THE MOTOR ACTIVITY OF THE SMALL INTESTINE AND ITS RELATION TO ACETYLCHOLINE,

with additional Papers on

THE ACTION OF UREA, ATROPINE AND ESERINE ON THE SMALL INTESTINE.

By

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INTRODUCTION.

Within recent years considerable interest has centred around the transmission of nerve impulses from nerve endings to effector organs by chemical substances. Among such, acetylcholine, as the chemical mediator of the parasympathetic system has received most attention. Recent work has indicated that in addition to this important rôle acetylcholine plays a part in the regulation of the tone and rhythmicity of the visceral muscle of the gastro-intestinal tract.

The present research is an attempt to show that acetylcholine is constantly formed in the tissues of the small intestine; that it exerts a powerful excito-motor effect on the visceral muscle of the intestine at the site of its formation, and that it is then hydrolysed, by the choline-esterase, into choline and acetic anhydride. I have also been able to demonstrate that atropine, eserine and urea influence this acetylcholine mechanism and so alter the motor activity of the bowel.

Before describing my own experimental work, I propose to present a review of the literature on the subject, which may be conveniently classified under the following seven heads:-

A. The methods of examining the movements of the small intestine and the influence of environmental conditions upon them.

B. Choline and its relation to intestinal motor activity.

C. The destruction of acetylcholine and the choline esterase.

D. The action of eserine and other substances on choline esterase activity.

E. The biological identification and assay of acetylcholine.

F. Acetylcholine: its history and general biological significance.

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HISTORICAL.

A. METHODS OF EXAMINING THE MOVEMENTS OF THE SMALL INTESTINE AND THE INFLUENCE OF THE ENVIRONMENTAL CONDITIONS UPON THEM.

The small intestine of man and mammals exhibits three types of movements, segmental movements, pendular and peristaltic movements. Various methods have been elaborated to make possible the observation of these movements. van Braam Houchgeest (1872) observed the intestinal movements directly. He immersed the body of an animal, excluding its head, in a bath containing a physiological saline solution warmed to body temperature; he then opened the abdomen by cutting through the linea alba and observed the intestinal movements.

Cannon (1902) studied the movement of the intestine by the Röntgen rays. The animal was given a meal containing bismuth subnitrate; the intestinal contents were thus rendered opaque to the rays and the movement could be observed in the intact animal.

The influence of the nervous system on the intestinal movement has been studied closely. Bayliss and Starling (1901) noted, in the rabbit, that the writhing pendular movements and true peristalsis were present after the animals had been pithed and both the vagus and splanchnic nerves had been divided. Cannon (1906) divided both the vagus and splanchnic nerves in a cat. He noted, after the preliminary shock of the operation had passed off, that although the transit of food through the intestine was slower than in the normal animal, yet the rhythmical segmentation of the intestinal contents was present and active. It was

concluded by these and other observers that the intestinal movements can occur after all the extrinsic nerves to the intestine have been severed.

Magnus (1904, a) elaborated a new method for the examination of intestinal movements. He found that a segment of bowel removed from an animal immediately after death, and suspended in a physiological fluid, will exhibit rhythmic contractions and real peristalsis. This work elicited and emphasised the all important fact that the gastro-intestinal tract is largely autonomous. Magnus (1904, b) further demonstrated that the rhythmic movements will continue in a segment of intestine after both the mucosa and serous membrane had been removed. He concluded, therefore, that the rhythmic movements were not dependent on afferent stimuli arising in the sense organs of these structures.

The movements are due then to the inherent property of the visceral muscle of the intestine, or the nerve cells and fibres which lie in contact with it. This final question is still an open one, although it is generally accepted that the rhythmical movements are myogenic, that is, a property of the visceral muscles itself (Alvarez, 1922 and 1928).

The environmental conditions affecting the segmental movements have received a considerable amount of attention. Evans and Underhill (1923) and McSwiney and Newton (1927) studied the effects of changes of the hydrogen ion concentration of the bath fluid on the rhythmical activity of smooth intestinal muscle.

They found that a pH of 7.2 was the optimum and that, within a narrow range, a decrease of the hydrogen ion concentration caused an increased tone and amplitude of movement. An increase in the hydrogen ion concentration caused a reduction of tone, frequency and amplitude. A sustained contracture resulted if a large decrease or increase was effected.

The need of the tissues exhibiting tone and undergoing rhythmical contractions, of oxygen was emphasised by Garry (1928).

Prasad (1935, a and b), studied the carbohydrate metabolism of intestinal muscle. He demonstrated that the visceral muscle has only a small store of readily available carbohydrate (0.25%). This he did by poisoning an intestinal segment with iodoacetic acid, under aerobic and anaerobic conditions. He also showed that the asphyxial arrest which can be induced is not due to the accumulation of lactic acid, but to the exhaustion of the labile store of carbohydrate.

Apart from such extraneous factors, there exists in the tissues of the bowel itself certain substances which influence the motor activity of the bowel. The similarity of action of certain tissue extracts and known chemical substances, has led to the isolation of these chemicals in the tissues. Certain such chemical substances contained in the tissues of various organs exert a powerful influence on the organ of which they form a part. This process has been termed "Autopharmacology." A general review of this subject is given by Dale (1933).

The research which I have conducted is an attempt to

study one aspect of the autopharmacology of the intestine, and to assess its influence on the motor activity of the intestine. In doing so, I have not prepared tissue extracts of the bowel tissues, by chemical means. I have attempted rather to study, and identify substances formed by the living intestinal segment during its motor activity. The environmental conditions affecting the bowel segment were kept as near the physiological ideal as the circumstances of the experiment would allow. By this method it has been possible to show that the isolated bowel of rabbits and other animals liberates an acetylcholine like substance into the bath fluid which bathes it.

B. CHOLINE AND ITS RELATION TO INTESTINAL MOTOR ACTIVITY.

In 1908 Magnus suggested that a chemical substance mediated the control of intestinal motor activity. At that time there was no direct evidence in support of this suggestion.

Wieland (1912) conducted experiments which awakened interest in this subject. He noted that the oxygenated mammalian Ringer solution in which an isolated segment of rabbit's jejunum had been suspended contained a substance which had a stimulating action on another segment of bowel suspended in a similar manner. He concluded that a substance had diffused through the serosa of the isolated bowel and that this substance had an excito-motor effect on the bowel. He referred to the substance as a biodialysate, and noted that the active constituent was soluble in alcohol, not destroyed by boiling and that its action was antagonised by atropine.

Le Heux (1918) working in the laboratory of Professor Magnus at Utrecht undertook the further identification and isolation of the biodialysate. He succeeded in isolating the active factor of the dialysate as pure crystalline choline. The isolated substance was tested biologically. It was found to yield the same reactions, qualitatively and quantitatively, as did choline on the isolated intestine of rabbits, the isolated frog's heart and the mammalian blood pressure preparation. He also demonstrated that if the active residue was treated with glacial acetic acid a great increase in its pharmacological activity resulted. It had been shown by Hunt and Traveau (1911) that the action of

choline is similarly activated, due to the formation of acetylcholine, which is much more powerful than choline. Le Heux was able in several instances to isolate from a dialysate at least 75% of the original activity, as pure crystalline choline. He found that 3-4 mg. choline diffused into the bath fluid in one hour if the whole small intestine of a rabbit was immersed in it.

It appeared from these experiments that choline was the chief excito-motor substance present in the bath fluid, and that the choline was present in a free diffusible state in the tissues of the bowel wall. Choline is a powerful stimulant of the visceral muscle of the bowel wall and it is present in the bowel in concentrations sufficient to stimulate the excitable structures of the intestine. Le Heux, therefore, concluded that choline provided one of the conditions for the automatic intestinal movements and referred to choline "als Hormon der Darmbewegungen."

The source of the intestinal choline is not known. That it is not formed by post-mortem decomposition of the mucous membrane was shown by Sawasaki (1925). He demonstrated that the intestinal muscle taken from the living narcotised animal, without injuring the mucosa and submucosa, contained the same amount of free choline as did a complete segment. The quantity of choline was not diminished by starvation and therefore was not derived from the food.

The amount of free choline present in the intestine is kept remarkably constant. Arai(1922,b)noted that after long chloroform anaesthesia, laparotomy, peritonitis and diarrhoea the choline content of the small intestine was unchanged. Under

experimental conditions, however, the amount of choline can be reduced by repeatedly draining away the fluid in which the intestinal segment is suspended. Girndt (1925) has shown that if this be done the surviving intestine is unable to regenerate the choline.

Following on the basic work showing the relation between choline and intestinal motor activity further investigations were carried out to determine what use could be made of this substance as a therapeutic agent. Le Heux(1921,a) found that the intravenous injection of choline to a normal animal resulted in increased pendular movements and peristalsis of the small intestine. Although the movement of this and other regions of the gastro-intestinal tract were increased yet their character was unchanged, no spasms or abnormal movements being produced.

Von Kuhlewein (1921) showed that the gastro-intestinal paralysis which results after prolonged chloroform anaesthesia in the cat could be alleviated by the intravenous injection of 15 mg. choline hydrochloride per kg. of weight. Similarly Arai (1922,a) noted that the intestinal paresis produced by a laparotomy and handling of the bowels in a cat could be prevented by the intravenous injection of choline. In all his experiments there was defaecation within 24-36 hours when choline was given whereas this did not occur in the control animals.

The therapeutic application of choline to man was next attempted. Klee and Grossman (1925) found that a dose of 10 mg. per kg. intravenously was active and innocuous to a human subject provided that it were given slowly 0.6 mg. per kg. per minute.

They obtained favourable results with this method in the treatment of paralytic ileus.

Wolf and Canney (1926) also report favourable results with this method of treatment in cases of paralytic ileus. Despite these and other favourable reports choline has not been extensively used as an excito-motor agent of the gastro-intestinal tract. This is in part due to the difficulty in preparing and maintaining a stable preparation of the substance.

In 1925 Abderhalden and Paffrath confirmed Le Heux's finding that choline is present in the bath fluid in which an isolated segment of intestine is suspended. They also studied the rate of appearance of the choline. They showed that a segment of bowel, which was filled with Tyrode Bayliss solution and was thus rendered very active, yielded in the first hour three to four times the amount of choline yielded by a non active segment of equal weight. The bath fluid containing the active segment continued to yield the greater amount of choline during the first 3-4 hours of the experiment. Later however the fluid obtained from the bath containing the less active segment contained the greater amount. The unfilled segment yielded its largest quantities between 5-8 hours, that is, when the tissues were approaching death. The total amounts obtained from the active and non active segments were the same. They thus showed that the activity of the intestinal muscle was related to the amount of choline which could be demonstrated in the bath fluid containing it.

These observers were able to demonstrate that a segment of intestinal muscle, stripped from the nerve elements of Auerbach's

plexus, also yielded choline to the bath fluid and that the rate of appearance resembled that of the ordinary segment of bowel in that the maximum yield was obtained between the 5-8 hours. They concluded that although the nerve elements of Auerbach's plexus were not responsible for the appearance of the choline they nevertheless regulated the amount given off at any time.

The further investigation of this subject focussed attention on the highly active esters of choline and their possible relationship to intestinal motor activity.

Rona and Neukirch (1912) investigated the action of a large number of organic substances on the isolated intestine. The great majority of these substances were found to be inactive, or to have only a slight effect on the motor activity. Two sugars (d-glucose and d-mannose) and the sodium salts of some aliphatic acids, especially acetic and pyruvic acids, were found to be active.

Le Heux(1921,b)was struck by the fact that of the sodium salts of all the aliphatic acids those of acetic and pyruvic were the most active. He had then recently demonstrated the presence of choline in a free diffusible state in the bowel wall, and he considered the possibility that these salts acted through the formation of the active acetic and pyruvic esters of choline. Acetylcholine was known to be 1,000 to 10,000 times as active as choline and Le Heux showed that pyruvylcholine was 100 times as active. The relative activity of the sodium salts of these two acids in equal molecular concentrations corresponded with the relative excito-motor activity of equal molecular concentrations of the corresponding choline esters. The sodium salt of succinnic

acid was without any activity on the bowel and Le Heux showed that succinnylcholine stimulated the bowel a little less than an equal weight of choline. The sodium salt of succinnic acid could not therefore be expected to have an excito-motor effect on a segment of bowel which is under the influence of choline. He also showed that if the choline was removed from the bowel segment by repeated washing then the activity of the organic salts was reduced or absent. He concluded from these experiments that the isolated bowel was able to synthesise active choline esters under these conditions.

Other workers have supported Le Heux's original work in this subject: their work will be referred to later. It may be stated here that the demonstration of the presence of acetylcholine in the bowel in the intact animal and in the isolated segment did not follow for some considerable time because the body tissues and fluids contain an enzyme which catalyses the hydrolysis of acetylcholine. The hydrolysis of acetylcholine and other choline esters was studied in detail after Loewi (1921) put forward his classical theory that an acetylcholine-like substance mediated the transmission of parasympathetic nerve impulses. It was then confirmed that the hydrolysis of acetylcholine was catalysed by an enzyme and that this enzyme activity was inhibited by eserine sulphate and other closely related substances. Eserine sulphate has been extensively used in this respect.

C. THE DESTRUCTION OF ACETYLCHOLINE AND THE CHOLINE ESTERASE.

Dale (1914, b) emphasised the evanescent nature of the effects produced by the intravenous injection of acetylcholine. He suggested at that time that this was due to its hydrolysis into the less active choline and acetic anhydride, and pointed out that the extreme rapidity with which this occurred in alkaline solutions at room temperature. In the blood he thought that an esterase contributed to the rapid removal of acetylcholine.

Loewi (1921 and 1924) published his classical papers on the transmission of the effects of vagal stimulation from one frog's heart to another by Ringer's fluid which was perfused from the donor to the recipient preparation. He postulated the presence of a 'vagus stoffe' which he said had been formed during the vagal stimulation and which had been carried to the second heart preparation and reproduced the vagal effects.

These experiments aroused great interest and the work was repeated and amplified by numerous observers. Uniform confirmation however did not result. Bain (1932) was able to repeat the experiment successfully. He used a modified Kroenecker canula by means of which the fluid from the donor heart was rapidly transferred to the recipient preparation. He thus emphasised the rapid destruction of the 'vagus stoffe' and suggested that the failure of some workers to repeat Lowei's experiment successfully was due to the fact that they used a slow method of perfusion and that the activity of the mediator was destroyed during its transference.

Loewi and Navratil(1926,a) compared the mechanism of the

destruction of acetylcholine and the 'vagus stoffe' activity. They showed that there is in the frog's heart a mechanism which rapidly destroys both the 'vagus stoffe' and acetylcholine at closely the same rate. Witanowski (1925) had previously observed that both acetylcholine and the 'vagus stoffe' were rapidly hydrolysed by watery extracts of the frog's heart, especially if these were alkalinised. The similarity in the destruction of these two substances was taken as evidence that they were closely related bodies.

Loewi and Navratil showed that the hydrolysing activity of these aqueous extracts of frogs' hearts was thermolabile, heating to 56°C. destroying its activity on both acetylcholine and the 'vagus stoffe.' They also noted that, as mentioned heretofore, the activity is inhibited by eserine and by ergotamine in high concentrations.

Clark (1927) confirmed these results; he also noted that the serum obtained from the blood of the frog had as powerful an action in destroying acetylcholine as the frog's heart (0.001 c.c. serum per minute and 0.1 mgm. moist ventricle per minute destroyed approximately equal amounts). Clark concluded that the destruction of acetylcholine was due to an enzyme hydrolysis. He noted that the enzyme was widely distributed in the frog's tissues and not confined to those tissues on which the drug produces its specific effects. He was of the opinion that as regards the frog's heart the enzyme was intracellular because repeated and prolonged washing of the heart (24 hours) did not remove the power of an emulsion of the heart to hydrolyse acetylcholine.

Galehr and Plattner (1927, a & 1927,b) conducted a detailed study of the rapid destruction of acetylcholine by mammalian blood. They found that defibrinated blood, taken from a number of species, was able to destroy the activity of a solution containing acetylcholine. They noted, however, that this destructive activity did not disappear after defibrinated blood had been subjected to a temperature of 58°C. or to ultra violet light. Further, they compared the destructive activity of the red blood cells and the serum on acetylcholine and found that the activity of the former was the more marked. They concluded from the above results that the destruction of acetylcholine and presumably of the 'vagus stoffe' by mammalian blood was not brought about by enzyme activity but was due to the catalytic hydrolysis of acetylcholine at the surface of the red cells.

Englehart and Loewi (1930) critically examined the work of Galehr and Plattner. They found that the resistance of the hydrolytic agent in mammalian blood to heat and ultra violet light was only relative, and was due to the presence of protective substances in the blood serum. They showed that the hydrolytic agent, present in the extracts of frog's heart, could be similarly protected by the addition of mammalian serum.

Plattner and Hintner (1929 and 1930) accepted this explanation of the results obtained by Galehr and Plattner and agreed that the hydrolysis of acetylcholine by mammalian blood serum is catalysed by an enzyme present in the serum. They also examined a large number of tissues taken from different animals and found that the esterase existed in all the tissues examined.

Further, Ammon and Voss (1935) have been able to show that, although in men and rabbits the destructive activity of the blood serum on acetylcholine was less than that of the whole or haemolysed blood, the activity of the haemolysed blood was equal to that of the whole blood. It follows, therefore, that the destruction of acetylcholine by mammalion blood is not due to a catalytic hydrolysis at the surface of the red cells.

Stedman, Stedman and Easson (1932) were the first to point out that the enzyme responsible for the hydrolysis of acetylcholine was probably a specific one. Esterases acting on fats, phosphoric and simple esters were known and it had hitherto been thought that the enzyme present in the liver, which hydrolysed tributyrin, was also responsible for the hydrolysis of acetylcholine. These workers showed that whereas liver esterase and pancreatic lipase, if carefully prepared, were without any appreciable action on esters of choline, there was present in the blood serum of horses an enzyme which readily hydrolyses such choline esters. This enzyme had little effect on simple esters such as methyl butyrate. In order to emphasise the specific nature of its activity, the new enzyme was termined choline-esterase. They prepared an enzymatic concentration from horse's serum which had an activity on the choline esters seven times that of ordinary horse serum. This concentrate had only a minor action on methyl butyrate.

Stedman, Stedman and White (1933) extended these observations; the blood sera of a number of animals were examined for the presence of enzymes which hydrolyse choline esters, methyl butyrate and tributyrin. Tabulation of the results showed that

little relation existed among the sera from different species with respect to their relative activities towards the three substances. They concluded that at least two enzymes, choline esterase and esterase were present in the blood sera. The preparations of the choline esterase used in these experiments were still not specific and did exhibit some activity on methyl butyrate. The blood sera of man and monkey were found to be amongst the most active in respect of choline esterase activity.

Ammon (1933) confirmed the finding of the Stedmans and their co-workers and stated that a specific enzyme, the choline esterase, exists in the blood and the tissues of various species.

The degree of specificity of choline esterase was established in 1935 by Stedman and Stedman. They were then able to obtain from the blood serum of a horse an enzymatic concentration which had five hundred times the activity of the original serum on choline esters, and was without any effect on other simple esters. They thus established the absolute specificity of choline-esterase. One may state therefore that a specific enzyme is present in the blood and tissues of a large number of animals which hydrolyses acetylcholine into choline and acetic acid.

The work of Bernheim and Bernheim (1933) suggests another method of destruction of acetylcholine. They found that the excised liver of rats has an increased oxygen consumption in the presence of acetylcholine. However, eserine inhibited this increased oxidation. It would appear, therefore, that the hydrolysis of the ester linkage must first take place before the oxidation can occur.

D. THE ACTION OF ESERINE AND OTHER SUBSTANCES ON THE CHOLINE ESTERASE ACTIVITY.

The pharmacological action of eserine is given as generally resembling that of acetylcholine. Its effect is, however, usually preceded by a latent period. Although it is included in the group of parasympathetico-mimetic drugs, the exact manner in which it exerts its effect has not been fully established. It can be shown that an important aspect of its activity is represented by its powerful inhibitory effect on the activity of the choline esterase. It is because of this inhibitory effect that eserine has been used extensively in the biological detection of acetylcholine.

Loewi and Navratil(1926,b)noted that the destruction of acetylcholine and the 'vagus stoffe' by emulsions of frog's heart was inhibited by the addition of eserine. They also noted that the diminution in rate and amplitude of contraction of the frog's heart on vagus nerve stimulation was potentiated by the presence of eserine. They ascribed this to the fact that eserine delayed the destruction of the 'vagus stoffe' which was formed when the vagus nerve was stimulated.

Eserine sulphate ($1:10^4$) and ergotamine, which also inhibited the choline esterase, were found to sensitise the frog's heart to the action of both acetylcholine and the 'vagus stoffe' in addition to the stimulation of the vagus nerve itself. This was considered to add weight to the hypothesis that stimulation of the vagus nerve produced the 'vagus stoffe' and that the 'vagus stoffe' was either acetylcholine or a substance closely allied to it.

Englehart and Loewi (1930) noted that the choline esterase present in the blood of various animals was also inhibited by eserine in concentration as low as $1:40.10^6$.

Matthes (1930) studied the inhibition of the choline esterase by eserine and other substances. He noted that the rate of destruction of an acetylcholine solution by the blood esterase was halved by the addition of eserine in a strength of $1:30.10^6$. He observed that with low concentrations of eserine the inhibitory action was slow and depended on the length of time that the eserine was in contact with the blood serum prior to its addition to the acetylcholine solution. He found that the percentage destruction of acetylcholine by the blood serum was inversely proportional to the length of time the eserine solution and the blood serum had been in contact.

Plattner and Hintner (1930) showed that eserine also inhibits the choline esterase present in the tissues of the animals examined, e.g. rabbit and cat.

As mentioned above, the choline esterase is a specific enzyme. The inhibition of this enzyme by eserine is in no way specific. Eserine has been found to exert an inhibitory influence on the activity of other enzymes, e.g. lipases, etc. Further, other substances have been prepared which have an inhibitory action on the choline esterase equal to that of eserine. Thus Matthes (1930) found that the synthetic urethane, miotine, prepared by Stedman (1929) had an inhibitory action of this degree.

Prostigmim, a synthetic urethane tested by Ammon (1934) has also an equal activity in this respect. Its effect upon the

heart rate and blood pressure is however less marked than that of eserine. It has thus been used clinically in the treatment of myasthenia gravis and intestinal atony in preference to eserine. Feldberg (1933) first used it in experimental work for the inhibition of the choline esterase activity and the detection of acetylcholine.

Other substances have the power of inhibiting the activity of the choline esterase. Ergotamine, ergotoxine, quinine, fluorides, various narcotics, atoxyl and muscarine all have this power. The activity of these substances however is exhibited in such low dilutions as to preclude their use in this respect both in clinical practice and in experimental work.

Although the action of the choline esterase is inhibited by eserine and its allied synthetic urethanes, it is not destroyed by these substances. Matthes (1930) showed that if the eserine was removed from an enzyme concentrate by dialysis the activity of the concentrate was fully restored.

In addition, and partly because of its inhibitory enzyme action, eserine sensitises various test preparations to acetylcholine and vagus stimulation. Führer (1918, a) found that the sensitivity of the dorsal muscle of the leech to acetylcholine was greatly increased by the previous addition of eserine. As mentioned above Loewi and Navratil (1926) found that eserine sensitised the frog's heart to the action of acetylcholine, 'vagus stoffe' and vagus nerve stimulation. It would appear that eserine increases the sensitivity of these and other preparations

to acetylcholine by preventing the destruction of this unstable choline ester. It is of interest to note, in this respect, that Feldberg and Rempel (1932) found that the action of the stable choline ester, carbaminoyl choline, on the leech muscle was not increased by the previous addition of eserine.

Eserine sensitises the leech muscle to substances other than unstable choline esters; thus Vartiainen (1934, a) noted that eserine sensitises the dorsal muscle of the leech to Barium and Potassium ions.

Dale and Gaddum (1930) were the first to use eserine to aid the preservation and detection of acetylcholine. Following upon their work eserine has been used almost routinely in the demonstration of acetylcholine-like substances in venous effluents and other biological products. Brown and Eccles (1934) made the interesting observation that the concentration of eserine in the perfusion fluid in 'in vivo' experiments was the same as that found by Matthes (1930) to be necessary in 'in vitro' experiments, i.e. eserine $1:30.10^{6}$ halved the rate of destruction of acetylcholine.

In summary one may state that there is present in the tissues and blood of a large number of mammals, including cats, rabbits and man, a highly specific enzyme, the choline esterase. This enzyme catalyses the rapid hydrolysis of the unstable choline esters. Acetylcholine is thus hydrolysed to choline and acetic acid and in this way its physiological activity is greatly reduced.

Eserine and other synthetic urethanes have a powerful, though not specific, inhibitory action on the choline esterase.

They have been extensively used experimentally in order to render the unstable choline esters more stable and thus more easily demonstrable. Eserine also increases the sensitivity of various biological test preparations to acetylcholine and is thus used in the detection of this substance.

E. THE BIOLOGICAL IDENTIFICATION AND ASSAY OF ACETYLCHOLINE.

The investigation of the endocrine system of the animal body and the isolation of autocoids such as adrenaline, insulin and pituitrin has necessitated the use of biological methods of identification and standardisation of these and other substances of biological significance. These substances exist in nature in such minute quantities that chemical and physical chemical methods of identification are as a rule inapplicable.

The general principles of the methods of biological assay are given by Sir H. Dale in his introduction to "The Methods of Biological Assay" by Burn (1928). It may be stated here that the methods are all essentially comparative, i.e. they are made with reference to an accepted reaction produced by the known product. The product yielding the same qualitative and quantative reactions as a known substance on more than one biological test object can have its identity and potence expressed with some confidence.

A large number of different biological preparations are affected by small doses of acetylcholine. In the work to be reported here biodialysates of bowel were tested for the presence of acetylcholine. In view of the fact that these biodialysates contain many other pharmacologically active substances besides acetylcholine, only the more specific and sensitive tests for this substance could be used and are described in detail here.

(a). The rectus abdominis muscle of the frog and the dorsal muscle of the leech.

Führer (1918, a and b) noted that the dorsal muscle of the leech (Hirudo medicinalis) when suspended in a physiological saline solution responded to the addition of comparatively small amounts of acetylcholine with a slow contraction. He also noted that the addition of eserine to make a concentration of 1:100,000 in the saline twenty minutes prior to the application of the acetylcholine increased the sensitivity of the muscle one hundredfold.

Reisser and Neuschloss (1921) found that the rectus abdominis muscle of the frog (Rana Temporaria; R. Esculenta) also responded with a slow contraction to the application of acetylcholine and other choline esters. The sensitivity of this preparation was increased four to seven times by the previous addition of eserine to the bath fluid.

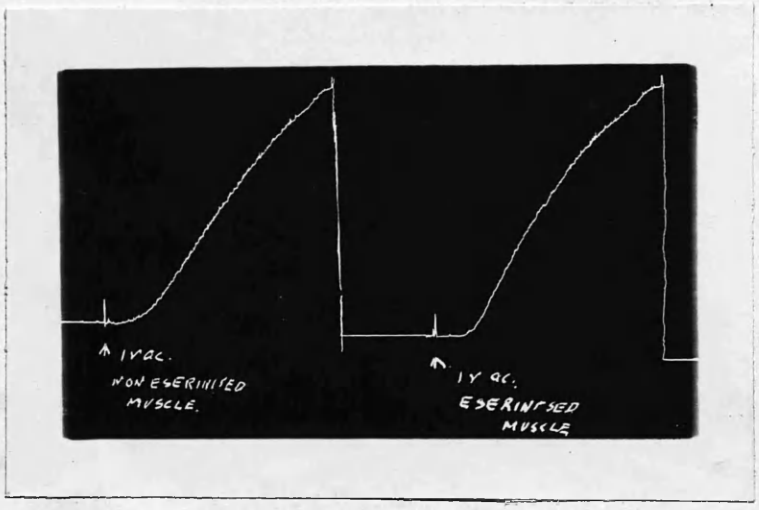
Minz (1932, a and b) conducted an extensive enquiry into the reaction of the dorsal muscle of the leech to choline esters and other substances present in the body tissues and fluids, in order to establish the specificity of the contraction of these muscles to acetylcholine. He was able to establish this specificity. Thus histamine, adenosine and adenylic acid, all commonly present in crude tissue extracts, were shown to be without action on the leech muscle in concentrations up to 1:100,000.

Choline itself was effective only in concentrations of 1:20,000 to 1:200,000. It is significant also that eserine does not sensitise the muscle preparations to choline. Minz noted and

Franel (1935) later confirmed that the presence of choline in a concentration of $1:2 \times 10^6$, although itself inactive, augmented the action of acetylcholine on these muscle preparations. Because of its greater sensitivity Minz advised the use of the dorsal muscle of the leech in preference to the no less specific, but less sensitive, rectus muscle of the frog.

Chang and Gaddum (1933) discussed at some length the value of the different biological tests for the choline esters. They reviewed the action of tissue extracts containing choline esters on the isolated frog's heart, isolated intestinal segment of the rabbit, blood pressure preparation of the cat, the denervated gastrocnemius muscle of the cat, the rectus muscle of the frog and the dorsal longitudinal muscle leech. They found that the last two preparations were the most specific and sensitive to acetylcholine. Beznák (1934) also reviewed the methods of biological assay of acetylcholine. His conclusions in the main corresponded with those of Chang and Gaddum (1933). These workers have standardised the conditions of testing of the leech and frog muscle to acetylcholine, and the following data are taken from their work.

The eserinised frog's rectus muscle preparation is sensitive to 0.1 γ to 1.0 γ acetylcholine in 5 c.c. fluid. The eserinised leech muscle is sensitive to one tenth of these quantities of acetylcholine, e.g. 0.01 γ to 0.1 γ . These workers however recommend the use of the frog's muscle preparation. Although this preparation is less sensitive to acetylcholine than is the leech muscle preparation, the deviation between subsequent contractions is



TRACE NUMBER ONE.

less than in the case of the leech muscle. Further, the relaxation time of the frog's muscle is only about one quarter of that of the leech muscle.

The frog muscle preparation's sensitivity to acetylcholine is increased only four to seven times by the previous addition of eserine to the bath fluid. The leech muscle is sensitised one hundred times by this procedure. This represents one of the disadvantages of the frog muscle preparation. The sensitisation of the leech muscle to an unknown solution by eserine can be taken as evidence that the solution contains a highly active but unstable choline ester. With the rectus muscle the same conclusion cannot so readily be drawn because eserine sensitises the muscle only four to seven times.

During the course of the present investigation, I noted that after a frog's muscle preparation has been exposed to eserine solution for some time it maintains the increased sensitivity when suspended in normal physiological saline. Even after several washings with saline the muscle retains almost the maximum sensitivity it exhibited when suspended in Ringer solution containing eserine. Trace 1 shows the response of a rectus muscle (a) suspended in Ringer solution, but after having been in eserine; (b) suspended in Ringer solution containing eserine 1:100,000.

The action of acetylcholine on these muscle preparations is not abolished by atropine, but it does not occur in the presence of nicotine. It thus represents one of the central or "nicotine" actions of acetylcholine. Both preparations show increasing sensitivity to the same concentration of acetylcholine at the first

three additions. After these preliminary variations the sensitivity of the preparations remains constant: the estimations reported here were all performed on muscle preparations which had attained their maximum sensitivity.

Both preparations are sensitive to the ionic concentrations of the constituents of the Ringer solution in which they are suspended. Thus an ionic concentration of 1% NaCl diminishes their sensitivity to acetylcholine. A hypotonic solution with an ionic concentration of 0.45% NaCl increases the sensitivity and reduces the latent period of these preparations. It is of interest to note that the histamine response of the virgin uterus of the guinea pig is modified by changes in the ionic concentration of the Ringer fluid in the same manner (Dale, 1913).

The preparations are not very sensitive to changes in the hydrogen ion concentration of the fluid in which they are suspended. The rectus muscle gives its maximum response at a pH 6.8 $\pm$ 0.4; the leech muscle at pH 7.8.

Both preparations are sensitive to changes in the ionic concentration of potassium in the bath fluid. An increase in the potassium ions above 0.1% and up to 0.2% results in a slow contraction of the rectus muscle. Higher concentrations of the potassium ion are toxic to the muscle. Tissue extracts and biological fluids often contain concentrations of potassium ions sufficient to produce a contraction of the frog muscle preparations; in the testing of such extracts for the presence of acetylcholine by means of the rectus muscle this source of error must be kept in

mind. Further it must be noted that concentrations of the potassium ion (0.06%) which are themselves without action on the muscle preparation increase the reaction of the muscle to a given amount of acetylcholine.

The reaction of the muscle of the frog to acetylcholine, although highly specific, is not uniform from day to day. Thornton (1934) noted this variability and also that the sensitivity of the muscle taken from one frog may differ widely from that taken from another frog belonging to the same batch.

I have noted during the course of this work that the sensitivity of the rectus muscle, taken from both sexes, fell considerably during the breeding season. The sensitivity may fall from 0.1γ to 10γ acetylcholine in 5 c.c. fluid. Wachholder and Nothmann (1931) noted the general seasonal variation of the reaction; the sensitivity was lower during the summer months.

Gasser and Dale (1926) noted that the rectus muscle preparation becomes temporarily insensitive after exposure to acetylcholine. The sensitivity is rapidly regained. I would mention here that I have been able to keep preparations immersed in Ringer solution containing eserine for 24 hours at room temperature. The sensitivity of such preparations to acetylcholine was found to be only slightly reduced. However none of the quantitative estimations reported here were performed on preparations which had undergone such treatment.

Dale and Feldberg (1934, b) made an observation, attention to which must be paid by anyone working with these muscle preparations. They noted that, if a Ringer solution comes in contact

with the skin of the human hand, it acquires a stimulant action on the dorsal muscle of the leech. They are of the opinion that this reaction is not due to the presence of acetylcholine in the sweat. The rectus muscle of the frog is similarly affected. This observation imposes the necessity on workers with these muscle preparations of ensuring that at no time during the experiment do any of the fluids to be tested come in contact with the skin.

(b). The isolated heart of the frog.

Straub's canula preparation of the frog's heart has been used by various investigators for the detection of acetylcholine in biological products. The preparation is highly sensitive; 0.01γ acetylcholine produces a definite decrease in the amplitude of the heart beat. I have not used this preparation in this work because it is sensitive to eserine, altered ionic concentrations of the mammalian saline solutions and to alterations of the hydrogen ion concentrations of the fluids to be tested. An increase in the hydrogen ion concentration or an increase in the potassium ion concentration have each effects similar to that produced by acetylcholine. The preparation was thus unsuitable for testing mammalian Ringer solutions for the presence of acetylcholine.

(c). The isolated segment of intestine taken from a rabbit.

The isolated segment of rabbit's intestine under proper 'in vitro' conditions is extremely sensitive to acetylcholine. It responds with a definite augmentation of activity when acetylcholine

to the extent of 1:500 x 10^6 is added, i.e. 0.2γ in a bath containing 100 c.c. fluid. This preparation has the further advantage that it is insensitive to histamine which is so often present in tissue extracts and biological fluids.

However, it has the disadvantage that it is sensitive to eserine. Eserine sulphate is almost constantly used to prevent the rapid hydrolysis of acetylcholine and thus makes the use of this preparation, for the detection of acetylcholine, difficult. I have used the isolated intestinal loop taken from the rabbit as a subsidiary test for the presence of acetylcholine.

The difficulty caused by the presence of eserine in the fluid to be tested was overcome by maintaining a concentration of eserine in the bath fluid in which the bowel was suspended, equal to that present in the fluid to be tested for the presence of acetylcholine. However, one must not overlook the fact that the action of eserine on the bowel is a complex one and as will be shown later it inhibits the hydrolysis of acetylcholine which is normally formed by the tissues of the bowel wall itself.

Further, Euler and Gaddum (1931) have shown that an unknown substance termed by them the P substance can be extracted from the tissues of the bowel wall. This substance has a similar action to acetylcholine on the isolated segment of rabbit's bowel and also on the blood pressure of the rabbit. However, these reactions are not antagonised by atropine, as are those of acetylcholine. This P substance is also without effect on the frog's rectus muscle preparation.

Adenosine, adenylic acid and their derivatives detected in

tissue extracts by Bennet and Drury (1931) inhibit the activity of the intestinal segment and are thus not confused with acetylcholine by this test.

(d). The blood pressure preparation of the cat.

Hunt and Taveau (1906) first pointed out the marked action of acetylcholine in depressing the blood pressure of a cat when given intravenously. Dale (1914) noted the same fact and pointed out that the fall in blood pressure was due to the marked peripheral vasodilatation produced by the drug and not due to the action of acetylcholine on the heart. Hunt (1917, a and b) studied the effect of acetylcholine on the blood pressure of the cat closely. He noted that 0.000.000.0024 mg. acetylcholine per kg. of body weight produced a distinct fall in the blood pressure.

We see therefore that the blood pressure of the cat is very highly sensitive to the injection of acetylcholine. Furthermore, the sensitivity of the preparation can be still further increased by the injection of eserine prior to the acetylcholine. The effect of acetylcholine on the blood pressure is not evident if atropine sulphate is given to the animal prior to the test. The response of the blood pressure of the cat, although sensitive, is not in any way specific. Other constituents of tissue extracts have a powerful effect in lowering the blood pressure; thus histamine, adenosine and the P substance of Euler and Gaddum (1931) all have this property. However, it is only the depressor action of acetylcholine which is sensitised by eserine and abolished by atropine.

I have used preparations a, c, and d in the present investi-

:gation. As mentioned above these preparations all give typical reactions to the active choline esters, but other conditions must be fulfilled before one can say that acetylcholine, as distinct from the other active choline esters, is present in a given tissue extract. The conditions to be fulfilled were postulated by Chang and Gaddum (1933) and are as follows:-

(1) If the action of an extract or a perfusate is augmented by eserine then this alone justifies the belief that the observed effect is not due to choline, or any other substance actually identified in the tissues except acetylcholine.

(2) The activity should disappear rapidly when the extract is mixed with blood, but if the blood has been previously treated with eserine, this reaction should be greatly retarded.

(3) The active principle should be unstable in alkaline solution. If a portion of an extract is mixed with an equal volume of 2N.NaOH, kept for ten minutes at room temperature, and then neutralised, any acetylcholine which it contains is destroyed. Choline is unaffected by this procedure.

(4) The 'muscarine' effects, i.e., the peripheral autonomic effects of extracts containing acetylcholine, should be antagonised by atropine; e.g., blood pressure of the cat.

The 'nicotine' effects should be antagonised by nicotine or curare, e.g., contraction of the frog's muscle. The extracts should react to these drugs in the same way as acetylcholine itself.

(5) When the activity of the extract is estimated quantitatively in terms of acetylcholine, using several different pharmacological tests, the same result should be obtained in each case.

This quantitative correspondence with a known quantity of acetylcholine, when different test objects are used, is the only method by which acetylcholine can be differentiated from other active choline esters. Chang and Gaddum (1933) compared several different choline esters with acetylcholine by five different methods. The following table taken from the work of these authors shows the relative potency per molecule of choline and some of its esters measured by these different tests in comparison with acetylcholine, the potency of which is expressed as 100 in each case.

TABLE I.

SUBSTANCE.	RABBIT.		FROG'S RECTUS.		LEECH.
	Intestine	Blood pressure (depressor effect).	Normal	After eserine.	After eserine
Choline	0.075	0.005	0.14	0.035	0.015
Proprionylcholine	3.000	4.000	550.00	450.000	45.000
Butyrylcholine	0.240	0	90.000	115.000	90.000
Valerylcholine	0.200	0	25.000	30.000	0.900
Glycollylcholine	0.220	0.250	1.200	1.000	0.130
Pyruvylcholine	14.000	10.000	13.000	13.000	16.000
Carbaminoylcholine	80.000	15.000	18.000	5.000	12.000

Note:- Noll (1932) states that the action of carbaminoylcholine on the isolated intestinal segment taken from a rabbit is somewhat greater than that of acetylcholine, i.e., its activity should be represented as greater than 100 in a table such as the above.

All the esters given in the table, except carbaminoyl choline, are active unstable substances. Their effect on the rectus muscle is increased by eserine to approximately the same degree as that of acetylcholine itself. They cannot, therefore, be thus distinguished.

If an extract containing one of these substances is tested on the different test objects there is no difficulty in showing that its activity does not correspond to that produced by acetylcholine. Pyruvyl choline is an exception; the ratio of its activity to that of acetylcholine will be seen to be fairly constant. It would therefore be difficult, if not impossible, to differentiate it by biological tests.

The methods described above and the special tests described by Chang and Gaddum constitute the principles of the method I have used to demonstrate the presence of acetylcholine in the biodialysates obtained from intestinal tissues. The actual technique will be described in the experimental section of this report. I now propose to discuss briefly the history and general biological significance of acetylcholine.

F. ACETYLCHOLINE: ITS HISTORY AND GENERAL BIOLOGICAL SIGNIFICANCE.

Strecker (1849) was the first to isolate choline: he obtained it from the bile of pigs, and later (1862) he established its chemical constitution. Baeyer (1867) followed up this work and prepared the acetic ester of choline, acetylcholine. He studied its properties and chemical constitution. The latter is now given as follows:- $(CH_3)_3 \equiv \overset{H}{\underset{Cl}{N}} - CH_2 - CH_2O(CH_3CO)$

Hunt and Taveau (1906) noted for the first time the marked pharmacological activity of acetylcholine. Further interest in this hitherto synthetic chemical was aroused by the work of Ewins (1914). He was able to demonstrate the presence of acetylcholine in certain extracts of ergot. Dale (1914, a) studied the pharmacological properties of the active extracts of ergot obtained by Ewins.

Prior to the work of Ewins (1914) and Dale (1914, b) there existed a considerable amount of confusion in relation to choline, muscarine and "pseudo muscarine." Muscarine, the active principle of Amanita Muscaria, was about the most specific drug acting on the parasympathetic nervous system. It had been extensively used as a tool for the physiological differentiation of the types of smooth muscle innervation. Schmeideberg and Harnack (1877) studied the chemical structure of muscarine and attempted its synthesis. They produced an active substance by the supposed oxidation of choline which they presumed was identical with natural muscarine.

It was early recognised that marked physiological differences existed between the natural muscarine and the synthetic

muscarine of Schmeideberg and Harnack. The confusion that resulted is indicated by the special terms which were coined to describe the synthetic product. These were, "synthetic muscarine," "choline muscarine" and "pseudo muscarine." Dale (1914, b) clarified the situation by showing that the synthetic muscarine was the nitrous ester of choline, and by defining its physiological properties.

Dale (1914, a and b) pointed out that the physiologically active choline esters, among which he now included "synthetic muscarine," have two distinct types of action on the heart, circulation and other organs:- (a) A peripheral action causing depression of the blood pressure and cardiac inhibition. This action broadly reproduces the actions of the cranial and sacral divisions of the involuntary nervous system. It is unaffected by nicotine, but abolished by atropine. Dale termed it the "muscarine" action. (b) The second action is a pressor action. It is unaffected by atropine but abolished by nicotine. He termed this the "nicotine" action.

Dixon (1906) had been struck by the similarity of physiological action between muscarine and the cranial and sacral involuntary nervous system. He conducted experiments using the heart in which he tried to prove that stimulation of the vagus nerve was accompanied by the appearance of muscarine in the venous effluent from the perfused organ. He termed it 'inhibitin' in this communication.

Dale (1914, b) noted the resemblance between the actions of the choline esters and those of the cranio-sacral autonomic nervous system. He questioned the physiological significance of

the marked similarity of these actions. The question was not pursued further at that time because there was no evidence that any depôt of acetylcholine existed in the body corresponding to the adrenaline depôt in the medulla of the suprarenal gland or even that acetylcholine existed in the tissues of the animal body at all.

The detection of acetylcholine in the tissues of the animal body has resulted in an extensive study of its biological significance. During his early work Hunt (1899 and 1901) studied the physiological activity of extracts of the suprarenal glands. He obtained a preparation that exhibited a physiological activity some fifty times that of an equal weight of choline, and which readily yielded choline as a result of simple chemical manipulations. The active constituent of these extracts was most probably acetylcholine, but the actual identification was not accomplished.

The classical studies of Loewi (1921 and 1924) referred to on page 13 greatly stimulated the workers in this field. Witanowski (1925) was able to demonstrate that a substance with the pharmacological properties of an active choline ester could be obtained from the frog's heart by suitable extraction.

The actual isolation of acetylcholine from animal tissues in a chemically pure state was first performed by Dale and Dudley (1929). They extracted acetylcholine from the spleen of the ox and of the horse. The initial extraction process was carried out as rapidly as possible following the death of the animals. It is thus probable that acetylcholine occurs as such in the living tissues and is not a post-mortem product or an artifact of the

extraction technique. A mixed dichlorplatinate of choline and acetylcholine was isolated which exhibited one fifth of the acetylcholine like depressor activity of the original splenic extracts. Dale and Dudley accounted for the losses of activity in the various stages of the isolation in a systematic manner. Dudley (1929) studied the composition of the mixed dichlorplatinate of choline and acetylcholine. In 1931 he was able to demonstrate that the formation of such co-ordination product compounds is characteristic of choline and a number of choline esters, including acetylcholine.

The isolation of acetylcholine from the tissues of animals of various species continued apace after the original work of Dale and Dudley (1929). The work of Chang and Gaddum (1933) is the most complete of any reported on the distribution of acetylcholine in animal tissues. They used bio-assay methods for the detection of acetylcholine.

There has been a general confirmation by workers in this field that acetylcholine is widely distributed in animal tissues and that its distribution is not confined to organs receiving parasympathetic nerve fibres. A difference of opinion has arisen however as to the quantities of acetylcholine present in the tissues and especially to the amount in the blood.

Two schools of opinion have arisen. Kapfhammer and Bischoff of Freiburg report relatively enormous quantities of acetylcholine in the tissues they have examined. In the blood of cows Kapfhammer and Bischoff (1930) and again in 1931 reported the presence of 5-55 mgm. of acetylcholine equivalent per litre of blood.

Dale and Dudley and their co-workers at the national institute of Medical Research in Hampstead, London, find much smaller quantities of acetylcholine in the blood and tissues of the animals which they have examined. Dudley (1933) using the same technique as did the Freiburg workers was able to demonstrate only 0.1 mgm. per litre of blood whilst working in London. He was able to isolate much larger quantities when working in the Freiburg laboratory with Bischoff. No explanation can at present be offered for this most curious discrepancy, but at present the results of the Hampstead workers are the more universally accepted. As pointed out by Dale and Dudley (1931) it is improbable that acetylcholine exists in the blood stream of animals in the quantities suggested by the Freiburg workers because the intravenous injection of less than one per cent. of the quantities they report produces marked physiological actions.

Ettinger and Hall (1934) found acetylcholine in the blood of the ox and of the dog. In the blood of the ox they found an acetylcholine equivalent up to 0.52 mgm. per litre. Loach (1934) however was unable to detect any acetylcholine at all in the normal ox blood. He used the method of Kapfhammer and Bischoff.

The physiological importance of acetylcholine is derived at present mainly from the evidential proof that it is the chemical agent involved in the humoral transmission of the so-called parasympathetic autonomic nerves. The part played by acetylcholine in the transmission of preganglionic and certain post-ganglionic sympathetic nerve impulses has also been elicited. More recently,

Dale, Feldberg, and Vogt (1936) have shown that stimulation of the somatic motor nerves is accompanied by the appearance of acetylcholine in the venous effluent from the voluntary muscle so stimulated.

The question has been raised whether acetylcholine is involved in the transmission of impulses in the higher centres, that is, whether there is a liberation of this substance at synapses in the central nervous system. On this question no direct evidence has as yet been obtained. The work of Dikshit (1935) and of Henderson and Wilson (1936) is however suggestive. These workers were able to produce symptoms which are characteristic of those produced by stimulation of peripheral cholinergic fibres by the intra-ventricular injection of minute doses of acetylcholine. The doses they used were insufficient to produce any symptoms when given intravenously.

A review of the humoral transference of autonomic nerve impulses is not relevant to this report. I would mention however several classical reviews of this rapidly increasing and fascinating field which have recently been published. Dale (1929) gives a survey of the history and a summary of the proof of this subject in his Croonian Lecture to the Royal Society. Loewi in his Harvey Lectures (1932-1933) and later, in the Ferrier Lecture (1935) gives a comprehensive survey of the subject.

Cannon (1933) and Bacq (1933 and 1935) give more general reviews and discuss the mode of transmission of both the sympathetic and parasympathetic autonomic nerve impulses.

The part played by acetylcholine in intermediary metabolism

has not been seriously considered. However the more recent work on the humoral transference of autonomic impulses has raised the question as to the exact manner in which the humoral mediator is liberated and its source. At present no definite answer can be given to these questions. It is established that repeated and prolonged stimulation of the cholinergic nerves to an organ, e.g. a frog's heart, does not reduce or increase the amount of acetylcholine present in the tissues (Vartiainen, 1934, b). This has led to the supposition that acetylcholine is present in a non-active "hidden" form in the tissues. If this is the case the part that acetylcholine can play as a source of a particular carbonaceous or nitrogenous molecular grouping might not be negligible. The observation of Ellis and Weiss (1932) that as much as one gramme of acetylcholine chloride can be destroyed in man on intravenous injection over a ten minute period without cardiovascular effects also indicates that it may play a part in intermediary metabolism other than through its effect on the nerve muscle mechanisms.

G. ACETYLCHOLINE AND ITS PARTICIPATION AS THE "HUMORAL" AGENT IN INTESTINAL MECHANISMS.

Magnus (1930) reviewed to that date the development of the idea that choline or its derivatives play a part, as humoral agents in the regulation of the activity of the intestine. The early work in support of this thesis has already been referred to.

The work of Le Heux(1921,b)was especially important in this respect. The approximate parallelism in the relative intensities of the effects of a series of sodium salts of aliphatic acids and the corresponding esters of choline on intestinal motor activity, indicated that the intestine has the power of synthesising choline and acids to form esters. Le Heux suggested that the synthesis of these esters of choline by the intestine was an enzymatic process.

In 1926 Abderhalden and Paffrath were able to demonstrate the synthesis of acetylcholine by an enzymatic concentration obtained from the intestine. They showed that a 'press extract' obtained from the small intestine of the pig was capable of catalysing the synthesis of acetylcholine from choline chloride and sodium acetate. Comparatively high concentrations of the reagents were required and the reaction of the fluid had to be kept slightly acid or neutral. Under these conditions they obtained a synthesis of acetylcholine which represented 0.2-0.8% of the possible theoretical yield.

That an enzyme capable of bringing about the synthesis of acetylcholine under experimental conditions is present in the blood serum of horses and in certain embryonic extracts was shown by

Ammon and Kwiatkowski (1934). They obtained a yield of acetylcholine which represented 0.1-0.2% of the theoretical maximum.

The blood serum of the horse and the embryonic extracts used are both rich in the enzyme which brings about the rapid hydrolysis of acetylcholine. They thus agreed with Abderhalden and Paffrath in the conclusion that one enzyme is capable of catalysing both phases of this reversible action according to the environmental conditions. This enzyme is the choline esterase as defined by Stedman and co-workers (1932).

Eserine was found to inhibit the synthesis of acetylcholine in a similar manner to that in which it inhibits its hydrolysis.

We thus note that there is present in the intestinal tissues an enzyme which is capable of bringing about both the hydrolysis and the synthesis of acetylcholine. Further Plattner and Hintner (1930) in their study of the distribution of the enzyme noted that the intestine was among the organs which contained relatively large amounts of the choline esterase.

It appeared reasonable to expect therefore that acetylcholine would be found in the tissues of the intestine. Chang and Gaddum (1933) in their extensive study of the distribution of acetylcholine in the body tissues, found relatively large amounts in trichloracetic acid extracts of the small intestine. They found 4 γ and 2.8 γ acetylcholine per gramme in the small intestine of rabbits.

The biochemical evidence here presented, although suggestive, does not throw any direct light on the problem of the relation of acetylcholine to intestinal motor activity. The work

of Hoet (1925) was of interest in this respect. He noted that stimulation of the vagus nerve in the neck in a living, or recently killed, rabbit caused a change in the intestine of the animal. This change was evidenced by an increased tone and amplitude of segmental movement of an excised segment of bowel under proper 'in vitro' conditions as compared with a normal segment, or a segment of bowel excised before the onset of the vagal stimulation.

He also demonstrated that this increased activity was readily inhibited by atropine.

This relation of vagal activity to the content of free active agent in the intestine is of interest when one considers that it has been satisfactorily demonstrated that vagal impulses to the small intestine are accompanied by the appearance of acetylcholine (Maaske, Bunting and Meek, 1935).

Feldberg and Rosenfeld (1933) following up the work demonstrating the humoral transference of cranio-sacral nerve impulses to the heart, eye, lungs and submaxillary glands undertook a series of experiments to investigate if, in fact, the vagal impulses to the gastro-intestinal tract were transmitted in a similar manner.

They found that the portal blood of a monkey or a dog, which had received an injection of eserine, contained a substance pharmacologically identified as a choline ester and probably acetylcholine itself. The pharmacological activity of the portal blood was present <u>even before the vagus nerves were stimulated</u>. The method used will be described in some detail, because the results obtained by these authors are fundamental.

They used dogs in their first experiments. The animals were anaesthetised by the intravenous injection of Pernocton (a barbiturate). The vagus nerves were cut in the neck and a canula was placed in the trachea. The abdomen was then opened. The splenic pedicle and accessory veins were ligatured. A glass canula was tied into the main splenic vein, distal to the site of the ligature. The tip of this canula projected into the portal vein and it was through it that the portal blood was carried to the exterior. A ligature was placed round the portal vein proximal to its junction with the splenic vein. This ligature was not tied but was made taut by traction when it was required that blood should pass up the splenic vein and through the canula.

In order to obtain the venous blood coming from the small intestine alone a canula was placed in the vena mesenterica magna. Canulae were also inserted into the femoral artery, femoral and jugular veins. After all the canulae were tied in, Novurudin was injected intravenously to prevent the coagulation of the blood. Eserine sulphate was then given, by intravenous injection, in order to inhibit the activity of the choline esterase. Prior to its injection, atropine sulphate was injected intravenously in order to counteract the powerful inhibitor effect of the eserine on the heart. The blood samples were collected over a period of ten minutes. They were cooled to room temperature and then tested on the eserinised dorsal muscle of the leech and on the blood pressure of cats. No direct vagal stimulation was carried out at any time during the experiment.

The portal blood collected under these conditions contained a substance which gave pharmacological actions identical with those of acetylcholine on the preparations used. The blood from the femoral artery, femoral and jugular veins, did not give any acetylcholine-like reactions.

The portal blood, as collected in these experiments, consisted of the venous blood draining from the pancreas, stomach, small and large intestines. The blood collected from the vena mesenterica magna which drains only from the small intestine showed a marked acetylcholine-like activity. These workers were able to demonstrate that, although the blood coming from either the stomach or the large intestine alone had an acetylcholine-like activity, that coming from the small intestine showed this activity to the greatest degree.

They concluded that the gastro-intestinal tract has the power of building an acetylcholine-like substance, this substance being active at the site of its formation and then being rapidly hydrolysed to choline and acetic acid. It was, therefore, demonstrable only when the action of the choline esterase was inhibited by eserine.

They also suggested that the formation of the acetylcholine-like substance was a function of the numerous nerve cells present in the plexuses of the gastro-intestinal tract. They concluded that the choline isolated in the biodialysates of isolated intestinal segments of Le Heux (1918) was probably derived, in part at least, from acetylcholine which had undergone hydrolysis.

Donomae (1934), using cats, was able to repeat the experiments of Feldberg and Rosenfeld, and to demonstrate, more conclusively, that the pharmacological activity of the portal blood was due to acetylcholine.

These fundamental experiments led to the conclusion that acetylcholine is liberated in the intestinal tissue in close relationship to the visceral muscle cells, in the absence of all extrinsic nerve impulses. The powerful action of acetylcholine on intestinal muscle led to the supposition that its spontaneous liberation in the tissues of the bowel would influence, markedly, the motor activity of that organ.

It was evident that, before such a general conclusion, as the above, could be made with confidence, a critical review of the experiments and further supporting evidence were required.

In considering the possible sources of error in the experiments of Feldberg and Rosenfeld, one must first consider whether acetylcholine is present in general venous blood as well as in the portal blood; and if so, whether it is present in a sufficient amount to affect the biological preparations used by these authors. It is generally accepted that acetylcholine can be extracted from general venous blood by biochemical means. The work of Chang and Gaddum, Dudley, Bischoff and Kapfhammer on this subject has already been referred to. It is accepted that acetylcholine is present in venous blood to the extent of 0.1 mgm. per litre in oxen and cows.

These workers did not test the activity of blood directly,

on the test preparations. The question therefore arises, whether ordinary venous blood, to which eserine has been added, contains sufficient acetylcholine in 10 or 20 c.c. of blood to affect the dorsal muscle of the leech or a blood pressure preparation of a cat.

Gollwitzer-Meier (1934) noted that the blood of a cow which had not had a previous injection of acetylcholine showed an acetylcholine-like activity equivalent to 0.005-0.013 mgm. per litre when applied directly to the test preparation. This activity was demonstrable only after the blood had been haemolysed. He concluded that acetylcholine was stored in the red cells of the blood and maintained there in an inactive form. Using the blood of the dog and cat, Gollwitzer-Meier and Kruger (1934), however, have themselves been unable to detect any acetyl-choline like activity in the general venous blood.

Feldberg and Kwiatkowski (1934) eliminated this source of error present in the experiments of Feldberg and Rosenfeld. They perfused the bowel of a cat with a physiological solution containing eserine. The bowel was perfused through the superior mesenteric artery. The venous effluent was found to have an acetylcholine-like activity on the test preparations used. It can therefore be concluded that the acetylcholine so demonstrated was derived from the intestinal tissues themselves.

Prior to these experiments acetylcholine had been demonstrated only in eserinised venous effluents after stimulation of the bulbo-sacral and certain other autonomic nerves. In these experiments the workers claim that division of both vagus nerves in the neck precludes the possibility that any nervous stimulation

is responsible for the appearance of acetylcholine. The tissues of the gastro-intestinal tract were thus held to be solely responsible for the appearance of acetylcholine in the portal blood.

A survey of the innervation of the gastro-intestinal tract and of the physiological action of the nerves supplying it, leads one to consider that simple section of the vagus nerves in the neck is not sufficient to ensure that no autonomic nerve impulses may reach the bowel. In these circumstances it is possible that autonomic nerve impulses, other than those transmitted through the vagus nerves, may be responsible for the appearance of acetylcholine in the gastro-intestinal tract.

The standard description of the innervation of the small intestine of the human subject is given as follows:- (taken from Kuntz, 1934).

The small intestine is innervated by both the vagus and sympathetic divisions of the autonomic nervous system.

The Sympathetic supply.

The sympathetic fibres are derived mainly from the coeliac and superior mesenteric plexuses. The postganglionic fibres, which arise in the ganglia of these plexuses, enter the intestine through the mesenteric nerves which in general accompany the mesenteric arteries. The preganglionic fibres travel to these plexuses in the great splanchnic nerves. These nerves are formed by the union of several rami arising from the sympathetic trunk between the 5-9 or 10 thoracic ganglia.

The Parasympathetic supply.

The vagus supply to the intestine is derived mainly from the right vagus through the division of the nerve which joins the coeliac plexus. The fibres arise in the dorsal nucleus of the vagus which lies subjacent to the ala cinerea of the rhomboid fossa. These preganglionic fibres run through the coeliac plexus. After leaving the plexus they form bundles which either take independent courses in the mesentery or run along the larger blood vessels and usually enter the intestinal wall with the latter. They then penetrate the subserosa and longitudinal muscle layer and enter the myenteric plexus.

In addition to the above innervation Ken Kuré and his co-workers (1931) state that the so-called 'spinal parasympathetic' nerves play an important rôle in the innervation of the gastro-intestinal tract. According to these workers, the number of parasympathetic nerve fibres, fine medullated fibres, in the vagus nerves which run to the intestine varies according to the individual animal. In some instances they are scarce. They state that many fine medullated fibres, coming from the dorsal roots of the thoracic segments of the spinal cord, run in the splanchnic nerves. These fibres they termed the 'spinal parasympathetic fibres.'

They demonstrated (1935) fine medullated nerve fibres in the dorsal roots of the spinal nerves by the method of osmium staining. This they did, after the roots had been sectioned, proximal to the ganglion and the ordinary sensory nerves had degenerated. These spinal parasympathetic nerves are supposed to have a

motor function to the small intestine, and their impulses may possibly be accompanied by the appearance of acetylcholine. The existence of these spinal parasympathetic nerves is not universally accepted. Hukara (1934) was unable to find any histological or experimental evidence to support the suggestion that such fibres exist in the splanchnic nerves. Other workers have been unable to confirm the findings of Ken Kuré and his co-workers. The question is sub judice at present.

Examination of the physiological function of the autonomic nerves to the gastro-intestinal tract raises a more serious difficulty to the view that the vagal fibres to the small intestine are the only ones whose impulses are accompanied by the peripheral appearance of acetylcholine. Stimulation of the vagus nerve as shown by Bayliss and Starling (1899), Klee (1912) and Hukara (1932) is followed by a general augmentation of intestinal motor activity. Klee (1913), amongst others, has shown that stimulation of the splanchnic nerves inhibits intestinal movements as a rule.

Dale (1934) suggested a new classification for the autonomic nerves. Those nerve fibres, whose effects are mediated by the formation of acetylcholine, he termed "cholinergic." Those whose effects were mediated by the formation of adrenaline or sympathin, he termed "adrenergic." It has been shown by Finkleman (1930) and Cannon and Bacq (1931) that the sympathetic, i.e., thoracic autonomic, fibres to the small intestine are mainly adrenergic. Maaske, Making and Bunting (1935) have shown that the vagus fibres to the small intestine are cholinergic.

Recent work on the motor functions of the autonomic nerves to the gastro-intestinal tract has shown that the effects produced by stimulation are not so constant as hitherto thought. Thus, stimulation of the sympathetic supply to the stomach of the cat may cause either contraction or relaxation, depending on the type of stimulation employed (Brown, McSwiney and Wadge, 1930; McSwiney and Robson, 1931).

Similarly Finkleman (1930) has shown that stimulation of the periarterial sympathetic nerves to the small intestine at a slow rate (2-4 per sec.) may cause contraction and increased motor activity of an isolated intestinal segment.

Harrison and McSwiney (1936) have shown that the reversal effects of sympathetic stimulation to the cat's stomach are enhanced by eserine and abolished by atropine. They were able to demonstrate this, both in the intact animal and with isolated preparations. They concluded from this, that there are probably cholinergic fibres in the sympathetic nerves to the stomach. There is no direct evidence, at present, to show that there are cholinergic nerve fibres present in the sympathetic nerves to the small intestine. However, the demonstration of cholinergic sympathetic nerve fibres to the stomach leads one to consider that such fibres possibly exist in the sympathetic nerves to the small intestine.

It is clear therefore that the experiments of Feldberg and his co-workers are open to the objection that all the cholinergic nerve fibres to the gastro-intestinal tract were possibly not severed. The operative procedures, including the opening of the peritoneal cavity, would thus be ample to stimulate any cholinergic

fibres which had not been severed. It would thus be responsible for the appearance of an acetylcholine-like activity in the portal blood in the absence of vagal stimulation.

It is towards a more conclusive demonstration of the independence of acetylcholine liberation on extrinsic nerve impulses that my own experiments have been directed. As set forth in the subsequent pages, I have been able to show that an isolated segment of intestine taken from the rabbit, cat, or duck liberates an acetylcholine-like substance under 'in vitro' conditions. A liberation of acetylcholine under such conditions has been mentioned as occurring by Feldberg (1933) but as far as I know the results have never been published.

A. THE EXPERIMENTAL DEMONSTRATION OF THE INDEPENDENCE OF ACETYLCHOLINE ON EXTRINSIC NERVE IMPULSES.

The experimental work to be reported here may be conveniently divided into three sections:-

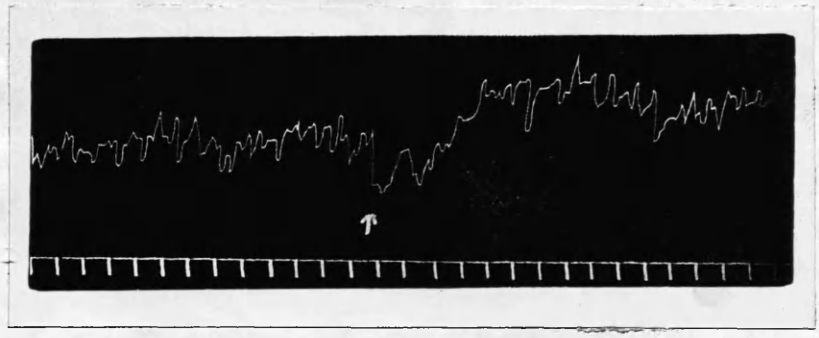
SECTION A.

The first series of experiments was performed to demonstrate that the fluid in which an isolated segment of small intestine had been suspended contained a substance which had a stimulant effect on the motor activity of a second segment of bowel.

Procedure. Two mammalian bowel apparatuses were used. The volume of fluid in each bath was 40 c.c. The physiological saline solution used for mammalian preparations in this and other experiments was Tyrode Bayliss. The composition was as follows:-

NaCl, 8 g. $NaHCO_3$, 1 g. KCl, 0.2 g.
NaH_2PO_4 0.05 g. MgCl, 0.1 g. $CaCl_2$ 0.2 g.
per litre. A pH of 7.4 was maintained.

The temperature of the bath fluid was maintained at 37°C. and the fluid was aerated by alveolar air supplied to it from an air bottle in which the air was stored under pressure. A rabbit was killed by first stunning it and then severing the great vessels in the neck. The abdomen was opened immediately after death. Two segments of jejunum, each about two inches in length, were excised. In all the experiments to be reported, the proximal part of the jejunum was used wherever possible. This was located by following



TRACE NUMBER TWO. TIME TEN SECONDS.

the course of the long U-shaped duodenum to its termination.

The segments of bowel, so removed, were cleansed by expressing saline solution through their lumen. A needle carrying a moistened thread was passed through the anti-mesenteric border at the proximal end. Another thread was passed through the mesenteric border at the distal end of the segment. Each segment was then attached to a bowel stand by one of the threads. The movements of the segment were recorded on a smoked drum by means of a light lever to one end of which the other bowel thread was attached.

A segment of bowel set up in this manner is usually inactive for some minutes. It recovers shortly from the drastic changes which have been made in its environmental conditions. It then commences to exhibit segmental movements, 8-10 occurring per minute.

This method, essentially that of Magnus (1904, a), has been described in some detail, because I have used it repeatedly during the course of the present work.

Having set up two bowel segments, as described above, nothing further was done for one hour. 10 c.c. of bath fluid was then withdrawn from one bath and added to the other. Care was taken to maintain at 37°C. the temperature of the fluid transferred.

Results. (See Trace 2).

The addition of the bath fluid was followed by a marked rise in the tone of the bowel segment without any appreciable increase in the amplitude of the segmental movements. A short latent period of 10-30 sec. is noted before the effect demonstrates

itself. This is due, probably, to the slow diffusion of the active factor in the bath fluid.

Heating of the fluid to 100°C. for five minutes, prior to its addition to the bath, did not destroy its excito-motor activity. The effect was antagonised by atropine.

It is concluded from this experiment that the bath fluid contains a substance, derived by biodialysis from the intestinal segment, which has an excito-motor effect on a second segment of bowel. It is noted also that the active constituent resembles choline in that it is not destroyed by heat and its action is antagonised by atropine. As reported on pages 7 and 10 the active constituent has been shown to be choline by Le Heux (1918) and Abderhalden and Paffrath (1925).

SECTION B.

The second series of experiments in this investigation was carried out in order to determine whether acetylcholine was liberated in the bath fluid. It was realised that the action of the choline esterase would require to be inhibited before any acetylcholine could be demonstrated. The esterase was inhibited by adding eserine to the bath fluid. The fluid was then tested for the presence of acetylcholine, on a second segment of bowel, and on the eserinised rectus abdominis muscle of the frog.

Procedure. A segment of jejunum four inches in length was set up in a mammalian bowel apparatus, as described on page 55. The bath contained 40 c.c. Tyrode Bayliss solution. Eserine Salicylas

(T. & H. Smith, Ltd.) as a freshly prepared solution in Tyrode Bayliss, was then added to the bath fluid. The solution was made up in a 1:15,000 strength some hours before the actual experiment, because eserine is only sparingly soluble in the saline solution. The eserine solution was then added to the bath fluid in divided doses to make an ultimate concentration of 1:4,000,000. If the solution was added in a single dose it was found that a marked rise of tone and abolition of segmental movement occurred.

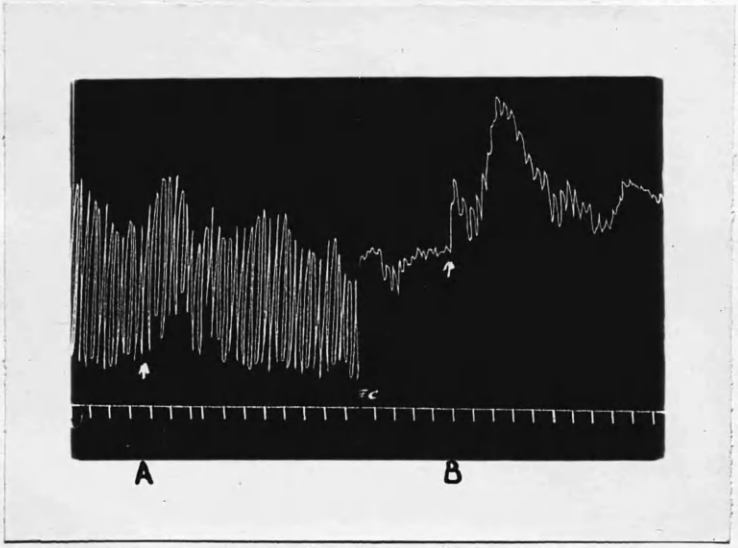
After a lapse of one hour the bath fluid was removed and tested for acetylcholine on (a) an isolated segment of bowel suspended in Tyrode Bayliss solution containing an equal concentration of eserine, and (b) the rectus abdominis muscle of the frog.

(a) The isolated segment of rabbit's bowel.

This test segment was set up at the same time as the donor preparation. The segments of bowel were taken from adjacent portions of jejunum of the one animal. The volume of the bath fluid was 40 c.c. in each case. Eserine solution was added to both baths at the same time. At the end of one hour the 10 c.c. of bath fluid removed from the first bath was added to the bath containing the recipient preparation. Prior to this 10 c.c. Tyrode Bayliss solution containing eserine 1:4,000,000 was added to the bath fluid as a control measure.

Results. (See Trace 3b).

The addition of the fluid was followed by a very marked rise in the tone of the test segment. No change in the amplitude of the segmental movements was noted. The reaction was antagonised



TRACE NUMBER THREE. TIME TEN SECONDS.

by atropine sulphate.

The control addition of Tyrode Bayliss solution containing eserine 1:4,000,000 was without any appreciable effect on the motor activity of the segment.

It is thus seen that the excito-motor effect noted on the addition of the bath fluid is not due to the eserine present in it, but to a factor whose action on the bowel resembles that of acetylcholine.

Histamine is without action on the intestinal segment taken from the rabbit and it plays, therefore, no part in the effects noted here.

In order to demonstrate the increase of excito-motor activity of a given volume of bath fluid, after the addition of eserine, a comparative experiment was performed. In this experiment the stimulant effect of 10 c.c. of bath fluid taken after the bowel had been suspended for one hour in Tyrode Bayliss solution was compared with that produced by 10 c.c. of bath fluid taken after the bowel had been suspended in Tyrode Bayliss solution containing eserine 1:4,000,000 for one hour. (See Trace 3, a and b).

The protocol of such an experiment is given below.

6/1/36. Rabbit. male. 1.2 Kg.

2 p.m. Rabbit killed and two segments jejunum, each two inches long, excised and washed with saline solution.

2.15 p.m. Each segment set up in a mammalian bowel apparatus. Volume of bath fluid 40 c.c.

3.15 p.m. 10 c.c. fluid taken from bath A and added to bath B.

3.30 p.m. Both baths drained of fluid. 40 c.c. fresh Tyrode Bayliss, warmed to 37°C. placed in each bath.

3.50 p.m. ½ c.c. eserine 1:150,000 added to each bath.

4.10 p.m. Above repeated.

4.30 p.m. Above repeated.

4.50 p.m. 10 c.c. fluid taken from bath A and added to bath B.

It is seen from this experiment that the addition of eserine to the bath fluid in which is suspended a segment of bowel causes a marked increase in the excito-motor activity of this bath fluid on a second segment of bowel. This action of eserine demonstrates that the excito-motor activity of the bath fluid is not due to choline alone, because the action of choline on the intestinal segment is not enhanced by the presence of eserine.

It would appear that the addition of eserine to the bath fluid prevents the hydrolysis, and thus preserves the activity of an acetylcholine-like substance which is present in the fluid.

One must not overlook the fact that eserine, besides inhibiting the choline esterase, has other actions on the intestinal segment. Thus Braecke and de la Cuesta (1933) have in fact shown that the addition of eserine to the bath fluid causes an increased quantity of choline to dialyse from a bowel segment. This fact will be referred to later when the further confirmatory tests of acetylcholine in the bath fluid are being considered.

(b) The effect of the eserinised bath fluid on the rectus abdominis muscle of the frog.

10 c.c. of the bath fluid was withdrawn, cooled and added

to the bath fluid in which a sensitised rectus abdominis muscle of a frog was suspended.

Results.

An acetylcholine-like contraction resulted. The activity of the fluid was very slight and did not allow of the carrying out of several essential confirmatory procedures.

It was not possible therefore to prove satisfactorily that acetylcholine was present in the bath fluid by this method. The results will not be considered further, because with an improvement of technique it was possible to obtain a bath fluid in which the acetylcholine-like activity was sufficiently pronounced to allow of confirmatory and quantitative tests being carried out.

SECTION C.

In the experiments described in Section B, eserine was used to prevent the hydrolysis of any acetylcholine which may have been present in the bath fluid. The isolated jejunal segment taken from a rabbit was used as a test preparation; because of this the concentration of eserine in the bath fluid was raised only to 1:4,000,000. Higher concentrations could not be used because regular segmental movements of the isolated bowel do not occur when the concentration of eserine is raised.

In the experiments to be described in this section, Section C, the rectus muscle of the frog and the blood pressure preparation of the cat have been used as the test objects. This has allowed of the raising of the concentration of the eserine in

the bath fluid. In addition to this the animals were given eserine intravenously a short time before they were sacrificed.

These two procedures brought about the more efficient inhibition of the choline esterase. The acetylcholine which was liberated in the bath fluid was not rapidly destroyed and was thus more easily demonstrable. Further the movements of the bowel segments were not recorded in the experiments of this section. It was thus possible to keep segments of greater length and weight in comparatively small quantities of bath fluid.

Procedure. Eserine salicylate solution in Tyrode Bayliss was made up before each experiment. 1.5 c.c. of the solution contained 1 mgm. of eserine. Doses up to 1 mgm. eserine per kg. of weight were given intravenously to rabbits. The effects produced by such an injection were striking. In general it was seen that the effects produced were all characteristic of over action of the cholinergic fibres of the autonomic nervous system.

Following upon the injection there was a latent period of one to three minutes during which the animal appeared quite comfortable. The heart rate, however, was found to be decreased within 10 secs. Fibrillary tremor of the muscles was next noted. This was especially noticeable in the gluteal and hamstring muscles. This was followed by incoördination of gait. The tone of the muscles could be felt to be greater than normal. This influenced the posture of the animal. The back was not so arched; the head was held unduly high and the limbs were prop-like. The normal crouching attitude of the rabbit was markedly altered. The hairs

over the body were kept erect. Defaecation occurred and excess salivation was an early and prominent feature. The pupils were markedly contracted and exophthalmos was noted. The pulse remained slow and regular but dyspnoea and noisy respirations resulting from excess bronchial secretion soon supervened. Paralysis of respiration followed in the fatal cases. This was followed by cardiac arrest and death. A dose of 1 mgm. eserine per kg. of weight was found to be fatal to the rabbit when given intravenously. Soma Weiss (1926) noted a similar train of symptoms in the cat when toxic doses of eserine were given. He found that the fatal dose was 0.8 mgm. per kg. of weight, given intravenously.

On opening the abdomen of a rabbit which had received a dose of eserine, one is struck by the marked engorgement of the splanchnic vessels with dark venous blood. The appearance of the gastro-intestinal tract also arrests one's attention. The segmental movements and occasional peristaltic rushes seen on opening the abdomen of an animal killed in the routine manner, are all absent. The whole tract appears tense. The muscular walls are firm to the touch and the pyloric sphincter is very prominent because of its spasm. No movements of any kind are observed. The pressure of the spastic muscles of the wall of the tract is so great that when one cuts into the lumen of the bowel the rapidly escaping gases make quite a considerable noise.

A large segment of jejunum was excised from the animal and thoroughly washed with saline. The ends of the bowel segment were closed with a gastro-enterostomy stitch to prevent the mucosa,

which tends to separate from the muscle, contaminating the bath fluid. The bowel segment was then weighed and placed in a mammalian bowel apparatus. After the segment was placed in the bath fluid it exhibited fairly active segmental movements. These were not recorded. Eserine salicylate solution was now added to the bath fluid to make a concentration of 1:150,000. At the end of one hour a sample of the bath fluid was withdrawn and tested for acetylcholine.

A protocol of this portion of an experiment is given herewith:-

Rabbit. Male. 2.1 Kg. . 11/2/36.

9.30 a.m. 1 c.c. 1:1,500 eserine solution given intravenously.

10.15 a.m. Additional 1 c.c. given.

10.30 a.m. Animal killed by stunning and then bleeding.

Bath A. 200 c.c. Tyrode Bayliss solution in the bath.
Weight of bowel segment placed in the bath: 6.5 g.

10.45 a.m. Bowel segment placed in the bath.

11 a.m. 2 c.c. eserine solution (1:1,500) added to the bath fluid.

12 noon. Bowel segment removed and the bath fluid retained for testing.

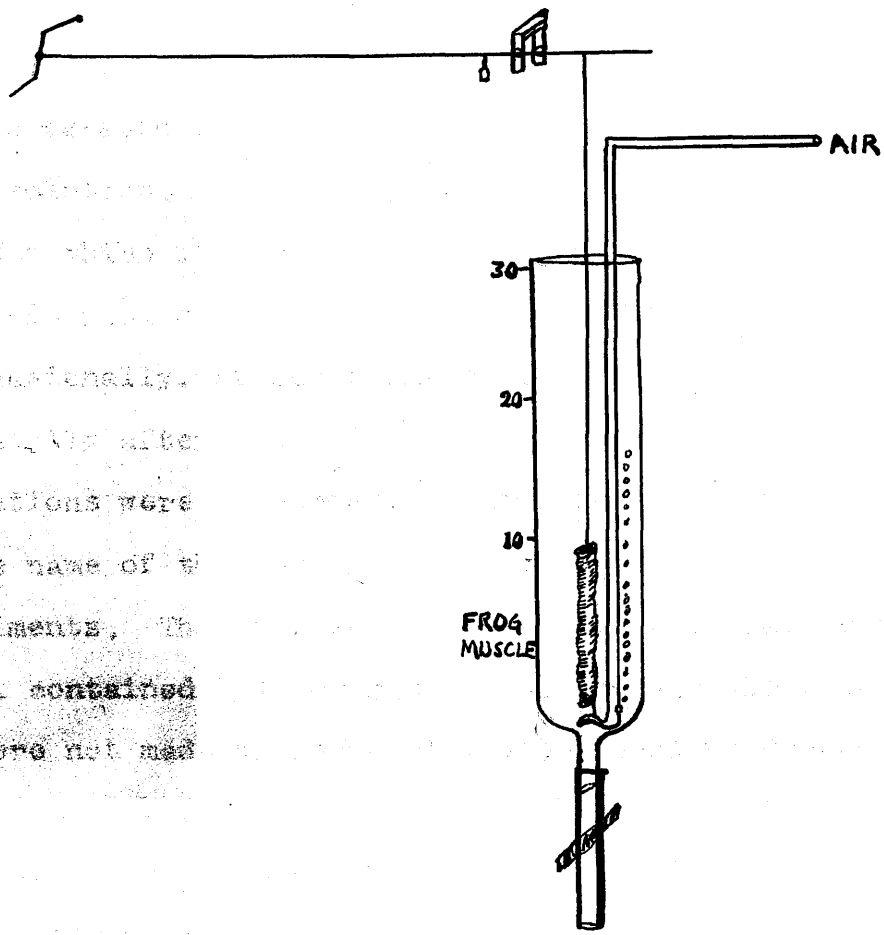
Using the bath fluid collected under these conditions it is possible to conduct a full biological detection of acetylcholine. The following test preparations were used:- (a) The rectus abdominis muscle of the frog, and (b) the blood pressure preparation of the cat. These tests will now be described.

(a) The effect of the bath fluid on the rectus abdominis muscle of the frog.

Procedure. A frog (Rana Temporaria) was killed by stunning, followed by decapitation. It was then pithed. The frog was laid out on a dissection board, ventral aspect being uppermost. A mid-line incision, through skin only, was made extending from the symphisis pubis to the mid sternal level. Transverse incisions were made on each side, above and below, and lateral flaps of skin were dissected back. The xiphisternum was cut from its attachment from the sternum. It was drawn up and the rectus abdominis muscle was thus put on stretch. The muscle was cut away from the other abdominal muscles, and freed from its attachment to the pubis. The excised muscle, with the xiphisternum attached, was placed in a Petri dish containing Howell Ringer solution. A heart clip, with a thread attached, was clipped on to the xiphisternum. A needle carrying a moistened thread, was passed through the caudal end of the preparation. The muscle was then attached to a stand by means of this thread. The thread attached to the xiphisternum was passed over the recording lever.

The hands of the operator were washed after killing and pithing the frog. The rest of the dissection, and the setting up of the preparation, was then performed without the skin of the hands coming in contact with the muscle or any of the solutions which were to bathe it. This precaution was taken because the human skin has the power of transmitting a stimulating property as regards the muscle, to solutions which come in contact with it.

The apparatus consisted of a 30 c.c. graduated bath, closed at its lower end by a clipped rubber tube. A muscle stand was placed in this bath and through the lumen of this stand the bath fluid was aerated and mixed: the contraction of the muscle was recorded on a smoked drum, by a frontal lever. This gave an approximately tenfold magnification of the muscle movements. The aeration of the fluid was carried out by means of an air bulb operated by hand.



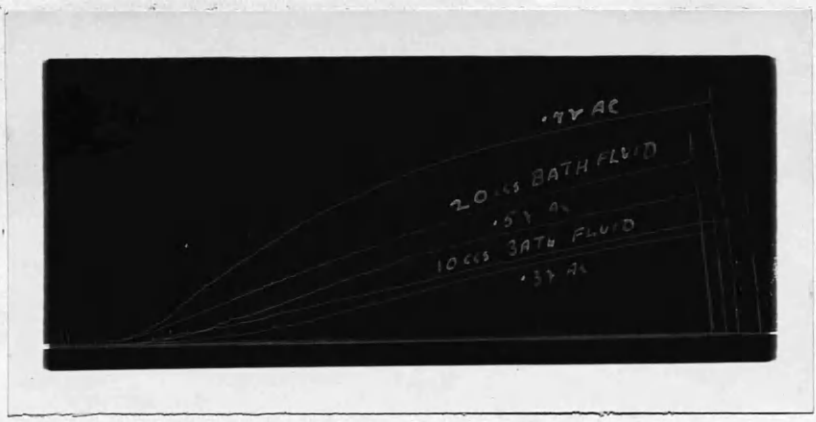
The Howell Ringer solution used has the following composition:- NaCl, 7 g. KCl, 0.3 g. $NaHCO_3$, 0.03 g. CaCl, 0.132 g. per litre. The pH of the fluid was maintained at 6·8.

The muscle preparation having been set up, it was washed three times by passing fluid through the bath. The bath was then filled to the 10 c.c. level. The diameter of the bath was such, that the muscle was completely covered by the 10 c.c. fluid. The preparation was left for ten minutes. The fluid was then drained away and replaced by fresh saline. 1 c.c. eserine solution 1:15,000 was added making a concentration in the bath fluid of 1:150,000. The fluid was aerated vigorously to ensure an even dispersion of the eserine solution. The preparation was then left for twenty minutes, after which it was found to be sensitive to acetylcholine. During all this period no spontaneous contractions of the muscle occur. Occasionally, preparations exhibit spontaneous contractions, especially after the addition of the eserine solution. Such preparations were discarded. Pragmoline (May and Baker) is the trade name of the acetylcholine used for standardisation in these experiments. This was suitably diluted with saline solution until 1 c.c. contained 1/1,000 mgm., i.e. 1$\surd$ acetylcholine. The solutions were not made up from solid acetylcholine because it is highly unstable in such a form. It was therefore not convenient to keep it and use as a standard.

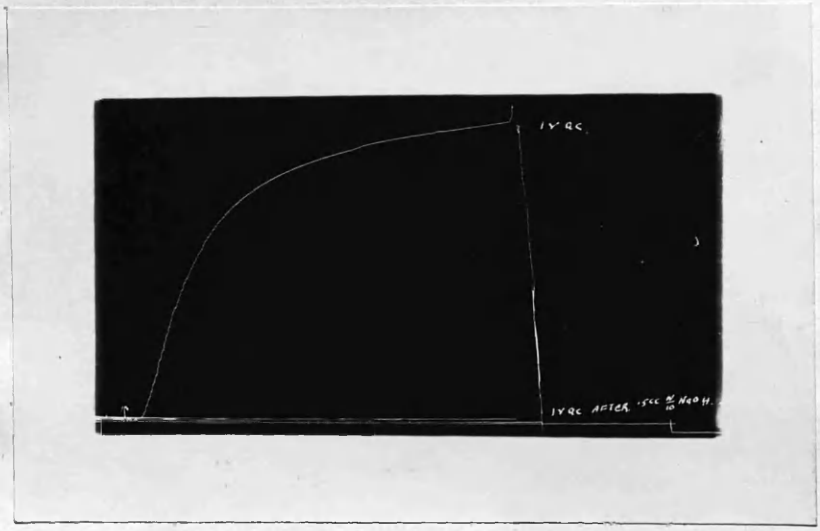
The drum on which the movements were recorded was set at its slowest speed. 1 c.c. of the acetylcholine solution, i.e. 1 $\surd$ acetylcholine, was added to the bath. The time of the addition

was marked on the trace. The slow contraction of the muscle in response to the acetylcholine was recorded. When the maximum contraction was reached the drum was stopped and the bath fluid was drained away. The muscle was now washed at least three times with Howell solution. The muscle then commences to relax and within 5-10 minutes this is completed. The relaxation was recorded on the stationary drum. The drum was then reversed by hand, and 1 c.c. eserine solution added to the bath. After 20 minutes, the muscle being now sensitive, the drum was set in motion and an additional acetylcholine response could be recorded. In all the subsequent tests the acetylcholine or test solutions were added at the same time and the drum was stopped at the same time. This makes the tracings strictly comparable. In preparing a muscle for use three separate applications of acetylcholine were first applied. It was only after the third contraction that the muscle attained its maximum and stable sensitivity. The average preparation gave a clear result to 0.2$\surd$ acetylcholine in 5 c.c. fluid during the winter and early spring months.

The frog muscle preparation, having been prepared and sensitised as described, was then used to test the bath fluid obtained from the mammalian bowel apparatus, for the presence of acetylcholine. 10 c.c. of the bath fluid was withdrawn, cooled to room temperature by placing in an ice bath, and then added to the frog muscle preparation. The cooling of the fluid is essential: Feldberg and Rosenfeld (1933) pointed out that mammalian blood or Ringer solution at body temperature causes a contraction of the muscle preparations even in the absence of acetylcholine.



TRACE NUMBER FOUR.



TRACE NUMBER FIVE.

Results. (See Trace 4).

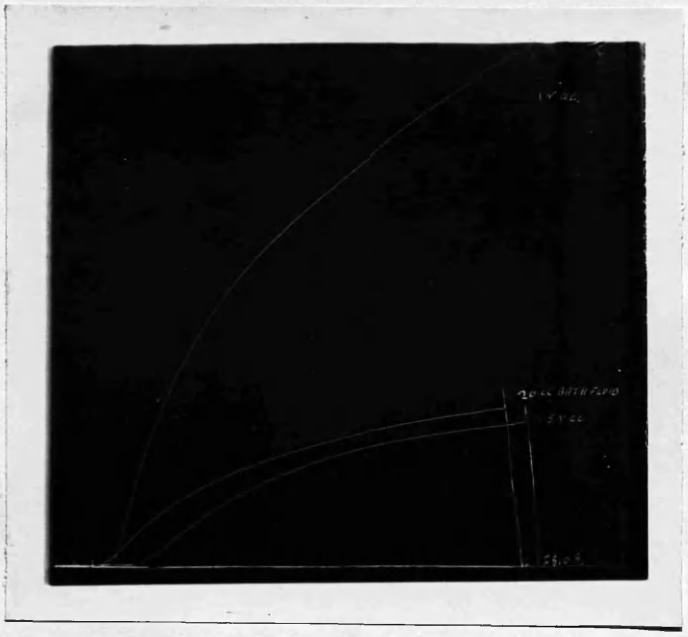
The addition of the cooled fluid caused a contraction of the rectus muscle comparable in every way to that produced by acetylcholine itself.

The following confirmatory tests were performed:-

(1) The fluid was tested on a muscle preparation without eserine being added to the bath fluid to sensitise the muscle. The contraction produced was somewhat less than that produced by the same quantity of fluid on the fully sensitised muscle. As pointed out on page 26, the frog's muscle does retain a considerable amount of its sensitivity to acetylcholine after being treated with eserine for some time. Thus the contraction brought about by the bath fluid was similar to that produced by acetylcholine on the non eserinised muscle.

(2) A bath fluid, known to be active, was allowed to stand at room temperature for 24 hours and then tested on the muscle. It was found that the acetylcholine-like activity had disappeared. Bath fluid stored for 24 hours in the refrigerator (4^{o}C.) was found to retain its activity.

(3) Acetylcholine is unstable in an alkaline medium. Trace 5 shows the activity of 1✓ acetylcholine before, and after treatment with 0.5 c.c. N/10 NaOH for 10 minutes, followed by neutralisation with 0.5 c.c. N/10 HCl. The bath fluid after such treatment was also found to lose its activity on the rectus muscle. Trace 6 shows the activity of 20 c.c. of the bath fluid before and after treatment with 2 c.c. N/10 NaOH followed in 10 minutes by neutralisation with



TRACE NUMBER SIX.

2 c.c. N/10 HCl (termed Ibid A on Trace 6).

(4) The activity of the bath fluid on the rectus muscle was antagonised by the addition of nicotine 1:1,000,000. It was not affected by the addition of atropine 1:1,000,000. *

(5) The contraction produced by a given volume of bath fluid was standardised against the activity of a known quantity of acetylcholine.

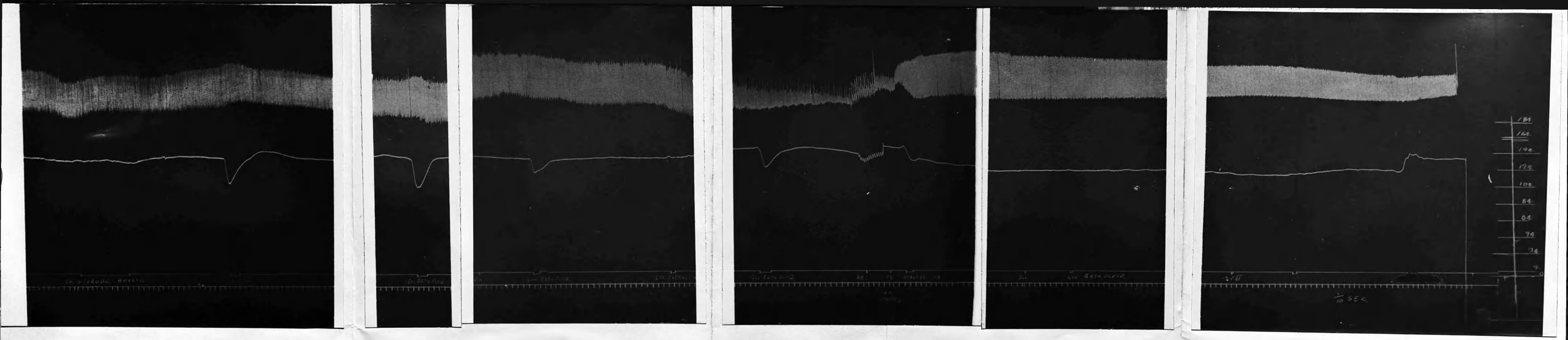
(b) The effect of the bath fluid on the blood pressure of a cat.

Procedure. A cat was anaesthetised with ether and a canula inserted into its trachea; the anaesthesia was then continued through the canula. The left carotid artery was dissected out and the blood pressure canula inserted into it. The intravenous injections were made into the right jugular vein. If the depth of anaesthesia was kept constant the blood pressure of the preparation remained constant. Variations in the blood pressure could thus be used to demonstrate the actions of certain drugs. Respirations were recorded by a stethograph placed on the chest wall. 0.1√ acetylcholine produced a fall of approximately 14 mm. Hg. in such a preparation.

The preparation, having been shown to react to the injection of acetylcholine, was then used for the detection of acetylcholine

* On several occasions the dorsal muscle of the leech (Hirudo Medicinalis) was used as a biological test for acetylcholine. The method of preparation of the leech was taken from Fuhner (1918) and Minz (1932). It will not be described in detail because I have not used it extensively. None of the results quoted in this work were obtained from experiments in which the leech was used. The preparation is highly sensitive to acetylcholine but it is less convenient to use than the rectus muscle of the frog because of its greater tendency to undergo spontaneous contractions and because of the greater length of time which it takes to relax after a contraction.

Trace Number Seven.


180
160
140
120
100
80
60
40
20
0
1/10 SEC.

in the bath fluid. The bath fluid was injected intravenously and the effects noted. The depressor effect produced by a given volume of bath fluid was standardised against the amount of acetylcholine necessary to produce a similar effect. Eserine was then injected intravenously; after the blood pressure had stabilised itself the effect of the same volume of bath fluid was then noted. The effects of the bath fluid and acetylcholine were then tested after the animal had received a dose of atropine sulphate.

The protocol of such an experiment is here presented:-

29/3/36. Cat. 1.9 kg. Chloroform induction; ether anaesthesia continued.

(1) Control. 10 c.c. Tyrode Bayliss. No effect.

(2) 0.2√ acetylcholine. Marked fall in B.P. 28 mm. Hg.

(3) Trachea cleaned out. B.P. rose during the temporary asphyxia.

(4) Drum stopped.

(5) 0.5 c.c. fluid from bath B. Marked fall in B.P. 36 mm. Hg.

(6) Drum stopped. Trachea cleaned out.

(7) 0.2 c.c. bath fluid. Fall in B.P. 16 mm. Hg.

(8) 0.5 c.c. eserine solution 1:1,500.

(9) 0.2 c.c. bath fluid. Fall in B.P. greater than in (7) 24 mm. Hg.

(10) Artificial respiration.

(11) Drum stopped.

(12) Atropine sulphate 1 c.c. 1:1,000 solution intravenously.

(13) Drum stopped.

(14) 0.2 c.c. bath fluid. No effect on blood pressure.

(15) 0.4 c.c. bath fluid. No effect on blood pressure.

(16) 0.2√ acetylcholine. No effect on blood pressure.

(See Trace 7).

Results. (See Trace 7).

It is seen from the protocol of the experiment quoted that the bath fluid has a powerful depressor effect on the blood pressure of the cat. 0.5 c.c. bath fluid had a depressor effect equal to that of 0.26√ acetylcholine, i.e. 36 mm. Hg.

The depressor activity of the bath fluid resembles that of acetylcholine in that it was enhanced by eserine and abolished by atropine.

0.2 cc. bath fluid depressed the blood pressure. 16 mm. Hg.

After eserine it caused a depression of 24 mm. Hg.

Using the two preparations described it was possible to determine quantitatively the amount of acetylcholine which was liberated and remained stable in the bath fluid. The results were calculated per unit weight of wet intestinal tissue. The results obtained using the frog's rectus muscle are compared in the table given below with those obtained when the blood pressure of the cat was used as a biological indicator. The calculations were carried out as follows:-

The frog's rectus muscle preparation.

Weight of rabbit's bowel used 6.5 g.

Volume of fluid in bath 100 c.c.

Acetylcholine equivalent, 10 c.c. fluid = 0.3√ acetylcholine (See Trace 4, p.68).

Acetylcholine equivalent, 100 c.c. fluid = 3√ acetylcholine.

6.5 g. bowel yielded 3√ acetylcholine.

1 g. bowel yielded 0.46√ acetylcholine.

Blood pressure of the cat.

Weight of rabbit's bowel used 6.3 g.

Volume of fluid in bath 50 c.c.

Acetylcholine equivalent, 0.5 c.c. = 0.26√ acetylcholine.
(See Trace 7, p.70).

Acetylcholine equivalent, 50 c.c. = 2.6√ acetylcholine.

6.3 g. bowel yielded 2.6√ acetylcholine.

1 g. bowel yielded 0.4 √ acetylcholine.

The frog's rectus muscle preparation. Cat's bowel used.

Weight of cat's bowel segment 14.4 g.

Volume of fluid in bath 200 c.c.

Acetylcholine equivalent, 20 c.c. = 0.6√ acetylcholine.
(See Trace 6, p.69).

Acetylcholine equivalent, 200 c.c. = 6√ acetylcholine.

14.4 g. bowel tissue yielded 6√ acetylcholine.

1 g. bowel tissue yielded 0.41√ acetylcholine.

TABLE 2.

Showing the acetylcholine equivalents per unit weight of wet intestinal tissue obtained in the experiments reported in this Thesis.

Method of standardisation.	Animal used.	Tissue.	Acetylcholine equivalent (√per g.of wet tissue)
Blood pressure of a cat	Rabbit	Jejunum	0.4
Frog's rectus muscle	Rabbit	Jejunum	0.46
Frog's rectus muscle	Cat	Jejunum	0.41

TABLE 3.

Showing the results obtained after biochemical extraction of the intestinal tissues.

Author quoted.	Animal used.	Tissues.	Acetylcholine equivalent (✓ per g. of wet tissue).
Chang and Gaddum (1933)	Rabbit	Jejunum	4, 2.8
Plattner and Tsudzimura (1935)	Cat	Jejunum	1.4

From these figures, the acetylcholine equivalent per gramme of wet intestinal tissue taken from the rabbit, corresponds in the two methods of estimation used. The activity of the bath fluid, thus, appears to be due to acetylcholine itself. Segments of bowel taken from cats and ducks also liberated acetylcholine into the bath fluid when treated in a similar manner. Quantitative estimations and comparisons were not carried out when the bowel of these animals was used. The acetylcholine equivalents of the intestine of rabbits and cats determined by Chang and Gaddum (1933) and Plattner and Tsudzimura (1935) after chemical extraction of the tissues are higher, and in no way comparable with those I have obtained in the experiments herein reported.

The final conclusion derived from these experiments is that the isolated segment of bowel liberates acetylcholine into the bath fluid in which it is suspended and that this acetylcholine can be demonstrated biologically if the action of the choline esterase be inhibited by eserine.

The data derived from these experiments in support of this

view may be summarised as follows:-

(1) The bath fluid has the power of producing a contraction of the eserinised muscle of the frog.

(2) This action is diminished if the muscle be not eserinised prior to the test.

(3) The activity of the fluid is lost on standing at room temperature (24 hours).

(4) The activity is lost if the fluid be treated with alkali.

(5) The activity on the frog's muscle is abolished by nicotine but not by atropine.

(6) The fluid has an excito-motor effect on the isolated segment of rabbit's bowel.

(7) The fluid depresses the blood pressure of the cat. This activity is enhanced by eserine and abolished by atropine.

(8) The quantitative standardisations of the acetylcholine equivalent per unit weight of bowel tissue correspond in the different methods used.

THE ACTION OF ESERINE SULPHATE ON THE BOWEL TISSUE AND ITS RELATION TO THE ABOVE CONCLUSION.

The action of eserine on the bowel tissues must be considered before the conclusions obtained in the preceding experiments can be unreservedly accepted. That eserine inhibits the choline esterase has been established and it was for this purpose that it was used. As mentioned on page 59 Braecke and de la Cuesta (1933) performed experiments in which they estimated quantitatively the amount of choline which diffused from a segment of bowel before, and after, the animal from which the bowel was taken had received a subcutaneous injection of eserine. Dogs were used, and eserine 0.5 mgm. per kg. was given subcutaneously. They noted that an increased amount of choline diffused from a segment of bowel after the eserine had been given. They used the isolated segment taken from a rabbit as their assay preparation. They did not test for the presence of choline, but acetylated all their products, and estimated the quantities of choline in terms of acetylcholine equivalent.

The results obtained in the experiments reported here were not due to this increased diffusion of choline from the bowel segment. This is shown by the following facts:-

(1) The non acetylated bath fluid was found to cause a contraction of the rectus muscle of the frog. Comparatively high concentrations of choline would be required to do this. The activity on the frog muscle was sensitised, somewhat, by eserine.

(2) The activity of the fluid on the frog muscle was lost on standing for 24 hours at room temperature: choline is stable under such conditions.

(3) The depressor activity on the blood pressure of the cat was sensitised by eserine. A choline effect is not so influenced.

(4) The activity on the rectus muscle disappeared after treatment with alkali. Choline activity is not so affected (Velhagen jr., 1931).

One may state, therefore, that the effects, which I have termed acetylcholine-like, given by the bath fluid, were not caused by choline from the bowel segment.

Acetylcholine was then present in the bath fluid. The question now arises: Was the eserine responsible for the actual appearance of acetylcholine or only for its preservation? Feldberg and Kwiatkowski (1934) have shown that if a physiological saline solution containing acetylcholine 1:20,000,000 be perfused through the superior mesenteric artery to the small intestine of a cat, then the venous effluent shows no acetylcholine-like activity. They thus further demonstrated that the bowel has the power of hydrolysing solutions of acetylcholine of a strength in excess of that found in the portal blood, or in the bath fluid, when eserine was added. Eserine thus appears to act by inhibiting the choline esterase.

The bowel wall contains within its substance the nerve plexuses of Meissner and Auerbach. Eserine could possibly have acted by stimulation of the nerve cells present in these plexuses and

caused the liberation of acetylcholine. No direct proof that this does not occur can be brought forward. However, one notes in the experiments demonstrating the presence of an acetylcholine substance in the venous effluents from the inferior mesenteric and superior cervical ganglia that in each case the ganglia were perfused with saline containing eserine. In no case did acetylcholine appear in the venous effluent until after stimulation of the preganglionic nerve fibres. One may conclude therefore that in the case of the bowel segment the eserine does not stimulate the ganglion cells and so cause the liberation of acetylcholine.

It would appear, therefore, that acetylcholine in minute amounts is normally built up in the wall of the intestine. The acetylcholine so formed is active at the site of its formation but is rapidly hydrolysed into the relatively inactive choline and acetic anhydride by the choline esterase present in the blood and intestinal tissues.

B. THE PHYSIOLOGICAL SIGNIFICANCE OF THE PRESENCE OF ACETYLCHOLINE IN THE TISSUES OF THE SMALL INTESTINE.

I now propose to consider the relation between the acetylcholine formed in the bowel wall and the motor activity of the bowel. Two subsidiary questions arise. These are:- (a) Which tissue present in the bowel wall is actually responsible for the formation or appearance of acetylcholine in the tissues? (b) Upon what tissue does acetylcholine act to produce its powerful excitomotor effect?

(a) Which tissue present in the bowel wall is actually responsible for the formation or appearance of acetylcholine in the tissues?

Feldberg and Rosenfeld (1933) held the view that the ganglion cells present in the nerve plexuses of the bowel wall were able to cause the liberation of acetylcholine in a similar manner to that in which stimulation of parasympathetic nerves causes its liberation.

The relation of the ganglion cells in the nerve plexuses of the bowel wall to the vagus and splanchnic nerves is a complex one. I propose to discuss this relation briefly, and consider the evidence showing that the ganglion cells in the enteric plexuses are really the excitor neurones of the bulbo-sacral division of the autonomic nervous system and therefore like them capable of causing the liberation of acetylcholine.

The autonomic nervous system is made up of three elements, in a similar manner to the somatic nervous system. These are, the

afferent neurone, the connector neurone and the excitor or effector neurone. The connector neurones in the autonomic system are situated in the central nervous system, whilst the excitor neurones are placed peripherally in the organism. The excitor neurones of the bulbo-sacral division are placed actually in the substance of the organsthey supply or closely applied to them. The ganglion cells present in the enteric plexuses of Auerbach and Meissner are regarded by some workers as representing the excitor neurones of the vagus and sacral nerves. Others hold the view that the two nerve plexuses form a separate peripheral nervous system, having connections with the rest of the autonomic system, but not forming an integral part of it.

Morphological and experimental evidence supports the former view. Thus Abel (1909) and Kunz (1910) has each been able to show that the nerve cells of the intestine in the early stages of development are not in the substance of the intestine but 'travel' into it in the course of development. Both workers state that all the cells which enter the intestinal wall are derived from vagal cells originally placed in the central nervous system. They state that no cells reach the intestine from the sympathetic (thoraco-lumbar) system. Alcock, working with Ammocoetes, found in the intestine groups of nerve cells of an ordinary type; these were visible without any special staining methods, and were in close connection with the fibres of the vagus nerves. These cells she considered to be the excitor cells of the vagus system, and analogous to the nerve cells found within the wall of the intestine in the higher forms of life. It would appear therefore that the

ganglion cells present in the bowel wall of the higher vertebrates are vagal in origin.

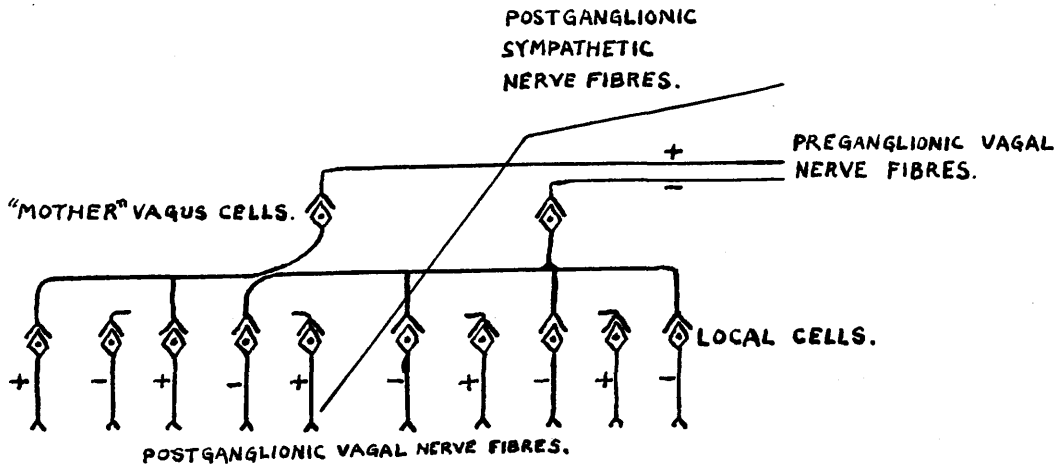
Johnson (1925) conducted experiments in which he produced degeneration of the extrinsic nerves of the small intestine in order to study their relation to the structure of the enteric plexuses and nerve cells. He noted two types of nerve fibres in the intercellular plexuses:- (1) Small extrinsic non medullated fibres. These he was able to trace to cell groups in both the myenteric and submucous plexuses. Here they formed fine intercellular plexuses similar to those found in other sympathetic cell groups. (2) The second type consisted of large fibres arising from the local ganglia. These he traced to terminations in the smooth muscle fibres of both the circular and longitudinal layers.

Johnson (1925) sectioned the vagus nerves of a cat and allowed them to degenerate. He then examined the nerve plexuses in the bowel. He found that the fibres of the second type were not affected, whereas those of the first type were degenerated. He considered that the former were the axons of the vagal excitor cells and the latter their preganglionic fibres. He also noted that fine non medullated nerve fibres were present. These he considered represented the postganglionic fibres of the thoraco-lumbar excitor ganglia. If the splanchnic nerves were sectioned, and given time for degeneration to occur, these fibres were no longer visible.

The conclusion reached is that the gastro-intestinal autonomic innervation is analogous with that of other organs. The preganglionic vagal fibres run from the fourth ventricle into the

substance of the bowel wall, and synapse with the ganglion cells present there. These cells are <u>all</u> excitor vagal neurones. The short postganglionic vagal fibres arise from these and travel to the visceral muscle cells. Only the postganglionic fibres of the thoraco-lumbar system enter the enteric plexuses.

Langley (1922) has suggested that 'mother vagus cells' exist in the bowel wall. The preganglionic fibres of the vagus synapse with these 'mother cells' and fibres arise from these and synapse with the very numerous ganglion cells present in the plexuses. The following diagram modified from Langley represents the view held of the structural identity of the nerve plexuses present in the bowel wall.



The ganglion cells in the enteric plexuses are thus seen to be vagal in origin. It was to be expected therefore that they would resemble the vagal cells in the heart and lungs and that the transmission of their nerve impulses would be accompanied by the liberation of acetylcholine. Dale and Feldberg (1934, a) and Maaske, Making and Bunting (1935) have, in fact, shown that stimulation of the vagus nerve to the stomach and intestine, respectively, is accompanied by an increased amount of acetylcholine in the venous effluents from these organs. Whether the ganglion cells in the bowel wall are responsible for the appearance of acetylcholine in the absence of all extrinsic stimuli cannot be definitely decided at present. It can only be noted that when extrinsic stimuli do affect these cells they are capable of causing the liberation of acetylcholine. One notes that, if the ganglion cells are capable of causing the liberation of acetylcholine, in the absence of all extrinsic stimuli, then they are unique, in this respect, in the animal body.

The other possibility which must be considered is that the formation of acetylcholine occurs in the intestinal tissues as a pure biochemical process. As will be detailed later, choline and choline esterase are both abundantly present in the tissues. One must consider whether the environmental conditions in the tissues of the bowel are favourable to the synthesis of acetylcholine. The synthesis of acetylcholine, from sodium acetate and choline in the presence of the choline esterase is possible (see page 42). The reaction is very slow and the yield very small. Beyond acting as an indicator, the laboratory synthesis of acetylcholine throws

no light on its presence and possible synthesis in the tissues of the bowel wall under physiological conditions.

(b) Upon what tissue does acetylcholine act to produce its powerful excito-motor effect?

If one accepts the view that the ganglion cells in the enteric nerve plexuses are responsible for the appearance of acetylcholine then it is evident that the acetylcholine demonstrated must be formed at the postganglionic synapse; it would thus act directly on the visceral muscle cells.

Generally, the actual proof that the postganglionic synapse is the site of formation or liberation of acetylcholine is very difficult. This is because of the shortness of the postganglionic fibres in the bulbo-sacral division of the autonomic nervous system.

The postganglionic fibres to the eye arise in the ciliary ganglion and are amongst the longest known. Englehart (1931) showed that impulses passing along these nerves to the constrictor muscle of the pupil were transmitted by an acetylcholine-like substance. The preganglionic synapses were not included in the perfused area and therefore he concluded that the acetylcholine substance found in the venous effluent was formed at the postganglionic synapses. He was not able however to stimulate the postganglionic fibres directly but used the preganglionic fibres present in the oculomotor nerve. The sequence in the transmission of the stimuli may be crudely represented as follows:-

Preganglionic fibre ⟶ synapse ⟶ postganglionic fibre ⟶ acetylcholine ⟶ effector cell.

When most other organs are perfused through the main artery, the venous effluent obtained has drained from an area which includes both the preganglionic and post-ganglionic bulbo-sacral synapses. It is not possible, therefore, to determine the exact site of liberation of any acetylcholine detected in the venous effluents.

The work of Feldberg and Gaddum (1934) and of Barsoun, Gaddum and Khayyal (1934) demonstrating that the excitation of the preganglionic fibres to the superior cervical and inferior mesenteric ganglia respectively liberates acetylcholine at these preganglionic synapses suggests the following sequence:-

Preganglionic fibre ⟶ acetylcholine; synapse ⟶ post-ganglionic fibre ⟶ effector cell.

If this sequence is generally applicable then it is seen that acetylcholine would not act on the effector visceral muscle cell directly but upon the preganglionic synapse.

The problem has been attacked from another angle. If acetylcholine be formed at the postganglionic synapse then it must, like

adrenaline, act directly on the muscle or effector cell. If it be formed at the preganglionic synapse then it must act on the effector cell through the mediation of the excitor neurone. As regards the bowel segment, it is of interest to know if acetylcholine acts directly on the visceral muscle in order to assess the importance of the neural elements present in the control of the intestinal movements. In the adult vertebrate it is almost impossible to denervate completely viscera of their bulbo-sacral autonomic supply.

Gasser (1926) prepared plexus-free preparations of visceral muscle taken from the intestine. He showed that such preparations were sensitive to acetylcholine and concluded that acetylcholine acts directly on the muscle cells. The method of separation of the visceral muscle from the nerve elements was that of Gunn and Underhill (1914). Although the preparations were all checked histologically for the presence of nerve elements the method is not considered absolutely reliable at present.

Plattner and Hou (1931) studied the reactions of the heart of the chick embryo to acetylcholine. In an embryo of 70-90 hours the vagus cells and postganglionic fibres have not yet migrated into the heart. In such a heart they noted a marked decrease in the heart rate when acetylcholine was applied. They concluded that acetylcholine acts directly on the effector cells and is produced at the postganglionic synapse.

This constitutes the generally accepted view and the deductions drawn from the experiments which I have performed are in accordance with this view.

The question cannot be taken as settled because the conclusions of several workers are at direct variance to this view. Markowitz (1931) tested the action of acetylcholine on explanted cardiac tissue in culture, taken from chick embryos. Explants from embryos less than six days old which had no innervation, she found, did not react to acetylcholine. Those of six days and over, being innervated, did react. Armstrong (1935) found that the injection of relatively large amounts of acetylcholine did not diminish the amplitude of contraction of the heart of Fundulus Majalis embryos, until the nerves had reached the auricle. He concluded, as did Markowitz, that acetylcholine produces its vagus-like effects by stimulating the vagus postganglionic fibres and not by acting directly on the cardiac muscle.

The most recent work on this subject is that of Cattell and Wolff (1935). They removed the ciliary ganglion in cats. After removal, the iris still responded, by contraction, to 0.1 c.c. 1:1,000,000 acetylcholine injected into the anterior chamber of the eye. Six to twelve days later, when the postganglionic nerves had degenerated, the pupil was constantly dilated, and the injection of the same quantity of acetylcholine still brought about the same qualitative and quantitative result. These workers were cognizant of the work of Armstrong, and they concluded by saying that "it would appear that the physiological properties of a muscle (adrenergic or cholinergic) are bestowed by the proximity of the special nerve fibres and that once acquired the specific sensitiveness is retained for a time after the degeneration of the nerve fibres."

Taking these general results into account it is seen that, as regards the small intestine, the conclusion that acetylcholine acts directly on visceral muscle fibres is in accordance with the main bulk of evidence on this subject.

Summarising one may state that acetylcholine is formed in the bowel tissues either at the postganglionic synapses or as a result of an enzyme catalysed synthesis and that it acts directly on the visceral muscle cells of the intestinal segment.

C. THE ACTION OF ACETYLCHOLINE ON THE GASTRO-INTESTINAL TRACT.

Dale (1914, b) noted the marked effect of acetylcholine on the gastro-intestinal tract. On the isolated intestine of rabbits he showed that a distinct augmentation of motor activity resulted to acetylcholine in a dilution of 1:500 x 10^6 in the bath fluid. Given intravenously acetylcholine, although most potent in depressing the blood pressure, had only a slight effect on the gastro-intestinal tract. This is because of the rapid inactivation which occurred by means of the choline esterase. This fact vitiates the claims put forward for acetylcholine in the treatment of paralytic ileus and allied intestinal conditions by the intravenous or intramuscular injection of this substance.

Frank, Zimmerman and Necheles (1934) demonstrated in dogs the slight and evanescent action of acetylcholine on the gastro-intestinal tract, when given intramuscularly. However, after an intramuscular injection of eserine (1 mgm. to a 25 lb. dog), itself insufficient to affect the tract, the same dose of acetylcholine given intramuscularly had a powerful excito-motor effect on the tract. Clinically this combination of eserine and acetylcholine is not to be recommended, the marked effect produced on the blood pressure being distinctly dangerous.

In attempting to assess the action of acetylcholine produced in the bowel itself, greater help is obtained from a consideration of the action of acetylcholine on the bowel segments under 'in vitro' conditions. The striking excito-motor effect of acetylcholine under these conditions leads one to believe that

acetylcholine formed in the bowel tissues themselves must play an important part in maintaining the tone and segmental movements of the small intestine. Prasad (1936) has shown that the action of acetylcholine on the intestine is a fundamental one and not markedly influenced by other metabolic changes in the tissues. Thus gut muscle, the movements of which had been abolished by asphyxia, under 'in vitro' conditions, still responded to acetylcholine. Gut poisoned by iodo acetic acid also responded to the addition of acetylcholine.

Acetylcholine also influences the absorptive power of the intestinal mucosa. Gellhorn and Northup (1933, a) studied the effects of acetylcholine on the absorption of glucose from the intestinal tract of the frog. They perfused an isotonic glucose solution through the lumen of the tract and perfused the intestinal blood vessels with Ringer solution, to which they added acetylcholine. The rate of perfusion was maintained constant. They found that the addition of acetylcholine, to make a high concentration in the saline (1:50,000 to 1:2,000,000), decreased the permeability of the cells and the absorption of glucose from the lumen of the bowel. Using low concentrations of acetylcholine (1:20,000,000 to 1:40,000,000) they found that the cell permeability and absorption of glucose were increased. Later (1933, b) they showed that stimulation of the vagus nerve to the intestine was capable of producing the same biphasic effect.

Acetylcholine, formed in the tissues of the bowel, possibly plays a part in the control of the blood vessels of the intestine. Necheles and co-workers (1936) investigated the effect of acetyl-

choline on the blood vessels of the stomach of the dog in both 'in vitro' and 'in vivo' preparations. They found that small doses of acetylcholine caused distinct and prolonged vasoconstriction and that the application of larger doses produced vasodilatation. The estimations were not carried out during gastric contractions. They considered that the smaller doses of acetylcholine used in the experiments were the more physiological and that the acetylcholine normally formed in the tissues would cause vasoconstriction. That acetylcholine is constantly present in the venous blood coming from the stomach was shown by Feldberg and Rosenfeld (1933). Necheles and his co-workers therefore suggested that the vasoconstrictor effect of acetylcholine may be an important factor in the genesis of ulcer in such areas in which end arteries are found, as in the distal part of the lesser curvature of the stomach and in the duodenal cap. As regards the intestine, Kohn and co-workers (1936) have found that perfusion of isolated segments of mesenteric arteries and veins from dog and man resulted in constriction when acetylcholine was added to the perfusion fluid.

The evidence presented above shows the action of acetylcholine when it is added to the physiological fluids batheing the intestine. I consider that one is justified in drawing conclusions from this evidence as to the effects of acetylcholine, normally formed in the bowel, on the intestine. These can be summarised as follows:- Acetylcholine formed in the tissues of the small intestine augments considerably the motor activity of the bowel.

It affects the cellular permeability of the intestinal mucosa and has a vasomotor effect on the mesenteric vessels. The amounts, which exist physiologically, will according to present evidence increase the permeability of the intestinal mucosa and the absorption of glucose and cause constriction of the blood vessels of the intestine.

As mentioned heretofore, acetylcholine is formed in the bowel wall even in the absence of all extrinsic nerve stimuli. Acetylcholine is constantly being produced in the bowel wall. As this is the only site in the body where this occurs, attention has been paid to the suggestion that the gastro-intestinal tract is the producer of this important 'hormone' for the whole organism. This could only occur, however, if acetylcholine was carried and stored in the body tissues in a hidden inactive state.

THE MYOGENIC OR NEUROGENIC ORIGIN OF THE SEGMENTAL MOVEMENTS OF THE SMALL INTESTINE.

Magnus (1904, b and c) suggested that the segmental movements exhibited by the intestine depended on the integrity of the nerve elements present in the intestine. Using an isolated segment of rabbit's bowel he stripped the longitudinal muscle from the circular muscle. The circular muscle was thus stripped of its nerve elements. He found that such a muscle preparation did not exhibit rhythmic contractions and thus reached the above conclusion. Gunn and Underhill (1914), Alvarez and Mahoney (1922) and Gasser (1926) have been able to obtain strips of circular intestinal muscle, which they considered free of nerve elements, and which exhibited rhythmic contractions. These workers concluded that the segmental movements of the intestine were exhibited as the result of the inherent power of the visceral muscle, i.e. that the movements were myogenic. v. Esveld (1928) has critically examined the results of these workers. He stated that the muscular strips accepted by the above workers, as being free of nerve cells, did in fact contain nerve elements. Their conclusion was, therefore, not to be relied upon. Further, he pointed out that the intestine contains a nervous syncytium, distinct from the elements of the bowel plexuses. This nervous syncytium is made up of the "Interstitielle Zellen" described by Cajal (1933). However, the weight of evidence at present appears to favour the view that the rhythmic intestinal movements are myogenic.

The fact that the isolated segment of bowel constantly

produces a powerful excito-motor substance, acetylcholine, may explain the power of intestinal muscle of contracting rhythmically. Magnus (1904, c) pointed out that the visceral muscle of the intestine has a quite considerable refractory period during which it will not respond to stimuli. It is thus appreciated that the intestinal muscle would respond to a constant stimulus, such as minute amounts of acetylcholine, by exhibiting rhythmic contractions. The respiratory centre in the fourth ventricle responds to the constant stimulus of carbon dioxide in the blood with rhythmic discharges in a similar manner.

It is not known whether intestinal visceral muscle, freed from nerve elements, is capable of liberating acetylcholine. No weight is therefore added to either the myogenic or neurogenic theories of the origin of the segmental movements of the intestine.

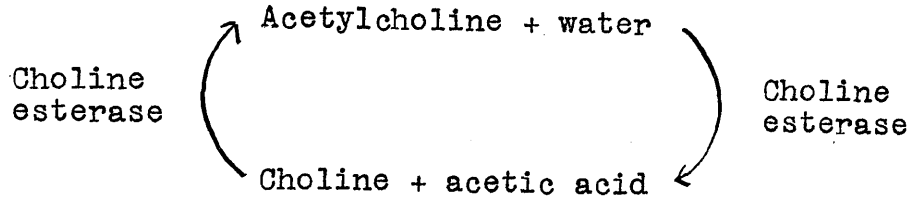
D. THE CHOLINE ESTERASE AND ITS RELATION TO INTESTINAL MOTOR ACTIVITY.

Choline was detected in crude extracts of intestinal tissue by Furth and Schwarz (1908). Le Heux (1918) found it in the bath fluid in which an isolated segment of rabbit's intestine had been suspended. Choline thus exists in the bowel tissues in a free and diffusible state.

Chang and Gaddum (1933) were able to demonstrate the choline ester, acetylcholine, in extracts of bowel tissue. Acetylcholine has also been demonstrated in the blood and physiological fluids which bathe the intestinal tissues. It, therefore, also exists in the tissues in a free and diffusible state. It is not considered that the choline found in the bath fluid by Le Heux was derived solely from the hydrolysis of this acetylcholine: it is admitted that it must, in part, have been derived in such a manner.

The hydrolysis of acetylcholine into choline and acetic anhydride is catalysed by the choline esterase. Plattner and Hintner (1930) have shown that this enzyme is abundantly present in the tissues of the bowel. Further, the work of Abderhalden and Paffrath (1926) has shown that an enzyme is present in the tissues of the intestine which facilitates the synthesis of acetylcholine from choline and acetic anhydride. They demonstrated that this enzyme also catalysed the hydrolysis of acetylcholine. It thus played a part in both phases of this reversible action.

It would appear from the facts given above that the following reversible enzyme catalysed reaction possibly occurs in the bowel tissues:-



Each of these reactions has a velocity constant under standard conditions and one must consider whether a state of equilibrium exists physiologically in the tissues. From the data at present available it would appear that no such equilibrium is attained in the bowel tissues, and that the tendency of the reaction favours the hydrolysis of acetylcholine considerably. Thus the experiments demonstrating the synthesis of acetylcholine (see page 42) have all been performed with comparatively high concentrations of the substrates, and even then, the yields were low. No information is available as to the velocity of this reaction. The velocity of the hydrolysis of acetylcholine is comparatively high.

If an equilibrium was reached one would expect to find in the tissues a constant relation between the quantity of choline and that of acetylcholine in the various organs of the body. Plattner and Tsudzimura (1935) studied this question. They found no such relation, and found no correlation between the amount of choline and that of the choline esterase present in any organ. One may conclude, therefore, that no acetylcholine:choline equilibrium exists in the tissues of the body and that the tendency of the reaction is towards the hydrolysis of acetylcholine. Acetylcholine is formed in the bowel wall and has a powerful excitomotor effect on the visceral muscle. It is rapidly inactivated by hydrolysis in the bowel tissues and is normally not liberated as such into the circulation.

EXPERIMENTAL INVESTIGATION OF THE EFFECTS OF THE CHOLINE ESTERASE ON INTESTINAL MOTOR ACTIVITY.

My own experiments have been directed towards investigating the effects on the acetylcholine:choline reaction present in the bowel tissues, of the addition of powerful enzymatic concentrations. The effects were assessed by the changes in intestinal motor activity. If the hydrolysis of acetylcholine is increased in rate then one would expect a diminution of motor activity and vice versa. The effect on the rate of hydrolysis of acetylcholine of increasing the concentration of the choline esterase has not been worked out. We can deduce from the general law of Michaelis and Menten (1913) that an increased concentration will cause an increased rate of hydrolysis as long as the quantity of the enzyme is small. To alter the tendency of the reaction towards the synthesis of acetylcholine one would require to increase the concentrations of the substrates.

In this series of experiments, changes in intestinal activity are taken as an indicator of the increased rate of hydrolysis or synthesis of acetylcholine. Certain evidence will be presented pointing to the conclusion that both reactions can be facilitated. The factors determining the direction in which the facilitation will occur, have not been determined. It is noted, however, that in the majority of cases the changes were such as to indicate that the rate of hydrolysis of acetylcholine increased.

Procedure. The choline esterase was obtained in concentrates of horse's serum, by the method described by Stedman, Stedman and

Easson (1932). 100 g. of ammonium sulphate was dissolved in 500 c.c. blood serum of the horse. The precipitate was removed in the centrifuge, washed with a small volume of an ammonium sulphate solution (20 g. to 100 c.c. water) and the mixture again centrifuged. The concentration of ammonium sulphate in the combined centrifugates, which measured approximately 600 c.c., was increased to 40 g. per 100 c.c. by the addition of 23 g. of the solid salt per 100 c.c. fluid.

The solution was rendered faintly acid to litmus paper by the addition of 35-40 c.c. of N/2 acetic acid. The solution was left overnight in the refrigerator. The precipitate which settled was centrifuged off, the liquid being rejected. The precipitate was then stirred in the centrifuge glasses with about an equal volume of a solution of 35 g. ammonium sulphate in 100 c.c. of water. After standing overnight, the suspension was again centrifuged, and the clear liquid poured into a flask. A crystalline precipitate, probably albumin, separated in the course of a few hours. This was removed and the concentration of ammonium sulphate in the liquid which measured 170-200 c.c. was increased to 40 g. per 100 c.c., by the addition of 5 g. of the solid salt. 2.5-3 c.c. of N/2 acetic acid was now added to render the solution faintly acid.

A precipitate separated: after 12 hours, this was removed and dissolved in a small volume of water. This was dialysed against Tyrode Bayliss solution for 24 hours. The dialysate was found to be free of sulphate after the dialysis. A concentrate of approximately 40 c.c. was obtained from 500 c.c. of serum.

Concentrates so prepared had a choline esterase activity seven times that of the original horse serum. The concentrates prepared were stored in the refrigerator until the time of use.

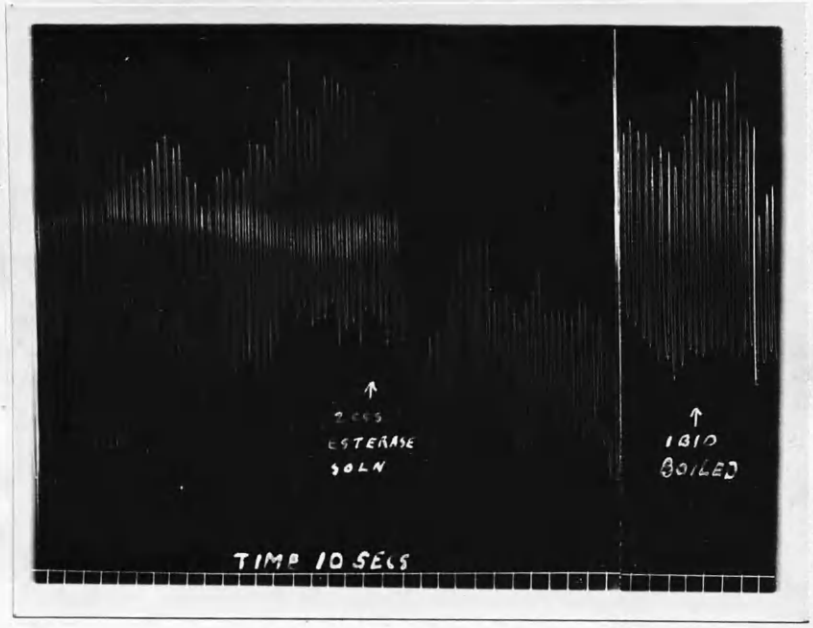
The effects of such concentrates on the motor activity of an isolated segment of bowel were tested as follows:-
A segment of jejunum two inches in length was taken from a rabbit and suspended in the bath of a mammalian bowel apparatus. The bath contained 50 c.c. Tyrode Bayliss solution, aerated with alveolar air and the temperature maintained constant at 37°C. The movements of the segment were recorded. The preparation was not used until one hour after the bowel had been suspended. This delay was necessary because the segmental movements and tone of the segment vary considerably during the first hour and were thus not suitable for testing purposes. After this delay the motor activity was stabilised and the effects of esterase concentrates added to the bath fluid could be investigated. The enzyme concentrates were warmed gently to 37°C. prior to their addition to the bath fluid.

In order to confirm that the results noted were due to enzyme activity of the concentrate, they were boiled to destroy their enzyme activity and then used. 5 c.c. of an enzyme concentrate was kept in a boiling water bath for fifteen minutes. It was then filtered, allowed to cool to 37°C. and then added to the bath fluid. The usual dose of the concentrate added to the bath was 1 c.c.

Results. (See Traces 8, 9 and 10).

Five different esterase preparations were tested and the results are tabulated below:-

First preparation.	5/11/35.	1 c.c. concentrate used. Marked diminution of tone and segmental movements. Applied twice.
	6/11/35.	1 c.c. used. Fall in tone and amplitude of movements again noted.
Second preparation.	19/11/35.	On three occasions after a preliminary rise in tone a fall in tone and amplitude of movements resulted.
Third preparation.	26/11/35.	On three occasions an increased amplitude of segmental movements resulted without any change in tone.
Fourth preparation.	5/12/35.	Slight diminution in the amplitude of movements.
	6/12/35.	Diminution of amplitude of segmental movements with each successive addition of esterase. When the amplitude was small, then further additions of enzyme concentrate resulted in an increase in the segmental movements.
	7/12/35.	When the amplitude of movement was large, addition of esterase resulted in diminution of the amplitude. When the amplitude was small, an increase resulted.
Fifth preparation.	14/12/35.	Marked fall in tone and amplitude of movements.
	15/12/35.	With three applications, marked decrease of motor activity. Boiling of the concentrate inactivated it.



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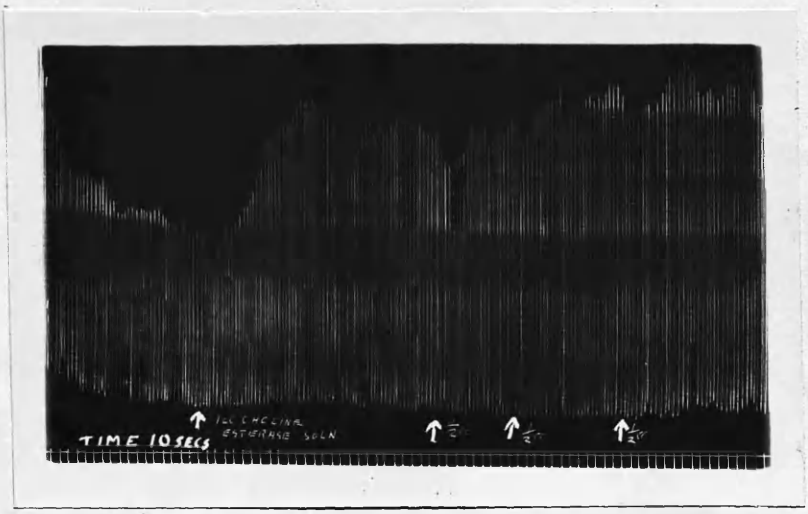

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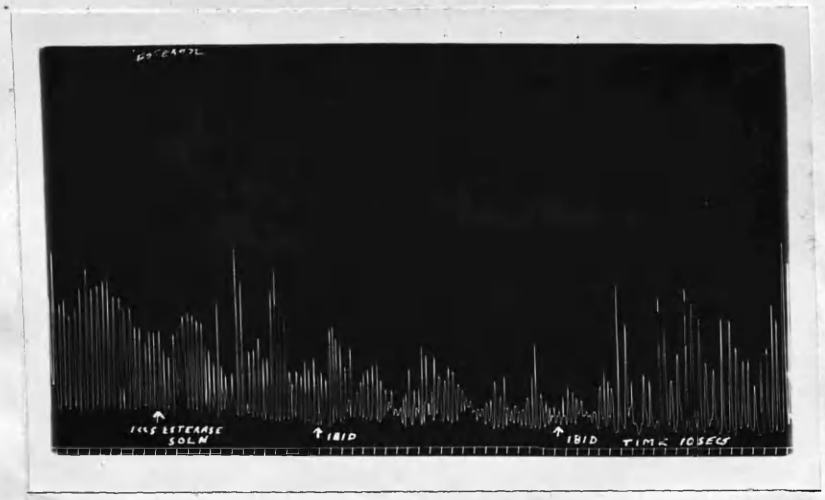


TRACE NUMBER TEN.

Fifth preparation (contd.)	17/12/35.	Above result obtained. Inactivated by boiling.
	18/12/35.	Fall in tone on two occasions (2 c.c.). 1 c.c. concentrate - no effect.

It was noted that of the twenty four additions of the esterase concentrate to the bath fluid, a diminution in motor activity of the intestinal segment occurred on seventeen occasions (See Trace 8). On six occasions an increase in motor activity was noted (See Trace 9).

Boiling of the enzyme concentrate destroyed the activity and is taken as proof that the activity noted in the other preparations was due to their enzyme content (See Trace 8). The concentrate, after being dialysed against Tyrode Bayliss solution, was isotonic with the bath fluid and could not influence the bowel by altering the ionic concentration of any of the salts in the bath fluid.

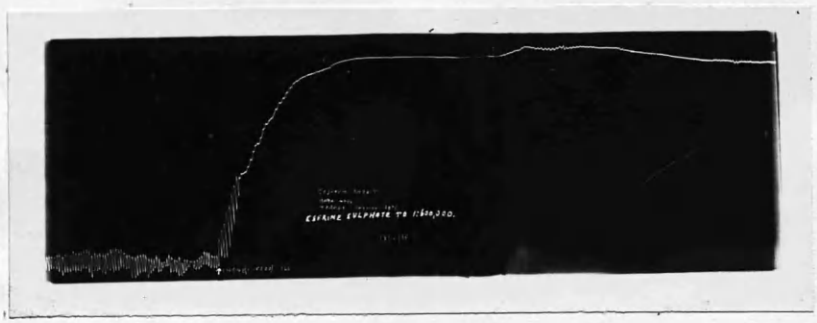
The different reactions of the bowel segment to the addition of the concentrates could not be accounted for by any differences in the five preparations used. As seen with the fourth preparation, the one preparation may cause different reactions with different segments of bowel, or with the same segments at different times (See Trace 10). The cause of the different reactions of the bowel segments to the addition of the choline esterase concentrates must lie in the bowel itself. The factors responsible are not known but it has been noted that, in general, when the tone of the segment was low, the concentrate increased the motor activity and vice versa.

The results show that in the majority of instances if concentrates of horse's serum rich in the choline esterase be added to the bath fluid in which a segment of bowel is suspended, then the rate of hydrolysis of acetylcholine is apparently increased. This is shown by the decrease in the motor activity of the bowel segment, this latter being strongly influenced by the acetylcholine, or absence thereof, present in its tissues.

E. THE ANTAGONISM BETWEEN THE ACTION OF ESERINE AND THE CHOLINE ESTERASE ON THE INTESTINAL SEGMENT.

The part played by the choline esterase in relation to intestinal motor activity can be demonstrated in another manner. The action of eserine on the bowel wall has already been referred to. Its main action is brought about through the inhibition of the choline esterase which it induces. The following experiments demonstrate that the presence of the choline esterase in the intestinal tissues is essential for the normal motor activity of the intestine. If its action be completely inhibited by eserine it is found that the normal movements are abolished; if in these circumstances concentrates rich in the choline esterase be added to the bath fluid then it is possible in this manner to restore the normal motor activity of an intestinal segment. Matthes (1930) has shown that eserine does not destroy the choline esterase and that there is a quantitative relation between the amount of eserine added to a concentrate and the degree of inhibition of the enzyme activity. It was thus possible to antagonise the effects of eserine on the bowel segment by the addition of serum concentrates, rich in the choline esterase, to the bath fluid.

Procedure. A segment of jejunum taken from a rabbit was set up in a mammalian bowel apparatus. The intestinal movements were recorded. The volume of the bath fluid was 40 c.c. Eserine solution was added to the bath fluid to make a final concentration of 1:600,000. The addition of the eserine as a rule caused such a great increase in the tone of the intestinal segment that the level of the recording



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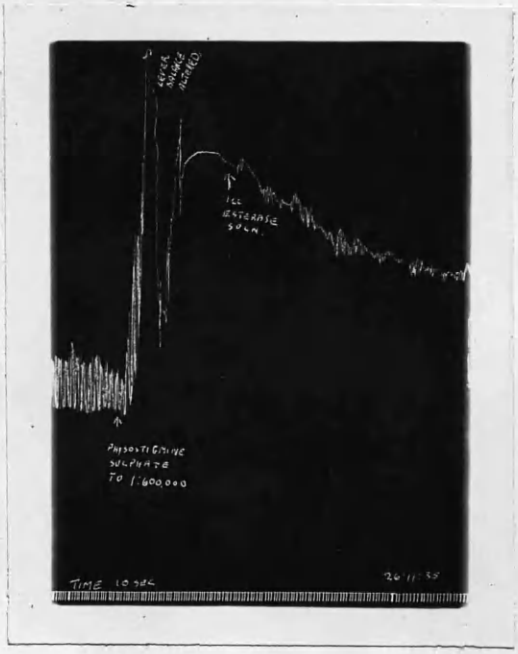


TRACE NUMBER TWELVE.

lever had to be altered and an increased balance weight placed. When the tone of the segment had stabilised itself the esterase concentrates were added to the bath fluid.

Results. (See Traces 11 and 12).

The addition of eserine solution to the bath fluid was followed by an increased amplitude of segmental movements and a great rise in the tone of the bowel segment. As the tone of the segment increased the movements rapidly diminished until finally they were abolished completely. In the control experiment it was noted that after such additions of eserine a bowel segment remained free of segmental movements and with the tone markedly increased for long periods (over 1½ hours) (See Trace 11). That the tissues were living was shown by their reaction to changes in the environmental conditions. Temporary asphyxia resulted in a sharp drop in the muscle tone. Under these conditions if 1 c.c. of the enzyme concentrate be added to the 40 c.c. bath it was found that a progressive fall in the tone of the intestinal segment resulted, and that this was followed by the reappearance of the segmental movements (See Trace 12).

One concludes from these experiments that the presence and activity of the choline esterase in the intestinal tissues is necessary for the maintenance of the normal tone and segmental movements. The action of eserine on the bowel must be exerted mainly in virtue of its inhibitory power on the choline esterase.

F. THE ACTION OF UREA ON THE SMALL INTESTINE OF RABBITS.

The action of various substances on the motor activity of the small intestine of rabbits and other animals can be more fully realised when one takes into account the marked influence of the normally present choline and acetylcholine on this activity. These substances are constantly present in the bowel tissues and exert a powerful influence on the motor activity. In this section of the work I propose to present evidence showing that the action of urea, atropine and eserine on the visceral muscle is brought about by the influence which these drugs exert on the acetylcholine:choline system in addition to any direct action which they possibly exert.

Le Heux(1921,b)repeated the work of Rona and Neukirch (1912), who had investigated the action of numerous organic substances on the motor activity of the isolated rabbit's bowel. As mentioned on pages 11 and 42 Le Heux suggested that the excito-motor activity of some organic salts, especially sodium acetate and pyruvate, was due to the formation of the corresponding choline esters in the bowel tissue. He noted that if the bowel segment was washed repeatedly the organic salts mentioned lost their excito-motor activity. He explained this by assuming that the repeated washings removed the choline from the bowel tissues and thus prevented the synthesis of the active choline esters. Since the work of Le Heux, the importance of acetylcholine in relation to bowel activity has received further attention and his results could be more accurately interpreted by saying that the addition of sodium acetate caused an increased formation of acetylcholine and thus a greater motor activity. Similarly

the addition of sodium pyruvate caused an increase in the quantity of active choline ester present. The work of Abderhalden and Paffrath (1926) also showed that a mechanism is present in the bowel tissues which is capable of causing the synthesis of choline esters.

Kreitmar (1932) prepared the carbamic ester of choline, carbaminoyl choline, and studied its properties. He found it to be a highly active substance with an action closely simulating that of acetylcholine. Chang and Gaddum (1933) compared the activity of carbaminoyl choline with that of acetylcholine (See Table 1, page 33). They confirmed the finding of Kreitmar that this active choline ester is stable in the animal body. It does not undergo rapid hydrolsis in the presence of the choline esterase. It has been suggested that such an ester might exist, naturally, in the body tissues, because of the known rôle that the liver plays in choline metabolism and its ability to form carbaminoyl derivatives, especially urea. No carbaminoyl compound of choline has, however, been detected in the tissues and at present there is no direct evidence in support of the suggestion. That the tissues of the bowel are capable of elaborating choline esters is beyond doubt. I investigated the action of urea on the bowel, in order to investigate whether any evidence exists that a carbaminoyl compound of choline is formed in the bowel tissues.

The pharmacological action of urea does not appear to have received a great deal of attention. Rona and Neukirch (1912) investigated the action of urea on the isolated segment of intestine of the rabbit. They reported that even with a urea concentration of 0.5-1% in the bath fluid, no appreciable effect was produced.

Bernheim (1933) noted the effect of urea on the isolated segment of intestine taken from a guinea pig. He found that if the tone of a segment was raised by the addition of histamine or pilocarpine, then the addition of urea to make a concentration of 1% in the bath fluid caused a marked relaxation in each case. He concluded that urea acted directly on the visceral muscle of the intestine.

THE EXPERIMENTAL INVESTIGATION OF THE EFFECTS OF UREA ON THE MOTOR ACTIVITY OF AN ISOLATED SEGMENT OF BOWEL.

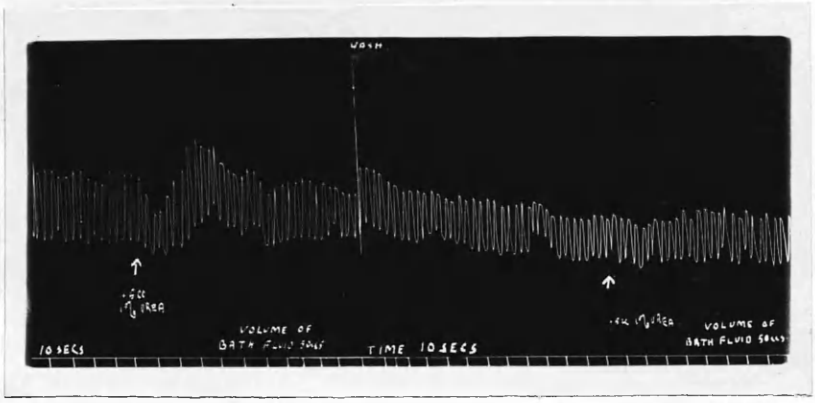
In the following experiments I have found that urea has a definite excito-motor effect on the isolated segment of the rabbit's intestine. It was also found that this effect was dependent on the choline content of the bowel.

Procedure. A segment of jejunum some two inches in length was suspended in a mammalian bowel apparatus. Urea recrystallised (May and Baker) was made up as a 1% solution in Tyrode Bayliss. To reduce the choline and acetylcholine content of the segment the bath fluid was removed and replaced with fresh fluid. This was repeated up till ten times. As choline is present in the bath fluid, and in certain circumstances acetylcholine also is present, the repeated washing of the bowel segment must remove these substances. It is admitted, however, that the repeated washing of the segment has probably other effects besides those mentioned above. To restore the activity of the bowel segment, choline chloride (B.D.H.) 0.5 mgm. was added to the bath fluid.

Results. (See Traces 13, 14, and 15).

A urea concentration in the bath fluid of 1:25,000 (4 mgm. per 100 c.c.) was without any appreciable effect on the motor activity.

A concentration of 1:10,000 (10 mgm. per 100 c.c.) produced a rise in tone and an increased amplitude of the segmental movements of the intestine (See Trace 13).



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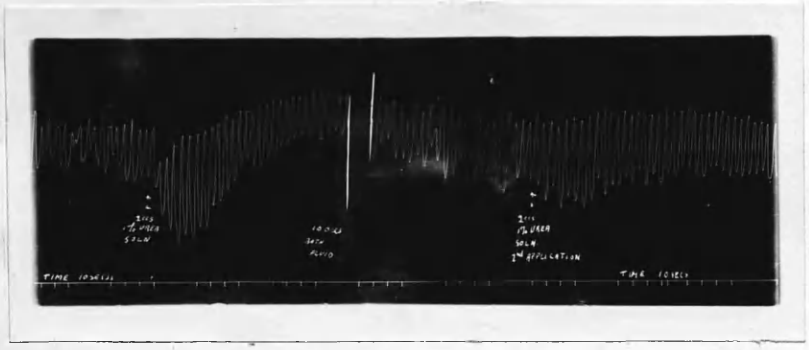

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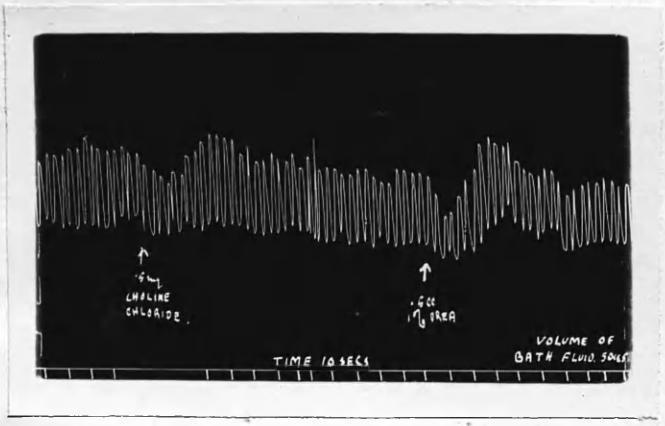

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A concentration of 1:5,000 (20 mgm. per 100 c.c.) produced a marked increase in the tone and the amplitude and frequency of the segmental movements (See Trace 14).

A second application of urea to the bath in a dose equal to that of the first application, i.e. 20 mgm. to 100 c.c. fluid, produced a much reduced reaction of the intestinal segment (See Trace 14).

After the bath fluid had been drained on several occasions, the segment was found to be insensitive to concentrations of urea which had previously produced definite responses (See Trace 13).

The addition of choline chloride to the bath fluid, 0.5 mgm. in 1 c.c. Tyrode Bayliss to 50 c.c. bath fluid, restored the sensitivity of the segment to the application of urea (0.5 c.c. 1% solution); (See Trace 15).

CONCLUSIONS.

Urea in doses of 10-20 mgm. per 100 c.c. of bath fluid has an excito-motor effect on the isolated intestinal segment taken from the rabbit. Repeated washing of the bowel segment removes its sensitivity to urea; the addition of choline chloride to the bath fluid under these circumstances, in addition to stimulating the bowel segment, restores its reactivity to the addition of urea. This leads one to suggest that the excito-motor activity of urea is possibly brought about through the formation, with the choline present in the bowel, of an active carbaminoyl ester of choline. Noll (1932) studied the action of carbaminoyl choline, prepared by Kreitmar(1932) on the intestinal segment taken from the rabbit. He noted that its

activity was somewhat greater than that of acetylcholine under 'in vitro' conditions. In the intact animal, given intravenously its action on the bowel was from 10-100 times as great as that of an equal dose of acetylcholine.

It is thus seen that the formation of a carbaminoyl ester of choline in the bowel tissues would be accompanied by an increased motor activity.

The effect of urea on the heart and blood vessels of the frog has been investigated, but as this subject is not relevant to this thesis, I do not propose to include it.

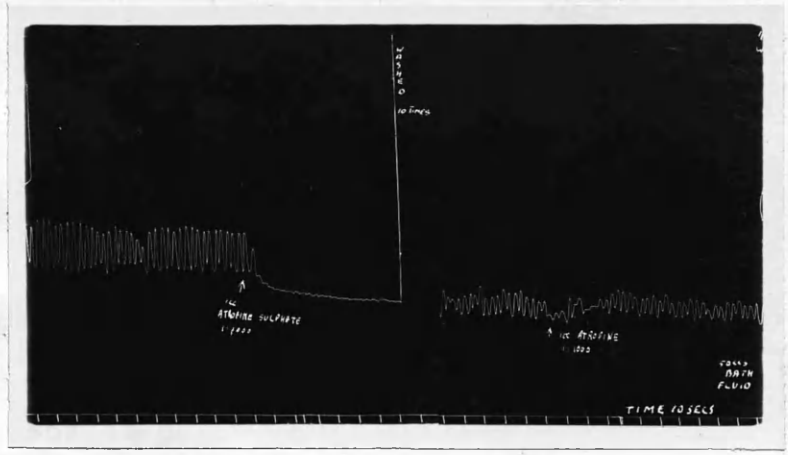
G. THE ACTION OF ATROPINE SULPHATE ON THE MOTOR ACTIVITY OF THE BOWEL.

Whilst working on the effect of urea on the isolated segment of the bowel I noted that the normal action of atropine on the bowel segment was reversed after the application of urea. I propose to discuss briefly the action of atropine on the bowel and its relation to the action of urea.

The effect of atropine on the intact and isolated intestine of vertebrates has received a considerable amount of attention. Various workers have published different results and even with the one worker the results have not always been uniform. Thus, Magnus (1905), using the isolated intestine of cats, found that comparatively large doses of atropine, 0.3% in a 200 c.c. bath, caused paralysis of the bowel; medium doses, 0.025-0.075% often stimulated the motor activity. This latter result was not constantly obtained. Magnus noted that it manifested itself especially when the bowel was in poor condition. Unger (1907) was able to confirm the observations of Magnus. Van Lidth de Jeude (1918), using the isolated segment of rabbit's bowel, found that with small and medium doses of atropine, inhibition of the tone and segmental movements was the more usual, but by no means universal, result. The medium doses, he found, occasionally stimulated the motor activity. Trendelenberg (1917) found that in the intact rabbit, the intravenous injection of atropine always produced paralysis of the bowel. Using isolated segments of bowel he found that the result varied, sometimes paralysis, and sometimes excitation occurred. No explanation of these varied results could be offered at that time.

Liljestrand (1919) found that the inconstant results produced by atropine on the isolated bowel were not due to the different proportions of the optical isomers of hyoscyamin which are present in the ordinary Galenical preparations of atropine.

Le Heux (1920) noted that if an isolated segment of rabbit's bowel was washed repeatedly, atropine in minute doses had no action, whereas medium doses had a constant excito-motor effect. The repeated washing of the bowel segment removed the free diffusible choline. Le Heux suggested that the varied actions of atropine on the bowel segment were related to the amount of choline present in the bowel tissues. He argued that the action of choline on the visceral muscle of the intestine is antagonised by atropine; when a sufficient amount of choline is present, then the intestinal movements are stimulated and the addition of even small amounts of atropine antagonises this action of choline. Inhibition of motor activity results. When sufficient choline is not present in the bowel tissues, then the antagonism is absent and atropine in moderate and large doses exerts a direct stimulant effect on the visceral muscle of the bowel. Le Heux was able to change the choline content of the bowel at will. He was thus able to change an inhibitory action of a given dose of atropine into an excitatory one. The addition of choline to the bath caused an increased motor activity of the bowel segment, and the application of atropine was then, once more, followed by a decrease in the tone and amplitude of rhythmic movements of the segment. I have repeated Le Heux's experiments.



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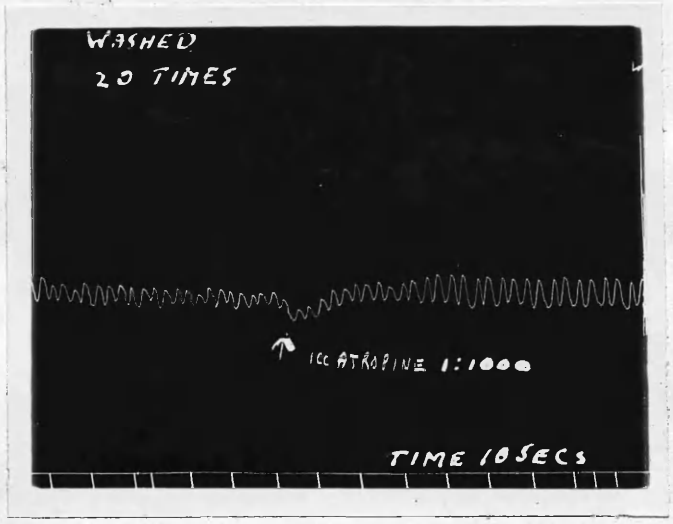

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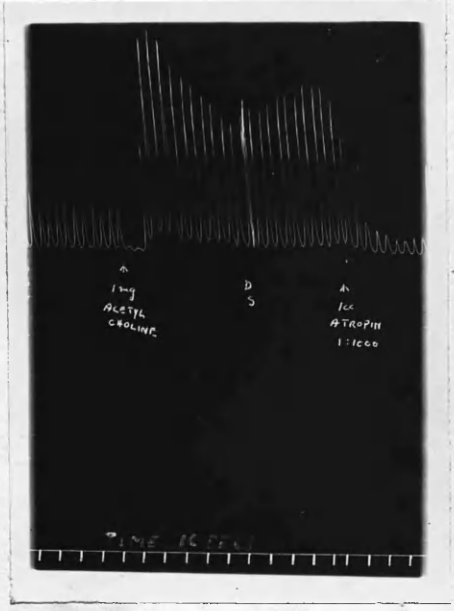

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Procedure. The bowel segment was set up in a mammalian bowel apparatus as has been previously described. Atropine sulphate was made up in 1:1,000 solution in Tyrode Bayliss solution. Acetylcholine was also made up in solution. 1 c.c. contained 1 mgm. The choline content of the bowel segment was reduced by repeatedly draining the bath fluid. The volume of the bath fluid was 50 c.c.

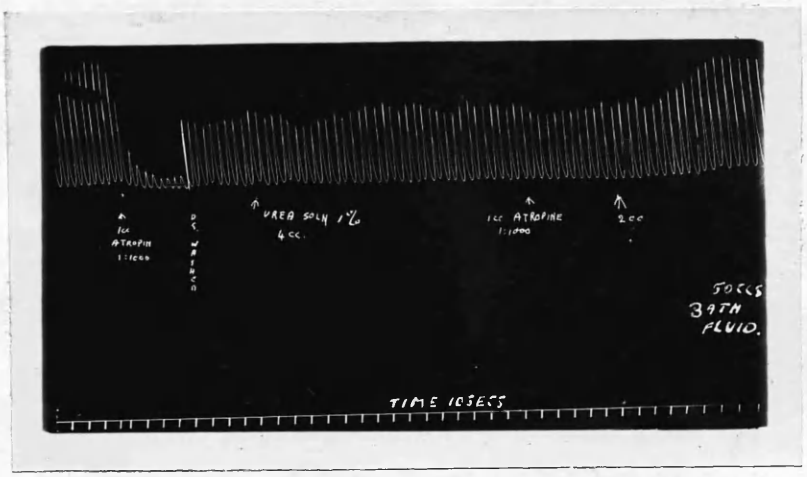
Results. (See Traces 16, 17, and 18).

1 c.c. of atropine solution 1:1,000 caused a marked diminution in tone and the abolition of the segmental movements of the preparation. After the bath fluid had been drained on ten separate occasions the addition of the same dose of atropine had only a very slight inhibitory effect on the bowel segment (See Trace 16).

After the bowel had been washed twenty times, 1 c.c. atropine 1:1,000 had no inhibitory effect and caused a slight increase in the motor activity of the segment (See Trace 17).

The addition of 1 mgm. acetylcholine caused a very striking rise in the amplitude of the segmental movements. The addition of the same dose of atropine as had been previously used now caused a marked diminution in the amplitude of the bowel segmental movements (See Trace 18).

Hoet (1925) criticised the work of Le Heux. He pointed out that the repeated washing of the bowel segment would have effects other than the removal of choline from the tissues. Hoet noted a relation between the inhibitory power of atropine and the vagal activity, prior to the excision of the bowel segment from a rabbit.



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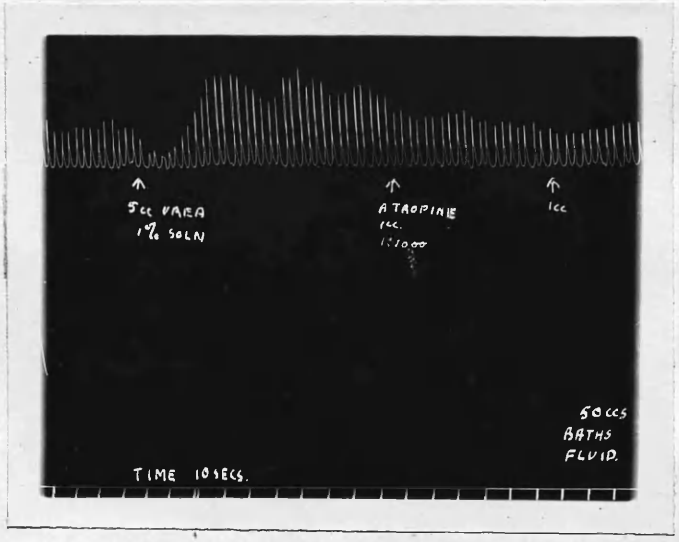


TRACE NUMBER TWENTY.

Thus, as reported on page 44 if the vagus nerves were stimulated prior to the excision of the bowel segment then the motor activity was increased and an inhibitory response to the application of atropine was constantly obtained. From what is now known of the cholinergic nature of the vagus nerve fibres to the small intestine, it will be seen that Hoet's work really demonstrated a relation between the inhibitory power of atropine and the free acetylcholine content of the bowel.

I have performed experiments which demonstrate that the inhibitory action of atropine on the isolated segment of rabbit's bowel can be reversed by the application of urea to the bowel segment.

Procedure. Urea and atropine solutions in Tyrode Bayliss were made up as before. A segment of rabbit's bowel was then set up in a mammalian bowel apparatus. The volume of bath fluid used was 50 c.c. A control addition of 1 c.c. atropine solution 1:1,000 was then made. The bath fluid was drained and replaced by fresh fluid. The urea solution was then added to the bath fluid and after its effect had demonstrated itself, further applications of atropine solution were carried out.

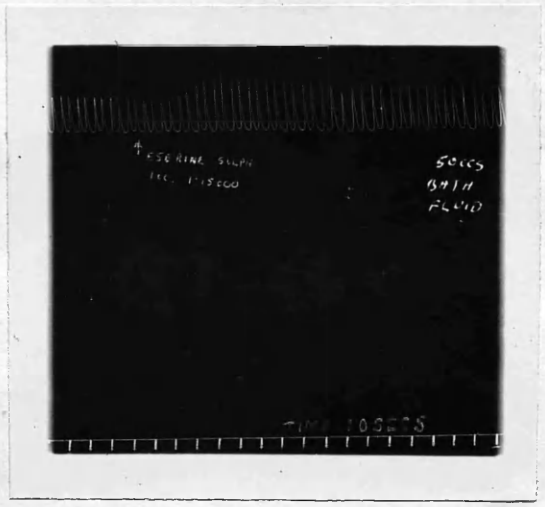
Results. (See Traces 19 and 20).

1 c.c. atropine solution 1:1,000 added to the bath caused a marked fall in the motor activity of the segment. The bowel was washed after this and thus we see that the addition of 4 c.c. 1% urea solution was without any marked effect on the motor activity; the further application of 1 c.c. atropine solution 1:1,000 was without any appreciable effect whilst the later addition of 2 c.c. of this

solution caused an increase in the tone and a marked rise in the amplitude of the rhythmic movements of the bowel segment (See Trace 19). Trace 20 shows the effect of 5 c.c. 1% urea solution on a fresh bowel segment; 1 c.c. atropine solution 1:1,000 is now without any inhibitory effect.

CONCLUSIONS.

One can conclude from these experiments that atropine sulphate has, in addition to the more commonly exhibited inhibitory action, the power of exciting intestinal visceral muscle. The mode in which the application of urea to the bowel segment promotes this excito-motor action is probably as follows. Atropine sulphate antagonises, almost specifically, the action of acetylcholine and choline on the bowel. However it would appear that the carbaminoyl compound of choline, prepared by Kreitmar, does not exhibit such a direct antagonism to atropine. Kreitmar (1932) noted that in the intact animal post-operative paresis of the bowel could be raised by the intravenous injection of 1√ carbaminoyl choline per kilo. body weight. The action was such that 20-30 minutes later, it was not possible to produce paresis of the bowel by doses of atropine, up to 500√ per kilo. body weight, given twice at short intervals. I would thus suggest that urea causes a reversal of the usual atropine effect on the bowel by the formation of carbaminoyl compound with the choline present in the bowel tissues. The action of this compound on the bowel is apparently not antagonised by atropine. Atropine thus acts directly on the visceral muscle cells and if applied in sufficiently large a dose causes an increased tone and amplitude of segmental movements.



TRACE NOMBER TWENTYONE.

H. THE EFFECT OF UREA ON THE ACTION OF ESERINE ON THE ISOLATED SEGMENT OF BOWEL TAKEN FROM THE RABBIT.

The effect of eserine on the intestinal segment can in the main be accounted for by the inhibitory action which it exerts on the choline esterase. The acetylcholine which is formed in the bowel tissues is consequently not destroyed and thus able to exert a prolonged effect on the intestinal muscle. Kreitmar (1932) noted, and Chang and Gaddum (1933) confirmed, that carbaminoyl choline is stable in the animal body. It is not hydrolysed by the choline esterase. If urea promotes the formation of a compound similar to that of the carbaminoyl choline of Kreitmar and if this compound be stable, then it would not be influenced by the enzyme inhibitory action of eserine. It is of interest to note that Minz (1932) noted that the action of carbaminoyl choline on the dorsal muscle of the leech is not sensitised by the previous addition of eserine. The following experiment demonstrates that the action of eserine on the bowel segment is greatly diminished if urea be first applied.

Procedure. Urea recrystallised (May and Baker) 1% solution in Tyrode Bayliss was used. Eserine Salicylas (T. & H. Smith, Ltd.) 1:15,000 solution was used. A bowel segment was suspended in a mammalian bath apparatus. Volume of bath fluid 50 c.c. 5 c.c. of the urea solution was added and four minutes later 1 c.c. of the eserine solution was added. This made a concentration of 1:750,000 of eserine in the bath fluid.

Result. (See Trace 21).

The addition of eserine after the bowel had been acted

upon for some time by urea, causes only a very slight increase in the amplitude of the intestinal segmental movement. This forms a striking contrast to the normal action of eserine in such concentrations (See Trace 11, page 95).

CONCLUSION.

The excito-motor action of eserine on a bowel segment taken from a rabbit is markedly diminished after the segment has been treated by urea. This is possibly due to the formation of a stable carbaminoyl compound of choline in the bowel tissues.

GENERAL SUMMARY AND CONCLUSIONS.

The main results which have been obtained in the experimental work reported in this thesis and the conclusions derived therefrom may be summarised as follows:-

Acetylcholine is normally present in the bowel tissues in a concentration sufficiently high to influence the motor activity of the bowel. It is normally rapidly hydrolised and inactivated by the choline esterase. It can be demonstrated in the bath fluid in which a bowel segment has been suspended only after the activity of the choline esterase has been inhibited by eserine.

The activity of the choline esterase is necessary for the maintenance of the normal motor activity of the small intestine. If the quantity of the enzyme be increased, then, in the majority of cases, the motor activity of an isolated segment of rabbit's bowel is diminished. If the activity of the enzyme be inhibited by eserine, then the segmental movements of the isolated bowel are abolished and the muscular tone is greatly and abnormally increased. It is possible to restore the normal motor activity of a bowel segment, under these conditions, by the addition of horse serum concentrates rich in the choline esterase.

The reversible, enzyme-catalysed acetylcholine:choline reaction occurring in the bowel tissues plays a very important part in the regulation of the normal tone and segmental movements of the small intestine; it also increases the permeability of the intestinal mucosa and causes constriction of the intestinal blood vessels.

Urea has a marked excito-motor effect on the isolated

segment of bowel taken from a rabbit. This occurs only when choline and acetylcholine are present in the tissues of the bowel segment. This action is probably due to the action of urea on these substances resulting in the formation of a carbaminoyl compound of choline.

The action of this compound upon the visceral muscle of the small intestine of the rabbit does not appear to be antagonised by atropine. When it is present in the tissues of the bowel wall atropine in moderate doses exerts a direct excito-motor effect on the intestinal visceral muscle.

This compound appears also to be stable. It is not affected by the choline esterase. In its presence the action of eserine on the bowel segment is thus greatly diminished.

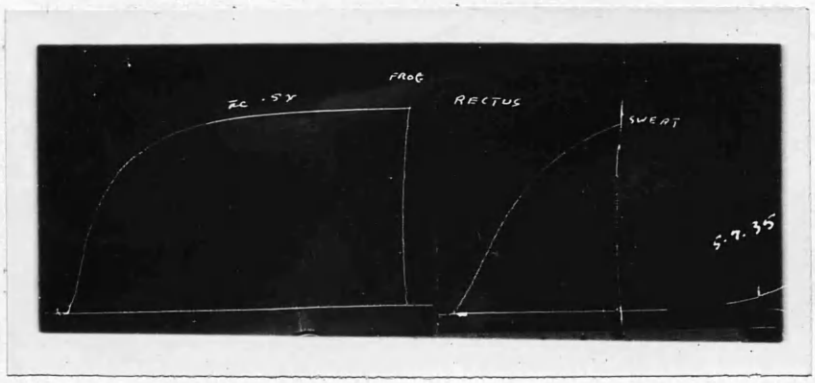
A note on the action of "Human Sweat" on the rectus abdominis muscle of the frog.

Dale and Feldberg (1934, b) noted that if a physiological saline solution came in contact with the human skin it acquired the power of producing a contraction of the dorsal muscle of the leech. They noted that although the contraction so produced resembled that produced by acetylcholine, the action of the saline solution on the muscle was not enhanced by the presence of eserine. It was their opinion that the activity acquired by the saline solution was not typical of that produced by acetylcholine.

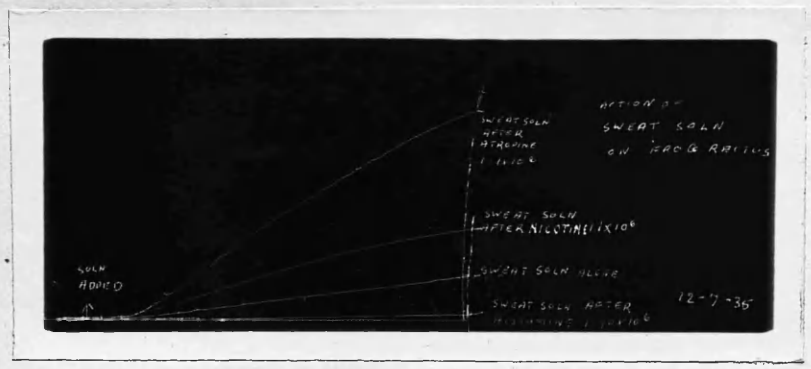
During the course of this work I noted that the rectus abdominis muscle of the frog was similarly affected by a saline solution which had been in contact with the human skin. In an attempt to determine the factor responsible for this action on the muscle a short series of experiments was undertaken. The work may be divided conveniently into two sections. In the first section, the relation of this action to various autonomic drugs was investigated and compared with that of acetylcholine. In the second section the various known constituents of human sweat were tested on the rectus muscle in order to determine the constituent responsible for the action on the muscle.

SECTION 1.

Procedure. The rectus abdominis muscle of a frog was set up as described on page 64. Special care was taken to ensure that the muscle preparation did not come into contact with the skin of the operator during the course of the dissection. The "sweat solution" was prepared by shaking 10 c.c. of glass distilled water in a test



TRACE NUMBER TWENTYTWO.



TRACE NUMER TWENTYTHREE.

tube using the thumb, fingers and palm of the hand, as stoppers, to prevent the loss of fluid. The experiments were performed as a rule when the temperature and humidity of the air of the laboratory were such as to favour the production of sweat. In some instances it was necessary to increase the amount of sweat by exercise. Such a "solution" was divided into 5 c.c. portions. This allowed of a comparison of the effects of the "solution" under different circumstances. The "sweat solution" was tested on the frog's rectus muscle under the following series of conditions:-

(a) In the absence of eserine sulphate in the bath fluid.

(b) After the muscle had been suspended in the bath fluid containing eserine for twenty minutes.

(c) When in addition to the eserine, atropine sulphate has been added to make a concentration of 1:1,000,000.

(d) When nicotine tartrate (B.D.H.) had been added to the bath fluid to make a concentration of 1:1,000,000.

(e) When histamine in ergamine acid phosphate (Burroughs and Wellcome) had been added to make a concentration of 1:10,000,000.

Results. (See Traces 22 and 23).

The frog's rectus muscle responded to the addition of 5 c.c. "sweat solution" with a contraction similar to that produced by acetylcholine (See Trace 22).

The responses obtained, under the test conditions, are tabulated below, and compared with those given by acetylcholine.

TABLE 4.

Conditions	"Sweat solution"	Acetyl-choline
No eserine added to the bath fluid	+	-
Eserine added to the bath fluid	+	+
" " " " " and nicotine	+	-
" " " " " and atropine	+	+
" " " " " and histamine	-	+ but reduced

+ indicates a contraction of the muscle.
- indicates no effect on the muscle.

(See Trace 23).

It is seen that the activity of the "sweat solution" in producing a contraction of the frog's rectus muscle differs from that of acetylcholine in that it is not sensitised by the addition of eserine, not abolished by nicotine and not evident in the presence of histamine.

SECTION 2.

The composition of human exocrine sweat is given as follows:-

Urea	0.24-1.12 mgm. per c.c.
Uric acid	0 up to 30 mgm. per 100 c.c.
Amino acids	1.6-6.4 mgm. per 100 c.c.
Ammonia	0.04-0.2 mgm. per c.c.
Lactic acid	15 mgm. per 100 c.c.
Sugar	2.8-50 mgm. per 100 c.c.

Taken from Mosher (1933).

Potassium 0.021-0.16 g. per 100 c.c.

Taken from Oppenheimer (1934).

Procedure. With the exception of the amino acids which are not specified I have tested the action of each of the constituents of sweat on the frog's rectus muscle.

Results.

Solutions containing potassium 0.1% produces a contraction of the frog's rectus muscle. This has already been shown by Chang and Gaddum (1933), Beznák (1934) and others. The other constituents of sweat, even in concentrations above those in which they normally exist, were found to be without action on the frog's rectus muscle. Cholesterol which is also present in sweat is without any action on the muscle.

Discussion.

v. Mégay (1935) has studied the acetylcholine-like action of sweat on the rectus muscle of the frog. He used undiluted sweat and also trichloracetic acid extracts of sweat. He found that it had an activity on the isolated heart of the frog, the rectus muscle of the frog, the dorsal muscle of the leech and the blood pressure of the cat similar in many respects to that of acetylcholine. He noted that the action on the frog's rectus muscle was not constant and that in the majority of cases was not sensitised by the presence of eserine. He also noted that the depressor activity on the blood pressure of the cat was not always antagonised by atropine nor was the activity abolished by the addition of blood, rich in the choline

esterase, to the sweat prior to testing. Quantitative standardisation against acetylcholine produced different results when different test objects were used. The average acetylcholine equivalent was 1 c.c. sweat = 0.2γ acetylcholine.

From these results v. Mégay concluded that the acetylcholine-like activity of sweat is not completely to be accounted for by the presence of acetylcholine in the sweat. He suggested that the anomalous results were due to the fact that the activity of the sweat was caused by the presence of acetylcholine in a solution containing potassium in a concentration sufficient in itself to affect the muscle or to affect the action of acetylcholine on the muscle.

That acetylcholine mediates the transmission of sympathetic nerve impulses to the sweat glands in a cat was shown by Dale and Feldberg (1934,c).Nevertheless it is difficult to accept v. Mégay's explanation of the activity of sweat. In the experiments which I have performed the sweat was collected in comparatively large amounts of distilled water. The resultant concentration of the potassium ions present in the sweat was thus reduced. In these circumstances the anomalous reactions of the sweat solution can not be accounted for by the presence of potassium.

I have thus been unable to determine which constituent of sweat is responsible for its acetylcholine-like activity on the frog's rectus muscle. It is my opinion that the assumption that acetylcholine, itself, is present in the sweat is not justifiable in the state of present knowledge. It is of interest to note that simple saline extracts of human hair and frog's skin have no acetylcholine like activity on the rectus muscle.

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REFERENCES.

Abderhalden, E. and Paffrath, H. (1925). Pflügers Arch., 207, 225.

Abderhalden, E. and Paffrath, H. (1926). Ferments Forsch., 8, 229.

Abel, Miss W. (1909). Proc. Roy. Soc. Edin., 30, 327.

Alcock, Miss. Quoted by W. H. Gaskell, 1916. The Involuntary Nervous System. Longmans, Green & Co.

Alvarez, W. C. (1922). and (1928) The Mechanics of the Digestive Tract. Paul B. Hoeber.

Alvarez, W. C. and Mahoney, L. J. (1922). Amer. J. Physiol., 59, 421.

Ammon, R. (1933). Pflügers Archiv., 233, 486.

Ammon, R. and Kwiatkowski, H. (1934). Ibid., 234, 269.

Ammon, R. and Voss, G. (1935). Ibid., 235, 393.

Arai, K. (1922, a) Ibid., 193, 359.

Arai, K. (1922, b) Ibid., 195, 390.

Armstrong, P. B. (1935). J. Physiol., 84, 20.

Bacq, Z. M. (1933). Extract. des Annals. de Physiologie, Tome 10, No. 4.

Bacq, Z. M. (1935). Ergebn. Physiol., 37, 82.

Baeyer, A. (1867). Ann. Chim., 142, 322.

Bain, W. A. (1932). Quart. J. exp. Physiol., 22, 269.

Barsoum, G., Gaddum, J. H., and Khayyal, M. A. (1934). J. Physiol., 82, 9P.

Bayliss, W. M., and Starling, E. H. (1899). Ibid., 24, 99.

Bayliss, W. M., and Starling, E. H. (1901). Ibid., 26, 125.

Bennet, D. W., and Drury, A. N. (1931). Ibid., 72, 288.

Bernheim, F. (1933). Arch. int. Pharmacodyn., 46, 169.

Bernheim, F., and Bernheim, M. (1933). Amer. J. Physiol., 104, 438.

Béznak, A. B. L. (1934). J. Physiol., 82, 129.

Braecke, M., and de la Cuesta, G. S. (1933). Arch. int. Pharmacodyn., 46, 397.

Brown, and Eccles (1934). J. Physiol., 82, 211.

Brown, G. L., McSwiney, B. A., and Wadge, W. J. (1930). Ibid., 70, 253.

Cajal, Ramon S. (1933). Histology. Ballière Tindall & Cox.

Cannon, W. B. (1902). Amer. J. Physiol., 6, 251.

Cannon, W. B. (1906). Ibid., 17, 429.

Cannon, W. B. (1933). Science, 78, 43.

Cannon, W. B., and Bacq, Z. M. (1931). Amer. J. Physiol., 196, 392.

Cattell, M., and Wolff, H. G. (1935). Science, 82, 106.

Chang, H. C., and Gaddum, J. H. (1933). J. Physiol., 79, 255.

Clark, A. J. (1927). J. Physiol., 64, 123.

Dale, H. H. (1913). Ibid., 46, 19P.

Dale, H. H. (1914, a). Ibid., 48, 3P.

Dale, H. H. (1914, b). J. Pharmacol., Baltimore, 6, 147.

Dale, H. H. (1928). Introduction to Methods of Biological Assay, J. H. Burn, 1928. Oxford Medical publication.

Dale, H. H. (1929). Croonian Lectures. Lancet, 1, 1179, 1233, 1285.

Dale, H. H. (1933). Johns Hopk. Hosp. Bull., vol. 53, 297.

Dale, H. H. (1934). J. Physiol., 80, 10P.

Dale, H. H., and Dudley, H. W. (1929). J. Physiol., 68, 97.

Dale, H. H., and Dudley, H. W. (1931). Z. physiol. Chem., 198, 85.

Dale, H. H., and Feldberg, W. (1934, a). J. Physiol., 81, 320.

Dale, H. H., and Feldberg, W. (1934, b). Ibid., 81, 40P.

Dale, H. H., and Feldberg, W. (1934, c). Ibid., 82, 121.

Dale, H. H., Feldberg, W., and Vogt, M. (1936). Ibid., 86, 353.

Dale, H. H., and Gaddum, J. H. (1930). Ibid., 70, 109.

Dikshit, B. B. (1934). Ibid., 80, 409.

Dixon, W. E. (1906). Brit. med. J., 21, 1807.

Donomae, I. (1934). Pflügers Arch., 234, 318.

Dudley, H. W. (1929). Biochem. J., 23, 1064.

Dudley, H. W. (1931). J. chem. Soc., London, 1, 763.

Dudley, H. W. (1933). J. Physiol., 79, 249.

Ellis, L. B., and Weiss, S. (1932). J. Pharmacol., Baltimore, 44, 235.

Englehart, E. (1931). Pflügers Arch., 227, 220.

Englehart, E., and Loewi, O. (1930). Arch. exp. Path. Pharmak., 150, 1.

Esveld, v. L. W. (1928). Z. mikr. Anat. Forsch., 15, 1.

Ettinger, G. H. and Hall, G. E. (1934). J. Physiol., 82, 38.

Euler, v. U. S., and Gaddum, J. H. (1931). Ibid., 72, 74.

Evans, C. L., and Underhill, S. W. F. (1923). Ibid., 58, 1.

Ewins, A. J. (1914). Biochem. J., 8, 44.

Feldberg, W. (1933). Arch. exp. Path. Pharmak., 170, 560.

Feldberg, W., and Gaddum, J. H. (1934). J. Physiol., 81, 305.

Feldberg, W., and Kwiatkowski, H. (1934). Pflügers Arch., 234, 333.

Feldberg, W., and Rempel, B. (1932). Quoted by Minz, 1932.

Feldberg, W., and Rosenfeld, P. (1933). Pflügers Arch., 232, 212.

Finkleman, B. (1930). J. Physiol., 70, 145.

Franel, L. (1935). Arch. int. Physiol., 42, 2, 256.

Frank, R., Zimmerman, L., and Necheles, H. (1934). Proc. Soc. exp. Biol., N.Y., 32, 255.

Fuhner, H. (1918, a). Arch. exp. Path. Pharmak., 82, 51.

Fuhner, H. (1918, b). Biochem. Z., 92, 347.

Fürth, O.V., and Schwarz, C. (1908). Pflügers Arch., 124, 361.

Galehr,O., and Plattner, F. (1927,a).Pflügers Arch., 218, 488.

Galehr,O., and Plattner, F. (1927,b).Ibid., 218, 506.

Garry, R. C. (1928). J. Physiol., 66, 235.

Gasser, H. (1926). J. Pharmacol.,Baltimore, 27, 395.

Gasser, H. and Dale, H. H. (1926). Ibid., 29, 53.

Gellhorn, D.,and Northup, E. (1933, a). Amer. J. Physiol., 105,684.

Gellhorn, D.,and Northup, E. (1933, b). Ibid., 106, 283.

Girndt, O. (1925). Pflügers Arch., 207, 469.

Gollwitzer-Meier, K. (1934). Arch. exp. Path. Pharmak., 174, 456.

Gollwitzer-Meier, K., and Kruger, E. (1934). Ibid., 176, 642.

Gunn, J. A., and Underhill, S. W. F. (1914). Quart. J. Physiol., 8, 275.

Harrison, J. S., and McSwiney, B. A. (1936). J. Physiol., 87, 79.

Henderson, W. R., and Wilson, W. C. (1936). Quart. J. exp. Physiol., 26, 83.

Hoet, J. E. (1925). J. Physiol., 60, 10P.

Houchgeest, v. Braam (1872). Pflügers Arch., 6, 266.

Hukuhara, (1932). Ibid., 229, 311.

Hukuhara, (1934). Quart. J. exp. Physiol., 24, 37.

Hunt, R. (1899). Amer. J. Physiol., 3, 18P.

Hunt, R. (1901). Ibid., 5, 6P.

Hunt, R. (1917, a). Amer. J. Physiol., 45, 197.

Hunt, R. (1917, b). Ibid., 45, 231.

Hunt, R., and Taveau, R. de M. (1906). Brit. med. J., 2, 1788.

Hunt, R., and Taveau, R. de M. (1911). Hyg. Labor. Bull., No. 73, Publ. Health Serv. U.S., Washington, 1911.

Jeude, van Lidth de (1918). Pflügers Arch., 170, 523.

Johnson, S. (1925). J. comp. Neurol., 38, 299.

Kapfhammer, J., and Bischoff, C. (1930). Z. physiol. Chem., 191,179.

Kapfhammer, J., and Bischoff, C. (1931). Ibid., 199, 153.

Klee, Ph. (1912). Pflügers Arch., 145, 557.

Klee, Ph. (1913). Pflügers Arch., 154, 552.

Klee, Ph., and Grossman, O. (1925). Münch. med. Wschr., 72, 251.

Kohn, R., Levitsky, P., Strauss, A., Strauss, S., and Necheles, H. Arch. Internat. de Pharmacodyn, 1936 (in press), quoted from Necheles (1936).

Kreitmar, H. (1932). Arch. exp. Path. Pharmak., 164, 346.

Kuhlewein, von M. (1921). Pflügers Arch., 191, 99.

Kunz, A. (1910). J. comp. Neurol., 20, 283.

Kuntz, A. (1934). The Autonomic Nervous System. Ballière, Tindall & Cox.

Kuré, Ken., Ichiko, K., and Ishikawa, Ky. (1931). Quart. J. exp. Physiol., 21, 1.

Kuré, Ken., Okinaka, S., and Kajiyama, M. (1935). Ibid., 25, 195.

Langley, J. W. (1922). J. Physiol., 56, 39P.

Le Heux, J. W. (1918). Pflügers Arch., 173, 8.

Le Heux, J. W. (1920). Ibid., 179, 177.

Le Heux, J. W. (1921, a). Ibid., 190, 301.

Le Heux, J. W. (1921, b). Ibid., 190, 280.

Liljestrand, G. (1919). Ibid., 175, 111.

Loach, J. V. (1934). J. Physiol., 82, 118.

Loewi, O. (1921) Pflügers Arch., 189, 239.
and (1922) Ibid., 193, 201.

Loewi, O. (1924). Pflügers Arch., 203, 408.
Ibid., 204, 361.
Ibid., 204, 629.

Loewi, O. (1932-1933). Harvey Lectures, Baltimore, p. 218.

Loewi, O. (1935). Proc. Roy. Soc. Biol. Sect., 118, 299.

Loewi, O., and Navratil, S. (1926, a). Pflügers Arch., 214, 678.

Loewi, O., and Navratil, S. (1926, b). Ibid., 214, 689.

McSwiney, B. A., and Newton, W. H. (1927). J. Physiol., 64, 144.

McSwiney, B. A., and Robson, J. M. (1931). Ibid., 71, 194.

Maaske, C. A., Bunting, H., and Meek, W. J. (1935). Amer. J. Physiol., 114, 100.

Magnus, R. (1904, a). Pflügers Arch., 102, 123.

Magnus, R. (1904, b). Ibid., 102, 349.

Magnus, R. (1904, c). Ibid., 103, 525.

Magnus, R. (1905). Ibid., 108, 1.

Magnus, R. (1908). Ergebn. Physiol., 7, 27.

Magnus, R. (1930). Stanford Univ. Publ. med. Sci., vol. 2, No. 3, p. 73.

Markowitz, C. (1931). Amer. J. Physiol., 97, 271.

Matthes, K. (1930). J. Physiol., 70, 338.

Mégay, v. K. (1935). Pflügers Arch., 236, 159.

Michaelis, L., and Menten, M. L. (1913). Biochem. Z., 58, 315. Quoted from Enzyme by J. B. S. Haldane (1930). Longmans, Green & Co.

Minz, B. (1932, a). Arch. exp. Path. Pharmak., 167, 85.

Minz, B. (1932, b). Ibid., 168, 292.

Mosher, H. (1933). J. biol. Chem., 99, 781.

Necheles, H., Levitsky, P., Kohn, R., Maskin, M., and Frank, R. Amer. J. Physiol., 114, 330.

Noll, H. (1932). Arch. exper. Path. Pharmak., 167, 158.

Oppenheimer's Handb. Biochem. (1934), 2, 535. Quoted by v. Megay (1935).

Plattner, F., and Hintner, H. (1929). Pflügers Arch., 222, 395.

Plattner, F., and Hintner, H. (1930). Ibid., 225, 19.

Plattner, F., and Hou, Ch. L. (1931). Ibid., 228, 281.

Plattner, F., and Tsudzimura, H. (1935). Ibid., 236, 175.

Prasad, B. N. (1935, a). J. Physiol., 85, 239.

Prasad, B. N. (1935, b). Ibid., 85, 249.

Prasad, B. N. (1936). Ibid., 86, 425.

Riesser, O., and Neuschloss, S. M. (1921). Arch. exp. Path. Pharmak., 91, 342.

Rona, P., and Neukirch, P. (1912). Pflügers Archiv., 146, 371.

Sawasaki, H. (1925). Ibid., 210, 322.

Schmiedeberg, and Harnack (1877). Arch. exp. Path. Pharmak., 6, 101.

Stedman, E. (1929). Amer. J. Physiol., 90, 528.

Stedman, E., and Stedman, E. (1935). Biochem. J., 29, 2563.

Stedman, E., Stedman, E., and Easson, L. H. (1932). Biochem. J., 26, 2056.

Stedman, E., Stedman, E., and White, A. C. (1933). Ibid., 27, 1055.

Strecker, A. (1849). Ann. Chim., 70, 149.

Strecker, A. (1862). Ibid., 123, 353.

Thornton (1934). J. Physiol., 82, 14P.

Trendelenberg, P. (1917). Arch. exp. Path. Pharmak., 81, 55.

Unger, M. (1907). Pflügers Arch., 119, 373.

Vartiainen, H. (1934, a). J. Physiol., 80, 21P.

Vartiainen, H. (1934, b). Ibid., 84, 282.

Velhagen, jr. K. (1931). Arch. exp. Path. Pharmak., 161, 697.

Wachholder, K., and Nothmann, F. (1931). Pflügers Arch., 229, 120.

Weiland, W. (1912). Pflügers Arch., 147, 171.

Weiss, S. (1926). J. Pharmacol., Baltimore, 27, 181.

Witanowski, W. R. (1925). Pflügers Arch., 208, 694.

Wolf, C. G. L. and Canney, J. R. C. (1926). Lancet, 1, 707.
