Sinclair, R., (1956). Biochem. J. 63, xviii.

Levine, R., Goldstein, M.S., Huddlestun, B., and Klein, S., (1950). Amer. J. Physiol., 163, 70.

Liebermann, I., and Ove, P., (1957). Biochim. biophys. acta, 25, 449.

Lipmann, F., (1932). Biochem. Z., 244, 177.

Lowell, F.C., (1944). J. clin. Invest., 23, 233.

Lu, K.H., and Winnick, T., (1954). Exp. Cell Res., 7, 238.

McIlwain, H., and Buddle, H.L., (1953). Biochem. J., 53, 412.

Maitland, M.C., and Maitland, H.B., (1933). Biol. Abstr., 7, no.1304.

Metzger, J.F., Fusillo, M.H., Cornman, I., and Kuhns, D.M., (1954). Exp. Cell Res., 6, 337.

Morgan, J.F., Morton, H.J., and Parker, R.C., (1950). Proc. Soc. exp. Biol., N.Y. 73, 1.

Morgan, J.F., and Morton, H.J., (1957, a). J. biophys. biochem. Cytol., 3, 141.

Morgan, J.F., and Morton, H.J., (1957, b). Canad. J. Biochem. Physiol., 35, 785.

Morgan, J.F., (1958). Bact. Rev., 22, 20.

Morton, H.J., Morgan, J.F., and Parker, R.C., (1950). Proc. Soc. exp. Biol., N.Y., 74, 22.

Morton, H.J., Pasieka, A.E., and Morgan, J.F., (1956). J. biophys. biochem. Cytol., 2, 589.

Moscona, A., (1952). Exp. Cell Res., 3, 535.

Munro, H.N., (1956). Scot. med. J., 1, 285.

Niu, M.C., and Twitty, V.C., (1953). Proc. nat. Acd. Sci., Wash., 39, 985.

Niu, M.C., (1956). In, "Cellular Mechanisms in Differentation, and Growth", p. 155. Ed. by Rudnick, D. Princeton: Princeton Univ. Press.

Osborn, M.J., Chaikoff, I.L., and Felts, J.M., (1951). J. biol. Chem., 193, 549.

Owens, O. von H., Gey, M.K., and Gey, G.O., (1953). Proc. Amer. Ass. Cancer Res., 1, 41.

Park, C.R., Bornstein, J., and Post, R.L., (1955). Amer. J. Physiol., 182, 12.

Park, C.R. and Johnson, L.H., (1955). Amer. J. Physiol. 182, 17.

Parker, R.C., Morgan, J.F., and Morton, H.J., (1951). Proc. Soc. exp. Biol., N.Y.., 76, 444.

Paul, J., and Pearson, E.S., (1957, a). Exp. Cell Res., 12, 212.

Paul, J., and Pearson, E.S., (1957, b). Exp. Cell Res., 12, 223.

Paul, J., (1958). The Analyst, 83, 37.

Perry, V.P., (1956). Amer. J. Hyg., 63, 52.

Phillips, H.J., and Feldhaus, R.J., (1956). Proc. Soc. exp. Biol., N.Y., 92, 478.

Price, W.H., Cori, C.F., and Colowick, S.P., (1945). J. biol. Chem., 160, 633.

Puck, T.T., Cieciura, S.J., and Fisher, H.W., (1956). J. exp. Med., 106, 145.

Puck, T.T. Marcus, P.I., and Cieciura, S.J., (1956). J. exp. Med., 103, 273.

Rice, L., and Evans, E.A., Jr., (1943). Science, 97, 470.

Roffo, A.H., (1929). Buenos Aires Inst., med. exper. etud. trat. cancer Bol., 6, 24.

Sacks, J., (1945). Amer. J. Physiol., 143, 157.

Sacks, J., and Sinex, F.M., (1953). Amer. J. Physiol., 175, 353.

Sanford, K.K., Earle, W.R., and Likely, G.D., (1948). J. nat. Cancer Inst., 9, 229.

Sanford, K.K., Waltz, H.K., Shannon, J.E., Jr., Earle, W.R., and Evans, V.J., (1952). J. nat. Cancer Inst., 13, 121.

Schmidt, G., and Thannhauser, S.J., (1948). J. biol. Chem., 161, 83.

Schneider, W.C., (1945). J. biol. Chem., 161, 293.

Shaffer, B.M., (1956). Exp. Cell Res., 11, 244.

Shorr, E., and Baker, S.B., (1939). Biochem. J., 33, 1798.

Sidman, R.L., (1956, a). Anat. Rec., 124, 581.

Sidman, R.L., (1956, b). Anat. Rec., 124, 723.

Stadie, W.C., and Haugaard, N., (1949). J. biol. Chem., 177, 311.

Stadie, W.C., (1954). Physiol. Rev., 34, 52.

Stetten, D., Jr., and Boxer, G.E., (1944). J. biol. Chem., 156, 271.

Tong, C., Sinex, F.M., Deane, H.W., and Hastings, A.B., (1952). J. cell comp. Physiol., 39, 73.

Thomson, R.Y. Paul, J., and Davidson, J.N., (1956). Biochim, biophys. acta, 22, 581.

Trevelyan, W.E., and Harrison, J.S., (1952). Biochem, J., 50, 298.

Trowell, O.A., (1954). Exp. Cell Res., 6, 246.

Tyrode, M.V. (1910). Arch. int. Pharmacodyn, 20, 205.

Vallance - Owen, J., Hurlock, B., and Please, N.W., (1955). Lancet, 2, 583.

Verne, J., and Verne-Soubiran, A., (1939). C.R. Soc. Biol. Paris, 130, 1232.

Villee, C.A., and Hastings, A.B., (1949, a). J. biol. Chem., 179, 673.

Villee, C.A., and Hastings, A.B., (1949, b). J. biol.Chem., 181, 131.

Warburg, O., and Kubowitz, F., (1927). Biochem, Z., 189, 242.

Waymouth, C., (1955). Texas Rep. Biol. Med., 13, 522.

Waymouth, C., (1956). J. nat. Cancer Inst., 17, 305.

Wick, A.N., and Drury, D.R., (1951). Amer. J. Physiol., 166, 421.

Willmer, E.N., (1942). J. exp. Biol., 18, 237.

Wilson, H., Jackson, E.B., and Bruesm A.M., (1942). J. gen. Physiol. 25, 689.

APPENDIX.

BALANCED SALT SOLUTIONS.

Substance. (g. per litre).	Tyrode (1910)	Earle (1934)	Hanks (1946)
NaCl	8.00	6.80	8.00
KCl	0.20	0.40	0.40
$CaCl_2$	0.20	0.20	0.14
$MgSO_4.7H_2O$		0.20	0.10
$MgCl_2.6H_2O$	0.10		0.10
$NaH_2PO_4.H_2O$	0.05	0.125	
$Na_2HPO_4.2H_2O$			0.06
KH_2PO_4			0.06
Glucose	1.00	1.00	2.00
Phenol red			0.02
$NaHCO_3$	1.00	2.20	0.35

Preparation.

Double glass-distilled water was used. The calcium chloride was dissolved in 100 ml. water then stirred into the solution prepared by dissolving the other salts, by groups, in approximately 800 ml. water. The final volume was 1000 ml. The solutions were sterilized in the autoclave by maintaining 15 lb. pressure for 20 minutes. Before use the appropriate amount of sodium bicarbonate was added and Earle's solution was equilibrated with a 5% carbon dioxide / air mixture. When BSS was being used for the preparation of Eagle's medium, glucose was omitted from it.

Phosphorus free saline (isotonic salt solution).

Substance	Concentration (g. per litre).
NaCl	8.00
KCl	0.40
$CaCl_2$	0.2
$MgSO_4 \cdot 7H_2O$	0.2

This solution was not used for cell growth but only for washing the cells at the completion of an experiment. It was prepared in ordinary distilled water.

Calcium and Magnesium free saline.

Substance	Concentration (g. per litre).
NaCl	8.00
KCl	0.20
$NaH_2PO_4 \cdot H_2O$	0.005
$NaHCO_3$	1.00
Glucose	2.00

The salts were dissolved in a litre of double glass distilled water.

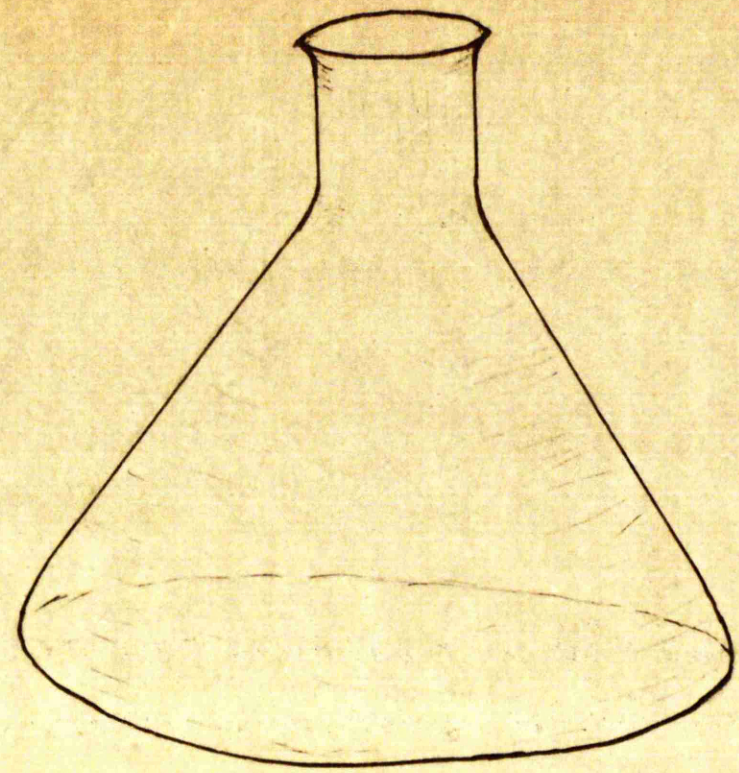
Eagle's medium.

All solutions (except No.2) were made up in Hanks BSS without glucose.

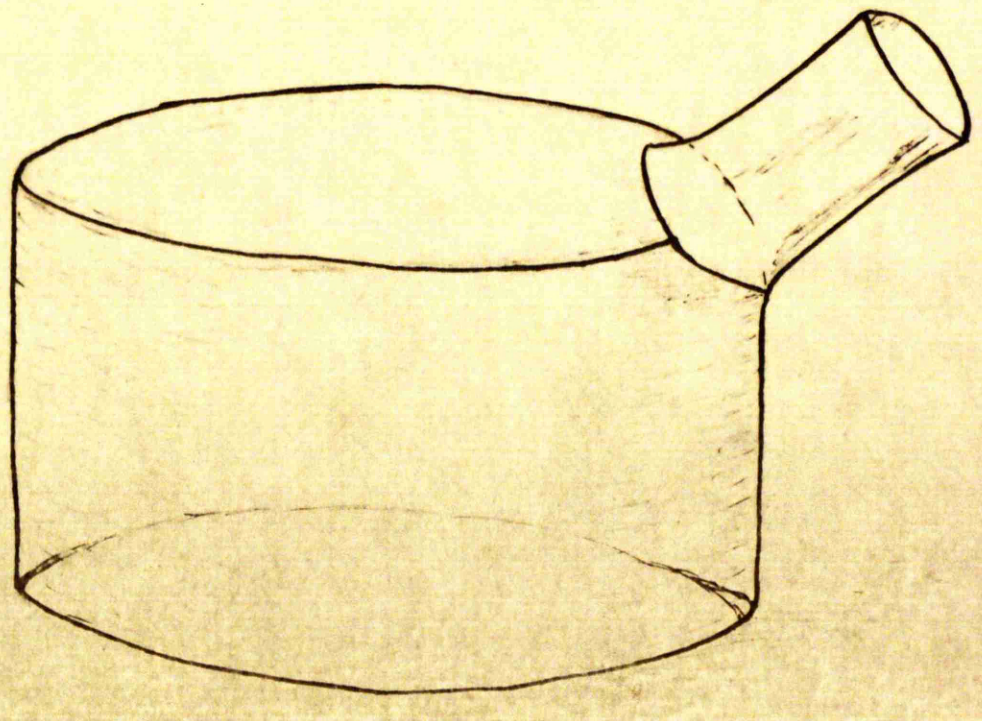
Solution 1.		Solution 2.		Solution 3.		Solution 4.		Soln 5	Soln 6.
Amino Acid	Conc.(mM)	Amino Acid	Conc.(mM)	Vitamin	Conc.(g./100ml	Vitamin	Conc.	Glucose	Glutamine
Arginine	0.1	Tyrosine	0.1	Choline	10^{-4}	Biotin	10^{-4}	20g./100 ml	1mM
Histidine	0.02	Cystine	0.05	Nicotinamide	10^{-4}	Folic Acid	10^{-4}		
Lysine	0.1			Pantothenic acid	10^{-4}				
Leucine	0.1			Pyridoxal	10^{-4}				
Iso-leucine	0.2			Riboflavin	10^{-5}				
Methionine	0.05			Thiamin	10^{-4}				
Phenyl-alanine	0.05								
Threonine	0.1								
Tryptophan	0.01								
Valine	0.1								
wt. x 100 dissolved in 100 ml. BSS.		wt. x 100 dissolved in 100 ml. 0.1 NHCl		wt. x 1000 dissolved in 100 ml. BSS. Dilute $\frac{1}{10}$ for use.		wt. x 100 dissolved in 100 ml BSS		prepared in BSS	wt x 100 dissolved in 100 ml BSS

To prepare 100 ml. Eagle's medium 1 ml. of each of the six solutions was added to 20 ml. serum and 74 ml. BSS without glucose. Antibiotics and bicarbonate were added as desired.

A large conical flask and a penicillin flask are shown opposite. They are used for routine cell cultures. The drawings are a half full size.

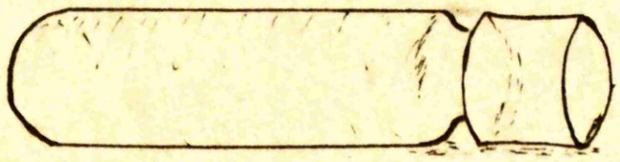


LARGE CONICAL FLASK.

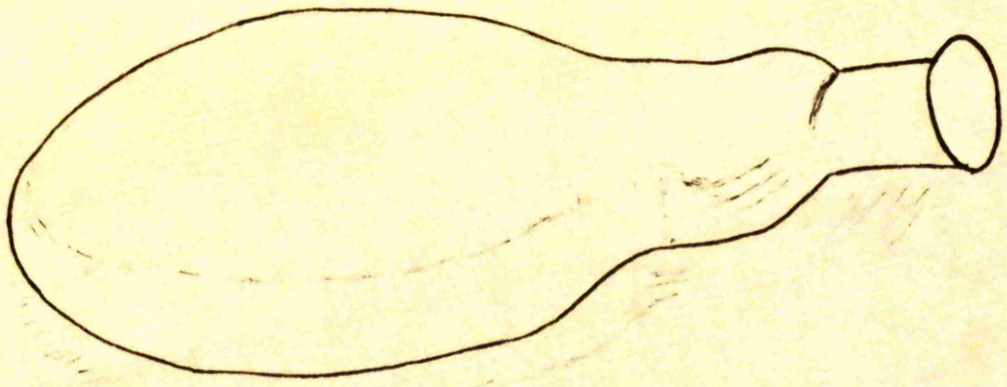


PENICILLIN FLASK.

A roller tube and a Kolle flask used in experimental work are shown opposite. The drawings are half full size.



ROLLER TUBE.



KOLLE FLASK.