# THE ACTION OF DRUGS ON THE CENTRAL NERVOUS SYSTEM,

## WITH SPECIAL REFERENCE TO ACETYL CHOLINE,

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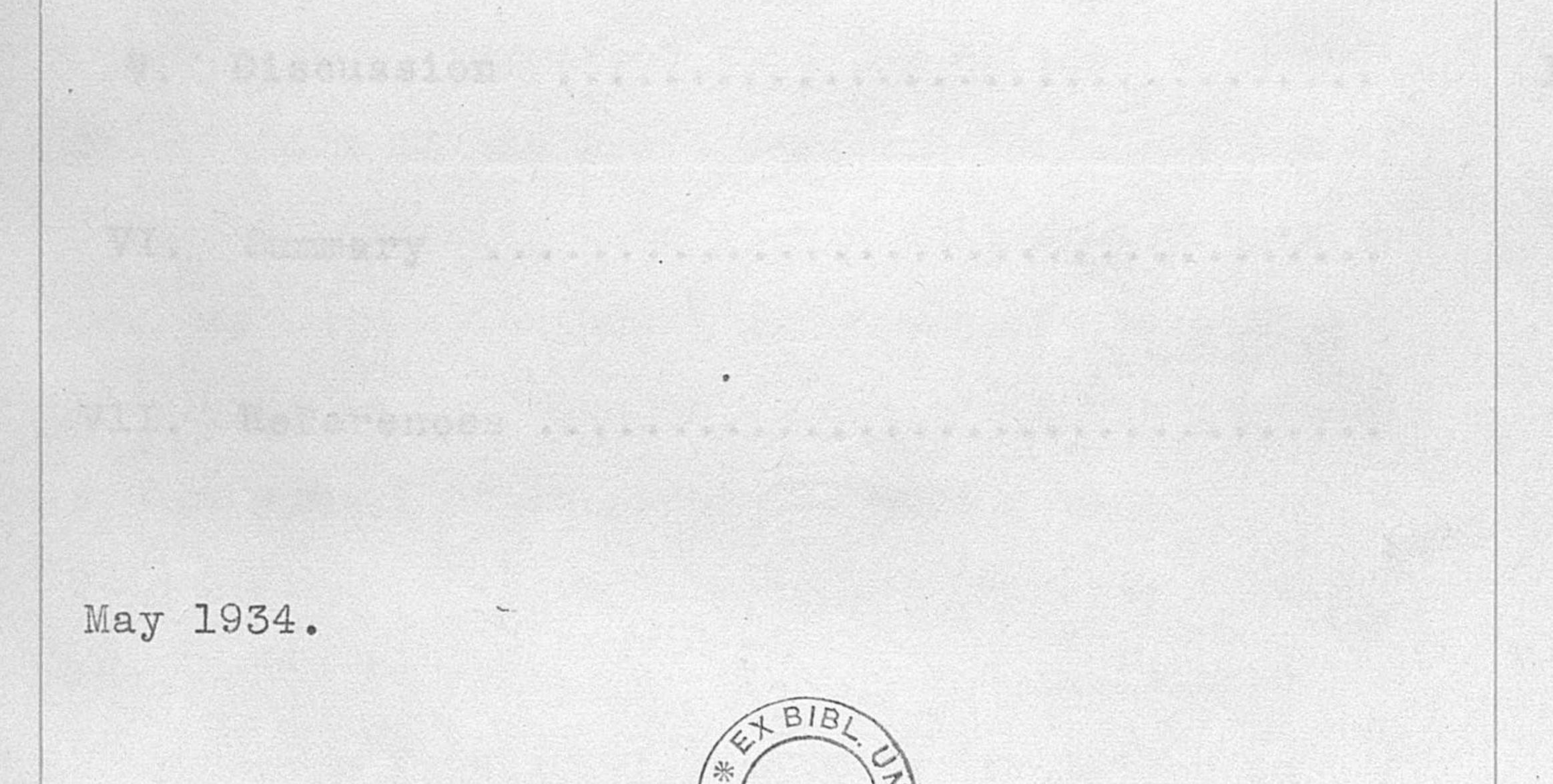
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presented for the Degree of Ph.D.,

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#### I. INTRODUCTION

The idea that effects of stimulation of certain nerves are due to liberation of a specific chemical substance at the endings of the nerve was conceived by scientists more than twenty-five Dixon (1906) for example, working vears ago. with the mammalian heart found that the heart contained a substance "pro-inhibition" which as a result of vagus excitation was converted into a chemical body "inhibitin", which by combining with the heart muscle resulted in cardiac standstill. Howell (1906) suggested that the effects of vagus stimulation on the heart were due to liberation of K ions and working with Duke (1908) showed that in perfused mammalian heart under certain conditions, there was an increase of K content in the perfusate to the extent of 29 per cent. during vagal stimulation. In explaining the probable events that occur in reflex excitation and inhibition Sherrington (1925) referred to a "state" or an "agent" being produced at the neurones "whose existence/

existence was outside the intrinsic properties of pure nerve fibre and with a so to say more chemical mode of origin and function than the nerve impulse per se". Sherrington's scheme put forward to explain excitation and inhibition, therefore, reduced the neurone to somewhat the character of secretory nerves.

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The work of Loewi (1921-29) on the amphibian heart has now established the theory of humoral transmission. He first showed that when an isolated frog's heart, containing a small volume of Ringer's solution, was subjected to inhibition by stimulation of the vagus nerve, a substance passed into the Ringer's solution, which, on transference to another isolated heart, reproduced there the effects of vagus stimulation. Since the publication of Loewi's results the humoral transmission of nerve impulses has been tested by many workers, and although there are various discrepancies to be explained, there appears to be a certain amount of evidence pointing definitely to such a transmission. The chemical substance liberated at the terminals during the stimulation of the vagus/

vagus nerve has been shown to be either acetyl choline or a substance with identical pharmacological properties.

Literature in support of Loewi's hypothesis has been accumulating during recent years. The work of Dale and his co-workers stands prominently amongst this. Dale and Dudley (1929) isolated acetyl choline from the normal tissues of a horse and thus showed that the choline ester is a normal physiological agent. This discovery materially strengthened the hypothesis of humoral transmission. Dale and Gaddum (1930) further showed that the effect of stimulation of the chorda tympani on the denervated mammalian tongue muscle, closely resembled the effect of an injection of acetyl choline. They observed that stimulation of the chorda tympani resulted in simple vaso-dilatation without any effect on the intact muscle and caused a tonic contraction of the denervated muscle and that acetyl choline produced both these effects, and pointed out the significant fact that acetyl choline was the only known chemical substance which had both these actions.

Brinkman/

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Brinkman and van Dam (1922) observed that the perfusate from frog's heart, collected during the stimulation of its vagus nerve, acquired a substance which had the property of stimulating the movements of the stomach of another frog,

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Englehart (1931) showed that a mechanism similar to the transmission of a chemical substance during vagal inhibition of the heart, existed in the oculomotor nerve also.

Bain (1932) found that stimulation of the peripheral end of the lingual nerve conferred upon the perfusing fluid a property of exciting the isolated rabbit's gut and stimulation of the vagosympathetic rendered it inhibitory to the isolated gut.

Gibbs and Szeloczey (1932) perfused the submaxillary glands of cats and dogs and found that during chorda stimulation a highly active substance was produced in the glands. They called this substance "chordastoff" and found that it had the same properties as acetyl choline.

Dale and Feldberg (1933) found that even though the effects of vagal stimulation on the muscular / muscular walls of the alimentary canal were not antagonised by atropine, such stimulation liberated a chemical substance in the portal blood which had all the properties of an unstable, atropine sensitive choline ester, indistinguishable from those of acetyl choline.

Further evidence as to the nature of the chemical transmitter at synapses in the sympathetic ganglia was offered by Feldberg and Gaddum (1933) who perfused the superior cervical ganglia of cats with salt solutions and tested the outflowing fluid with various biological tests for acetyl choline. Their results strongly supported the view that the substance was acetyl choline.

Feldberg, Minz and Tsudezimura (1933) found that a chemical transmission occurred in splanchnic fibres which cause output of adrenaline from the cells of suprarenal medulla and that the transmitting agent directly affecting the medullary cells had all the properties of acetyl choline.

It will be seen from the literature given above that there is a considerable evidence supporting the view of humoral transmission, not only with the vagus, but with other parasympathetic nerves/ nerves also, and that the bulk of evidence points to the chemical agent being acetyl choline. It will further be seen that most of the work in connection with the hypothesis of humoral transmission has been done in relation to the peripheral terminations of the parasympathetic nerves. Evidence is presented in this paper which suggests that humoral transmission may occur in the central nervous system as well.

The similarity between vagus stimulation and acetyl choline action is strikingly seen in the different organs of the body and is easily studied in relation to the peripheral terminations of the vagus. No attempt has so far been made to compare the effects of stimulation of the afferent fibres of the vagus and application of acetyl choline to the centres in the central nervous system.

Cushing (1931) first pointed out that injections of surgical pituitrin in the ventricles of the brain of a patient caused reactions totally different from those produced by an intravenous injection and very closely resembling the effects of/ of pilocarpine. In a series of publications (1932) he later showed that pituitrin thus introduced acted directly on the hypothalamic nuclei and was thus responsible for the effects produced. Cushing's work demonstrated the method of applying drugs directly to the higher centres and suggested the possibility of studying humoral transmission centrally.

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Vagus control of respiration has been a subject of considerable experimentation and controversy, but there is a certain degree of unanimity on the question of effects of stimulation of the central end of the vagus. Rosenthal (1865) first pointed out that stimulation of the central end of the vagus caused cessation of respiration and his observation has been confirmed by many writers since then. The effects depend upon the strength of the stimulus, but with a sufficiently strong current inhibition of respiration is always produced.

The effects of stimulation of the central end of the cut vagus were therefore compared with those of intraventricular injections of acetyl choline, on the respiratory movements.

II./

#### Part II. Similarity between central vagal

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stimulation and intraventricular

acetyl choline injection.

II. Similarity between the effects of central vagal stimulation and intraventricular acetyl choline injections.

#### (1) Methods.

Cats were used in all experiments. The animals were anaesthetised with paraldehyde and ether. Paraldehyde was given orally by a stomach tube and the dose varied from 0.8 to 1 c.c. per kilogram of body weight. Sufficient quantities of ether were administered to keep the animal fully anaesthetised. In some experiments urethane was used as an anaesthetic. The dose employed was 1.8 g. per kilogram of body weight and the drug was given by intramuscular injection.

When the animal was fully under the influence of the anaesthetic, a median incision was made in the neck, the trachea exposed and a glass tracheal cannula fixed in the trachea. The carotid artery on the right side was dissected and an arterial cannula inserted in it. Vagi on both sides were carefully dissected and cut in the middle of the neck. A venous cannula was also fixed in the femoral vein on the left side and attached to a burette/ burette containing warm saline. Intravenous injections, when required, were given by inserting the needle through the rubber connections into the venous cannula. Respirations were recorded by connecting the tracheal cannula to a rubber tambour and the blood pressure by connecting the arterial cannula to a mercury manometer. Half saturated solution of sodium sulphate was used as an anti-coagulant.

For artificial respiration a Palmer type of artificial respiration pump was used.

For giving intraventricular injections the following technique was employed: -

The skin on the scalp was incised by a longitudinal incision, the soft parts retracted and the junction of the coronal and sagittal sutures exposed. A small hole 4 mm. in diameter was trephined at the junction of these sutures, usually on the left side but occasionally on the right. Bleeding was controlled by lightly plugging the opening with cotton wool. The plug could be removed after a few minutes without any further bleeding occurring. For giving intraventricular injections a fine tuberculine needle was employed. The needle was held in its proper position by the/ the following methods: -

(i) A soft rubber cork which fitted exactly in the trephine opening was pierced through by the fine needle. The length of the needle sticking out was so adjusted as to be in the position of the lateral ventricle when the cork was inserted in the The cork when properly fixed had its under hole. surface flush with the outer surface of the dura. The position of the trephine opening, the size of the hole and the cork, the direction of the needle and its length were all standardised by repeated trials. The same trephine was used in all experiments so that the same cork, holding the needle could be fixed in the hole. The trephine opening was made in a fixed position so that by merely fixing the cork in the hole the position of the end of the needle was ensured to be in the lateral ventricle. This was found to be so by making a hemi-section through the skull and brain of cats and noting the position of the end of the needle when the cork was fixed in the usual way. For giving the injection, the cork holding the needle was held in the right hand, the needle pierced through the dura and other coverings of the brain, passed through the/

the substance of the brain in the direction of the lateral ventricle and the cork fixed in its proper position. A syringe was attached to the nozzle of the needle and the injections made by very slowly pushing the piston.

(ii) The needle was pierced through the coverings of the brain and its substance in the direction of the lateral ventricle and when the end of the needle was in the cavity of the ventricle, the needle was fixed in that position by plaster of Paris.

(iii) The needle, mounted on the syringe, was passed in the direction of the lateral ventricle and when it was in its cavity the injections were made by pushing down the piston. The needle was then taken out and a second injection when required was made in the same way. This method was usually employed when making injections in the third ventricle. At the end of such experiments some methylene blue was injected in the same manner as the drugs and a post mortem examination of the brain made to see the site of injection and the direction of the flow of the dye. After a few trials/ trials the position of the ventricles could be ascertained with a fair degree of certainty.

In all the procedures described above the position of the needle could be confirmed by the outflow of cerebro spinal fluid through the nozzle of the needle. The flow was not marked because of the small size of the needle and not more than one or two drops escaped.

The vagi were stimulated by induced currents, a du Bois Raymond type of coil being used for the purpose. A 2-volt accumulator was employed to supply the current. The cut central end of the right or the left vagus was placed on platinum electrodes and stimulated by a tetanising current. In some experiments both the nerves were placed on the electrodes and stimulated in the same way.

## (2) Effects of stimulation of the central end of the vagus.

As has been observed before Rosenthal first pointed out that stimulation of the central end of the vagus caused inhibition of respiration due to/

to a tetanus of the inspiratory muscles. Since then it has been shown by many observers (Schäffer. 1900) that the effect of weakest effective stimulus is inhibitory. It is not uncommon. however, to see an increase in the rate of respiration after a mild stimulation of the central end of the vagus. Schaffer (1932) found that stimulating the central end of the vagus provoked either increased or decreased activity of the respirations depending on the character and intensity of the stimulus. Traube (1847) first pointed out that there are two sets of afferent fibres in the vagi which have an antagonistic action on respiration, and this fact has been generally recognised since then (Lovatt Evans, 1932). The effect of central vagal stimulation should therefore depend on the preponderance of one set of fibres or the other resulting in either an increase or a decrease in respiration.

I stimulated the central end of one or both vagi in more than 50 experiments and found that in the great majority of cases the effect was inhibitory if a sufficiently strong stimulus was used. With a moderate stimulation an acceleration of/ of respiration is sometimes produced. In some of these experiments inhibition of respiration may come on after cessation of the stimulus. These delayed effects appear to be of some significance and will be discussed later. My results therefore agree with those of previous authors who observed that stimulation of central end of the vagus generally produces inhibition of respiration.

## (3) Effect of intraventricular injections of acetyl choline.

Injections of acetyl choline were given in the lateral or the third ventricle of cats as described before. The dose varied from 0.05 to 1 γ. The quantity of the fluid injected never exceeded 0.2 c.c. The possibilities of reactions due to mechanical effects of the injection were not therefore great. Control injections of 0.2 c.c.off Ringer's solution were given before and after injection of a drug, and it was found that such injections did not produce any appreciable change in respiration or the level of blood pressure of the animals.

Intraventricular injections of acetyl choline produced an effect on respiration and blood pressure of cats which was totally different from that produced/

produced by an intravenous injection. As is well known, acetyl choline given in small doses such as 0.5 to  $1^{\gamma}$  intravenously produces a very marked fall of blood pressure and a comparatively small effect on the rate or depth of respiration. Intraventricular injections of similar quantities of acetyl choline however had a marked effect on respiration and comparatively small effect on blood pressure. There was usually a marked depression or a complete cessation of respiration and an inconstant effect on blood pressure. The blood pressure showed a slight fall or a slight rise or no effect whatever. Some of the effects on blood pressure might be due to the mechanical effects of depression of respiration. The animals showed a considerable variation in their response to an intraventricular injection of acetyl choline but respirations were very commonly slowed or completely The following table summarises the stopped. results obtained in 29 experiments.

Table I /

## Table I.

The Effect of an Intraventricular Injection of Acetyl Choline.

No.	Wt.of animal in kilos.	Dose in $\gamma$ of acetyl choline given intra- ventricularly (Later- al, except otherwise stated)	Effect on respiration	Remarks.
1	2.3	1.0		Recovery
2	3.4	0.5		do.
3	3.3	0.1 to 1.0	?	
4	2.4	0.1		Recovery
5	2.1	0.1	-	do.
6	3.2	0.05 to 0.1	-	do.
7	5.1	0.1 to 10	+	
8	2.1	0.1	++	
9	2.6	1.0		
10	2.6	1.0(3rd ventricle)		Injury to A.C.
11	2.2	0.1		Recovery
12	2.9	0.1(3rd ventricle)		Recovery
13	2.0	0.1		do.
14	2.3	0.5(3rd ventricle)		do.
15	2.0	0.1(3rd ventricle)		do.
16	2.5	0.5		Convulsions and +++ later
17	1.8	0.1(3rd ventricle)		Recovery
18/				
			. /	

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## Table I. (contd.)

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No. Wt. of animal in kg.		Dose in $\gamma$ of acetyl choline given intra- ventricularly (Later- al except otherwise stated)	Effect on respiration	Remarks
18	1.9	0.5		
19	2.2	0.1	9 84 Chare 	
20	1.7	0.1	Cride A	
21	3.0	0.1		
22	3.2	0.1		
23	2.4	0.5		
24	3.4	0.05 to 1.0	+7	
25	2.7	1.0	?	
26	2.6	0.1	-	Recovery
27	2.1	0.1	1-9099	do.
28	2.6	0.5		
29	2.4	0.5(3rd ventricle)		

+ = Stimulation of respiration.

- = Depression of respiration.

A. C. = Apneustic centre.

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It will be seen from the table that the dose of acetyl choline varied from 1.0 to  $0.05 \gamma$ . Three animals out of 29 showed a definite stimulation of respiration after an intraventricular injection of the drug. In 2 there was no appreciable response even with 1  $\gamma$  doses and in the remaining 24 there was a depression of respiration varying from a slight diminution in rate and depth to a complete cessation of respiration. The effects were reversible and the injections could be repeated in the same animal. Acetyl choline given in small doses such as 0.05 to 1  $\gamma$  in the ventricles of the brain therefore was found to produce a marked depressant action on respiration. Fig. 1 shows the effect of an intraventricular injection of 0.5 of acetyl choline given in the lateral ventricle of a cat. There is a complete cessation of respiration and a very small fall in blood pressure. The latter may perhaps be due to the mechanical effects of cessation of respiration. The effect is transitory and the normal respirations are regained by the animal in less than 2 minutes.

This depression of respiration may be due to the drug acting locally on some of the nerve centres/

centres or acting after absorption in the circulation. It is doubtful whether drugs injected in the cerebro spinal fluid are absorbed with almost the same rapidity as given intravenously, but it is believed by some (Dixon and Halliburton, 1912) that they are absorbed very rapidly. As the nature of the actions produced by an intraventricular injection was different from that produced by an intravenous injection, it is obvious that the effects were not due to absorption into the circulation. In order to demonstrate, however, that the cessation of respiration was due to the action of acetyl choline on the central nervous system and to exclude the effects of interference with ventilation, the movements of a strip of the diaphragm were recorded by a modification of the technique described by Thomas and Frank (1928).

## Method of recording movements of the strip of the diaphragm.

The animal (cat) was anaesthetised with urethane or paraldehyde and ether, and the tracheal, arterial and venous cannulae inserted in appropriate places/ places as described before. The tracheal cannula was then connected to an artificial respiration pump and artificial respiration at the rate of 20 strokes per minute was employed. The volume of the air pumped in the lungs was adjusted by inserting a T-tube with rubber connections, between the tracheal cannula and the rubber tubing from the A screw clamp fixed on the open end of the oumo. T was adjusted in such a way that the volume of air going to the lungs was about the same as that required to inflate the lungs during normal An incision was made on the left respirations. side, about 2 cm. from the median line and extending from the 2nd left costal cartilage above to the left costal arch below. The cartilages were cut and the chest wall retracted by hooks, so that the left diaphragm could be easily seen with the left phrenic nerve entering it. A strip of the diaphragm 1 cm. wide and about 4 cm. long was then isolated in such a way that the phrenic nerve ran along the centre of the strip. The vessels accompanying the nerve were carefully dissected, ligated and cut. Any injury to the nerve was avoided as far as possible. The lower end/

end of the strip was then fastened to a fixed rod by silk thread and the upper end connected to a writing lever. The strip was held in its natural position as far as possible so as to avoid any undue tension on the nerve and was protected from the rhythmically expanding lungs by glass rods. Such a preparation if kept moist by Locke's solution contracts regularly for a considerable time. It is separated from all connections of the body except through the phrenics and its activity could be modified only by impulses going through this nerve. Administration of a drug by any channel will therefore produce effects on the movements of the strip of the diaphragm by acting through its nervous connection.

The action of acetyl choline on respiration was studied by this method. Injections were given intraventricularly as described before. It was found that acetyl choline when introduced into the lateral or the third ventricle caused a very marked depression or a complete cessation of the movements of the disphragm strip. The same effect was observed after central vagal stimulation.

Fig. 2 shows the striking similarity between central/

central vagal stimulation and an acetyl choline injection. The respirations were recorded in this experiment by the diaphragm strip method. At A. central end of the left vagus was stimulated electrically. The movements of the strip ceased during stimulation and became normal immediately after cessation of stimulation. The small movements seen in the figure during stimulation were due to the mechanical effects of artificial respiration. An injection of 0.1  $\gamma$  of acetyl choline was then given at B. in the third ventricle. There was a complete cessation of diaphragm movements which lasted for about 5 minutes, and the movements gradually returned after that interval.

The movements of respiration are controlled by a wide variety of physiological processes in the body acting on respiratory centre such as  $CO_2$  and  $O_2$  tension, hydrogen ion concentration of the blood, reflexes from different parts of the body such as the lungs, larynx etc. and also through aortic and carotid sinus nerves. In the above experiment, any of the factors mentioned above is unlikely to influence the activity of the respiratory centre. There was obviously no change in  $CO_2$  or  $O_2$  tension of the blood or in its hydrogen/ hydrogen ion concentration for the animal was under artificial respiration. Section of both the vagi prevented any reflexes through that nerve. There was no change in the level of blood pressure to initiate any reflexes from the aortic arch or the carotid plexus. It is significant therefore that under such circumstances the action of central vagal stimulation and application of small quantities of acetyl choline to the hypothalamic centres showed a remarkable similarity on the movements of the strip of the diaphragm.

## (4) Effect of atropine on the intraventricular injections of acetyl choline.

One of the important points regarding the pharmacological action of acetyl choline is the fact that most of its effects are antagonised by atropine. The nature of this antagonism appears to be uncertain. Straub (1907) suggested that atropine rendered tissues impermeable to parasympathetic drugs like acetyl choline. Clark (1926) however showed that although acetyl choline does enter the frog's heart, there is no regular relationship between the/ the amount entering the heart cells and the extent of the action produced. Clark (1926) suggested that atropine and acetyl choline are attached to different receptors in the heart cells and their antagonism was an antagonism of effects rather than of combination. While discussing the humoral transmission during vagus stimulation Dale (1929) explained the failure of atropine to antagonise the effects of vagus stimulation on the intestines by assuming that acetyl choline was liberated in cell substance by a nerve impulse in such intimate relation to the reactive structures that atropine was relatively ineffective in hindering its action. Whatever be the mode of action of this antagonism between atropine and acetyl choline, most of the effects produced by acetyl choline are antagonised by atropine.

Experiments were therefore made to see the influence of atropine on intraventricular injection of acetyl choline and also on the effect of central vagal stimulation. The procedure adopted was as follows:-

A small quantity of acetyl choline varying from/

from 0.5 to 0.1  $\gamma$  was introduced into the lateral or the third ventricle of cats and the effect on respiration noted. Doses of atropine were then given, intravenously in some cases, and intraventricularly in others. A few experiments were done in which atropine was administered both intravenously and intraventricularly. After such atropinisation of the animal, exactly the same quantity of acetyl choline was introduced in the ventricles as given before and the difference between the reactions produced by such injections of acetyl choline noted. The results are summarised in the table (Table II).

Table II /

#### Table II.

The Effect of Intraventricular Acetyl Choline

before and after Administration of Atropine.

		and the second	and the second state of the second state		And the second sec	and the second
No.	Wt. of animal in kg.	Dose of acetyl choline in $\gamma$ .	Effect on the res- pirations	Dose of atropine in mg.	Dose of acetyl choline in $\gamma$ .	Effect on resp.
l	2.1	0.1	- 14 - 14 <sup>-1</sup> -14-1	0.2 i.v.	0.1	-
2	2.4	0.1	-?	0.2 i.v.	0.1	-
3	2.9	0.1		0.2 i.v.	0.1	
4	2.9	0.5		0.02, 3rd vent.	0.5	
5 6	2.3	0.5		0.02, lat. vent. and 0.1 i.v.	0.5	-
6	1.8	0.5		0.2 i.v.	0.5	
7	2.2	0.1	No14	0.2 i.v.	0.1	-
8	3.5	0.5		0.2 i.v.	0.5	
9	1.7	0.1	shi was	0.2 i.v.	0.1	
10	2.6	0.1	10 - 10 -	0.1 lat. vent. and 1.0 i.v.	0.1	
11	2.5	0.1	<b>-</b>	0.05 lat. vent.	0.1	
12	2.9	0.5		0.1 lat. vent.	0.5	-
		And the second s	the second se			

- = Depression of respiration i.v. = Intravenous injection. 3rd vent. = 3rd ventricle of the brain. lat. vent. = lateral ventricle of the brain.

It will be seen from the table that the dose of acetyl choline varied from 0.1 to 0.5  $\gamma$  . Atropine was given intravenously in 7 cases. intraventricularly in 3 experiments and both intravenously and intraventricularly in 2 experiments. The dose of intravenous atropine was approximately 0.1 mg. per kilo. of body weight of the animals and that of intraventricular atropine about 0.01 to 0.05 mg. per kilo. of the body weight. The table shows that in only one experiment (No. 5) there was a slight decrease in the depression of respiration produced by an intraventricular acetyl choline injection when administered after atropine. In others there was either no difference at all between the two injections of acetyl choline given before and after administration of atropine or there was an actual increase in the depression of respiration produced by the injection of intraventricular acetyl choline given after atropine. It was found therefore that atropine did not antagonise the depressant action on respiration of intraventricular injections of acetyl choline.

Action/

Action of atropine on the effects produced by stimulation of the central end of the vagus.

The action of atropine on the effects of stimulation of the central end of the vagus was also studied. The vagus was stimulated centrally by a current just sufficient to produce an inhibition of respirations. Atropine was then administered both intravenously and intraventricularly as described before and the vagus stimulated with the same strength of current as before. Out of 30 such experiments it was found that in none did atropine modify the action of vagus stimulation on respiration.

(5) Action of Physidstigmine on the Effects produced by Intraventricular Acetyl Choline and Central Vagal Stimulation.

Another important point in the pharmacological action of acetyl choline is the fact that its action is enhanced by physostigmine. Similarly the effects of parasympathetic stimulation are also increased by physostigmine. There is a considerable evidence in the literature supporting the view put forward/ forward by Anderson (1905) that physostigmine increases the effects of parasympathetic stimulation. Winterberg (1907) and Loewi and Mansfeld (1910) supported this observation. Dixon and Ransom (1912) observed the same phenomenon while working with the broncholconstrictor nerves of the cat. Heinekamp (1925) working with the blood pressure of dogs found that a previous administration of physostigmine markedly increased the depressor response on blood pressure of vagus stimulation. Gibbs (1926) perfused the fowl's heart and found that physostigmine considerably enhanced the effects of vagus stimulation on the heart. Dale and Gasser (1926) while studying the action of acetyl choline on the denervated gastrocnemius muscle observed that small doses of physostigmine considerably lowered the threshold of stimulation of the muscle by acetyl choline.

Fuhner in 1918 first demonstrated the striking effect of physostigmine in enhancing the action of acetyl choline on the plain muscle of the leech. He found that soaking the muscle of the leech in a 1 in 1 million solution of physostigmine markedly increased the stimulant action of acetyl choline but/ but did not affect the action of pilocarpine, and further suggested that this effect was due to the action of physostigmine preventing the hydrolysis of acetyl choline by the tissues. Loewi and Navratil (1926) studied the hydrolysing action of the tissues using the frog's heart muscle for the purpose. They concluded that physostigmine increased the inhibitory action of vagus stimulation and also ofacetyl choline by its action on the ferment which hydrolyses acetyl choline. Englehart and Loewi (1930) found that physostigmine prevented the destruction of acetyl choline in the blood by inhibiting the action of the hydrolysing ferment, and working simultaneously Dale and Gaddum (1930) arrived at the same conclusion.

There is thus a considerable evidence in the literature pointing to the effect of physostigmine on both vagal stimulation and acetyl choline action. The effect of small doses of physostigmine was therefore studied on the action of intraventricular acetyl choline and also on the stimulation of the central end of the vagus.

The method employed was the same as was used to study the action of atropine. Physostigmine was/ was given in small doses intravenously and intraventricularly and the effect of acetyl choline injection and vagus stimulation before and after such administration of physostigmine compared. The results are shown in Table III.

Table III /

### Table III.

The Effect of Intraventricular Acetyl Choline

and Vagus Stimulation before and after

Administration of Physostigmine.

		AUTOTOT	N			
No.	Wt. of animal in kg.	Dose of acetyl choline in γ or vag.stim.	Effect on resp.	Dose of physos- tigmine in mg.	Dose of acetyl choline in $\gamma$ or vag. stim.	Effect on resp.
710	24.8	d0.		Date ways		÷.
1	2.2	d0.1.0		0.1 3rd vent	1.0	-
2	1.7	0.1	163 <b>-</b> 666	1.0 i.v.	0.1	-**
3	3.0	0.1		1.0 i.v.	0.1	
4	3.0	0.1		2.0 lat.	0.1	
5	2.4	0.5		vent. 0.1 lat. vent.	0.5	
20	3.5	40, 60	*	011 1.04	do.	-
6	2.7	0.1	-	0.1 3rd vent	0.1	?
7	2.7	do.1.0		0.1 lat. vent.	1.0	
8	1.7	stim. vag.		1.0 i.v.	stim. vag.	
9	3.0	do.		2.0 lat. vent.	do.	
10	2.7	do.	singelon :106 <sup>77</sup>	1.0 i.v.	do.	
11	2.7	do.	te of the bi priotic of th	0.1 lat. vent.	do.	
12	2.6	do.	-	0.2 lat. vent.	do.	-
13	2.6	do.		0.2 i.v.	do.	-
14/						

-35-

Table III (contd.)

No.	Wt. of animal in kg.	Dose of acetyl choline in γ or vag.stim.	Effect on resp.	Dose of physos- tigmine in mg.	Dose of acetyl choline in γ or vag. stim.	Effect on resp.
14	2.6	stim. vag.	The later	0.1 3rd vent	stim. vag.	
15	2.4	do.		0.1 i.v.	do.	
16	2.4	do.	n 7 experie netyl chell	0.1 lat. vent.	do.	-
17	2.1	do. 1991.	rere-eeupos	0.1 i.v.	do.do.	
18	2.1	do.		0.01 lat vent.	do.	-
19	2.8	do.		0.5 lat. vent.	do.	
20	3.5	do. 19 2 1	he etfeat :	0.l i.v.	do.	-
21	3.5	do.choline	protecting	1.0 i.v.	do.	-
22	2.7	do.ben phy		0.1 3rd vent	do.	
23	2.4	do.	se tont pb;	2.0 i.v.	do.	-

- = Depression of respiration i.v.= Intravenous injection. 3rd vent. = 3rd ventricle of the brain. lat. vent.= lateral ventricle of the brain.

superially after the response of respiration to

It will be seen from the table that physostigmine was given to 23 animals either intravenously or intraventricularly. In only one experiment (No. 3) it was given both intravenously and intraventricularly. The intravenous dose varied from 0.05 to 1 mg. per kilo. approximately and the intraventricular from 0.005 to 0.6 mg. per kilo. approximately. In 7 experiments the effect of equal quantities of acetyl choline given before and after physostigmine were compared. The dose of acetyl choline varied from 0.03 to 0.5 Y per Out of 7 experiments, in 3 there was a slight kilo. increase in the depressant action of acetyl choline on respiration. In 2 the effect was exactly opposite, acetyl choline producing less depression of respiration after physostigmine. In the remaining 2 experiments the effect was about the same. It would appear from these that physostigmine did not materially alter the response of respiration to an intraventricular injection of acetyl choline. The increase in the depression of respiration seen after physostigmine in the 3 experiments was not marked enough and in view of the fact that in 2 experiments/

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experiments there was an opposite effect it would appear that the slight increase in the action in the 3 experiments referred to above was not very significant.

The effect of central vagal stimulation before and after physostigmine was studied in 16 experiments. In 10 experiments the depression of respiration produced by central vagal stimulation was exactly the same before and after administration of physostigmine. In 5 there was a slight increase in depression of respiration after physostigmine and in 1 the effect was exactly opposite.

In one experiment (No. 20) central vagal stimulation caused an acceleration of respiration before physostigmine and a slight depression after it. In the 5 experiments which showed a slight increase in the depression of respiration, the effect was not marked at all. The conclusion one could draw from these experiments therefore was not very definite, but it would appear that as in the case of acetyl choline injection, physostigmine did not materially alter the response of central vagal stimulation.

(6) /

#### (6) Discussion.

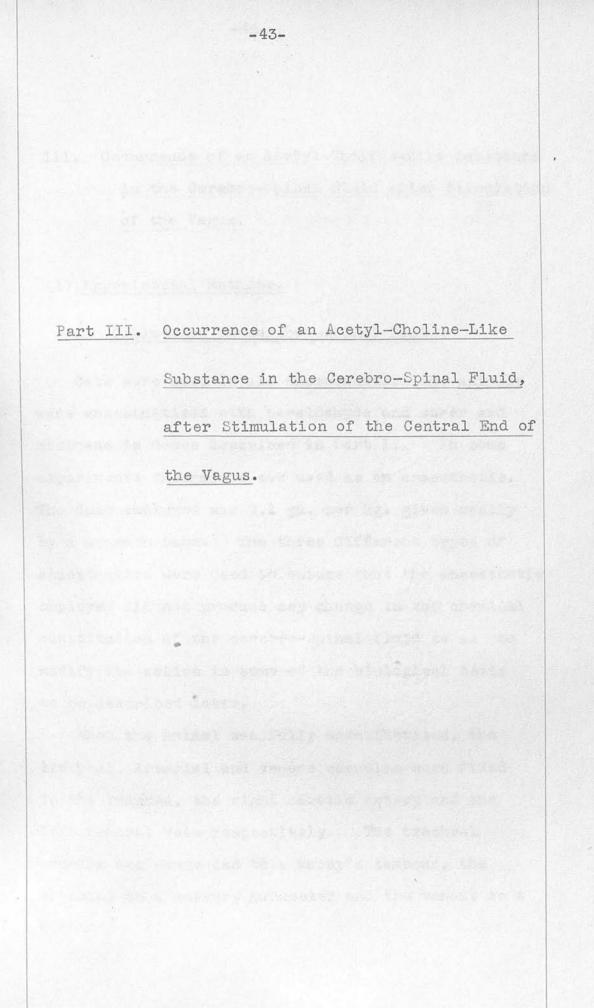
The experiments described above show the close similarity between the effects of central vagal stimulation and intraventricular acetyl choline injection on the respiration of cats. Both these procedures produce an inhibition of respirations. The inhibition thus produced is neither paralysed by atropine nor augmented by physostigmine. In view of the literature quoted in support of humoral transmission, a simple explanation of the similarity of these two will be to assume that central vagal stimulation produced a substance closely resembling acetyl choline in its action and liberation of such a substance acted on certain cells in the central nervous system and produced inhibition of respiration. Acetyl choline when injected in the ventricles of . the brain could reach these cells directly and thus produced a similar effect.

As has been discussed before, most of the actions of vagus stimulation and acetyl choline are antagonised by atropine. There are, however, various actions both of vagus stimulation and of acetyl choline which are not antagonised by atropine. The anomalies observed in regard to this antagonism by/ by atropine have been discussed by Dale (1929) in his Croonian lectures. The similarity between vagus stimulation and acetyl choline administration is complete in the case of the heart for the effect of both is antagonised by atropine. In the case of the intestines, however, atropine antagonises the action of acetyl choline, while it does not materially alter the effect of vagus stimulation. Similarly the vasodilator effects of acetyl choline are completely obliterated by atropine, but the vasodilator effects produced by stimulation of chorda tympani or the antidromic stimulation of the sensory nerves are not affected by atropine (Gasser. 1930). The chorda tympani if stimulated causes increased activity of the salivary glands and a dilatation of the arterioles, and both these effects are supposed to be due to liberation of an acetyl choline-like substance. Atropine completely paralyses the effect on the glands but does not modify the effect on the arterioles. The antagonism between atropine and acetyl choline on the contractions caused by the latter in certain normal muscles/

muscles of the frog has been well known (Reisser and Neuschloss, 1921). In the mammal, however. Dale and Gaddum (1930) found that atropine does not antagonise the effect of acetyl choline on the denervated muscle. Acetyl choline causes a contracture of the eserinised leech muscle (Fühner. 1918, a and b), but this reaction is unaffected by atropine (Chang and Gaddum, 1933). It can be argued from the foregoing that failure of atropine to abolish an action does not necessarily mean that the action is not due to acetyl choline. In my experiments the action of intraventricular acetyl choline was not abolished by atropine, nor was the action of central vagal stimulation. Failure of atropine to antagonise the action of vagal stimulation on respiration does not therefore necessarily mean that the action was not due to liberation of acetyl choline. especially because the action of acetyl choline itself was unaffected by atropine.

It has been observed before that the effects of stimulation of the central end of the vagus may be manifested during the stimulation or may come off after cessation of the stimulus. This has been found/ found to be true when the respirations were recorded by the diaphragm strip method. Interference with ventilation therefore cannot be a cause of these delayed effects, and these can be explained more easily by a secretory theory than by any other, for it can be argued that during the period of stimulation a chemical substance was liberated which persisted after cessation of the stimulus and thus produced respiratory inhibition.

It would appear from the experimental data given above that there is some reason to believe that an acetyl choline-like substance may be liberated in the brain during stimulation of the central end of the vagus. If it is liberated in the brain, it should eventually appear in the cerebro-spinal fluid. Attempts were therefore made to see if any such substance could be detected in the cerebro-spinal fluid after stimulation of the central end of the vagus.



III. Occurrence of an Acetyl-Choline-Like Substance in the Cerebro-Spinal Fluid after Stimulation of the Vagus.

(1) Experimental Methods.

Collection of cerebro-spinal fluid.

Cats were used in all experiments. The animals were anaesthetised with paraldehyde and ether and urethane in doses described in Fart II. In some experiments chloralose was used as an anaesthetic. The dose employed was 0.1 gm. per kg. given orally by a stomach tube. The three different types of anaesthetics were used to ensure that the anaesthetic employed did not produce any change in the chemical constitution of the cerebro-spinal fluid so as to modify its action in some of the biological tests to be described later.

When the animal was fully anaesthetised, the tracheal, arterial and venous cannulae were fixed in the trachea, the right carotid artery and the left femoral vein respectively. The tracheal cannula was connected to a Marey's tambour, the arterial to a mercury manometer and the venous to a burette/ burette containing warm saline. Artificial respiration was arranged as described before and the vagi on the two sides dissected and cut in the middle of the neck.

The head of the animal was flexed to open up the space between the atlas and the 2nd vertebra. The skin at the back of the neck was incised and the position of the occipital protruberance ascertained by palpating with the index-finger. A medium sized hypodermic needle with a sharp and short bevelled end was then introduced about 1.5 cm. below the occipital protruberance and pushed upwards and backwards till the atlanto-occipital ligament was felt. If the point of the needle was felt to impinge on the bony wall, it was slightly withdrawn and worked up or down till the atlanto-occipital ligament was felt. The ligament was pierced by sharp gentle jerk and the end of the needle was felt to be free in the cisterna magna. As soon as the cisterna magna was entered into, the cerebro-spinal fluid was seen gushing out through the needle. The nozzle of the needle was connected to a soft rubber tube about 2.5 cm. long and carrying a drawn-out glass tube at the other end. The fluid/

fluid was allowed to flow through these rubber and glass connections and collected in a glass capsule. The flow of the fluid could be stopped by applying a bulldog to the rubber tube. It was unnecessary to hold the needle in any kind of support for the soft tissues of the neck through which the needle passed held it quite firmly. Care was taken to see that the needle was not pushed too far forward and injury to the vital centres was thus avoided. The cerebro-spinal fluid collected was usually quite clear. Samples tinged with blood were discarded and were not used for biological tests.

The cerebro-spinal fluid gushed out as soon as the needle was free in the cisterna magna, but soon the flow became slower. The initial flow was about 0.5 to 0.75 c.c. and about 1.0 to 1.5 c.c. could be subsequently collected drop by drop. The total quantity of fluid that could be collected from an animal varied from 1 to 2.5 c.c. The maximum amount collected from one animal was 3.5 c.c.

Artificial respiration was employed in all experiments before the cisternal puncture was made. Changes in the chemical composition of the cerebrospinal/ spinal fluid that may possibly arise as a result of asphyxiation due to central vagal stimulation were thus avoided.

Both the vagi were stimulated simultaneously. The strength of the current employed was just sufficient to produce cessation of normal respiration. Duration of stimulation varied from 30 to 60 seconds. In most of the animals respiratory movements of a spasmodic character appeared after the first 30 or 40 seconds, and in some cases interfered with the collection of cerebro-spinal fluid.

The cerebro-spinal fluid was collected in two lots, one representing the normal sample and the other representing sample obtained after vagal stimulation.

The normal sample of cerebro-spinal fluid was collected in two ways. The first was to puncture the cisterna magna before any operative interference with the vagi. The second method was to collect a sample of cerebro-spinal fluid about 20-30 minutes after stimulation of the vagi.

The cerebro-spinal fluid representing the fluid collected after vagal stimulation was similarly obtained in two ways. The vagi in the neck were stimulated/ stimulated for about one minute and during or immediately after the stimulation the cisterna magna was tapped. The first sample obtained was therefore the sample after vagus stimulation. In the other method the vagi were stimulated after the normal cerebro-spinal fluid was withdrawn and the fluid obtained during or immediately after the vagal stimulation. In some of the experiments therefore the first sample was normal cerebro-spinal fluid and the second sample cerebro-spinal fluid after vagal stimulation, and in the others, the first sample was vagal and the second normal.

The cerebro-spinal fluid thus obtained before and after vagal stimulation was tested for the presence of an acetyl-choline-like substance on the cat's blood pressure, the rat's intestine and the frog's heart.

(2) Effect of cerebro-spinal fluid obtained before and after Central Vagal Stimulation on Cat's Blood Pressure.

Cerebro-spinal fluid collected before and after central vagal stimulation was injected intravenously either into the same animal or into another animal/

animal. The animals were anaesthetised with paraldehyde and ether, urethane or chloralose and exactly equal quantities of the two samples were injected through the cannula in the femoral vein. Some experiments were made where the sample obtained after vagal stimulation was injected first and the normal cerebro-spinal fluid injected afterwards. In other experiments the order was reversed, the normal cerebro-spinal fluid being injected first and that obtained after yagal stimulation injected afterwards. The speed of the injections was the same in both The quantity of cerebro-spinal fluid cases. injected varied in different experiments but exactly the same quantity of the two samples obtained before and after vagal stimulation was injected. The quantity of warm saline run through the burette was exactly the same. The following table gives the summary of the experiments done.

Table IV. /

## Table IV.

The effect of an intravenous injection of cerebrospinal fluid obtained before and after central vagal stimulation.

				1	
No.	Wt. of animal in kg.	Quantity of normal C.S.F. in- jected intra- venously.	Effect on blood pressure.	Quantity of C.S.F. ob- tained after vagal stim- ulation in- jected intra- venously.	Effect on blood pressure.
1	2.1	0.8 c.c.	?	0.8 с.с.	
2	2.7	0.5	-?	0.5	
3	2.8	0.8	- ?	0.8	-?
4	2.7	0.8	÷	0.8	÷
5	3.0	0.5	2	0.5	+
6	3.0	0.8	+	0.8	÷
7	3.0	0.85	?	0.85	?
8	2.6	0.7	?	0.7	?
9	3.2	0.7		0.7	-
10	3.2	0.8	?	0.8	+
11	3.5	0.7	+?	0.7	+?
12	3.0	0.5	?	0.5	?
13	2.4	0.5	+	0.5	+
14	2.7	0.7	+ .	0.7	?
15	3.1	0.8	+	0.8	+
16	4.4	1.0	- Xean	1.0	-
17	3.4	0.5	?	0.5	?
18	3.5	0.5	? -	0.5	-
19/					

Table IV (Contd.)

No.	Wt. of animal in kg.	Quantity of normal C.S.F. in- jected intra- venously.	Effect on blood pressure.	Quantity of C.S.F. ob- tained after vagal stim- ulation in- jected intra- venously.	Effect on blood pressure.
Citras,	78153-041	ees no offect s	2 (41)、 加速	如是市道。自己	
19	2.6	0.8c.c.	techad varia	0.8 с.с.	
20	2.0	0.5	?	0.5	?
21	2.5	0.25	+?	0.25	-?
22	2.5	0.25	?	0.25	?
23	3.2	0.7	Alertos 1 de	0.7	-
24	2.6	0.75	pen after vo	0.75	
25	1.8	0.5	?	0.5	?
26	2.3	0.75	+	0.75	+
27	2.0	0.5	after beat	0.5	
				And the second second	

+ = rise in blood pressure.

- = fall in blood pressure.



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It will be seen from the table that the effects produced by intravenous injections of cerebro-spinal fluid were very inconstant. Injection of normal cerebro-spinal fluid sometimes produced a fall in blood pressure, sometimes a rise, and many times no effect at all. The quantity of cerebro-spinal fluid injected varied from 0.25 to 1.0 c.c. and exactly the same quantities of the two samples obtained before and after vagal stimulation were injected. Out of 27 experiments done, in 18 there was no change in the chemical composition of cerebro-spinal fluid obtained after vagal stimulation as compared with the normal cerebro-spinal fluid. In 2 experiments the depressor effect of the cerebrospinal fluid was increased after central vagal stimulation, in 3 only a depressor effect appeared after vagal stimulation, and in 1 experiment the pressor activity of cerebro-spinal fluid disappeared after central vagal stimulation. These 6 experiments therefore suggest that a depressor substance sometimes appears in the cerebro-spinal fluid after central vagal stimulation. In the remaining 3 experiments, however, the results were exactly opposite. In one case the depressor activity of normal/

normal cerebro-spinal fluid decreased after the stimulation and in two cases a pressor activity appeared after such stimulation.

Fig. 3 shows the effect of an intravenous injection of the two samples of cerebro-spinal fluid obtained before and after vagal stimulation in one of the experiments.

# Discussion. All fluid, laisted later endeline

The effects of an intravenous injection of cerebro-spinal fluid have been a subject of controversy for some years. Cushing and Goetsch (1910) observed that human cerebro-spinal fluid injected intravenously in rabbits and dogs produced a haemodynamic action similar to that of posterior pituitary extract. Carlson and Martin (1911) denied this and showed that the cerebro-spinal fluid obtained from dogs had no pressor or depressor effect on canine circulation. It is significant to note in this connection that Cushing and Goetsch used concentrated human cerebrospinal fluid, while Carlson and Martin used undiluted canine cerebro-spinal fluid. Jacobson (1920) made a comprehensive study of the haemodynamic actions of the/ the cerebro-spinal fluid, human and bovine, and also studied the effect of an artificial cerebro-spinal fluid prepared according to the formula given by Halliburton (1889) and that given by Meztrezat (1912). He concluded that concentrated human and bovine cerbero-spinal fluid give a depressor effect probably due to histamine. Concentrated cerberospinal fluid human and bovine and artificial cerebro-spinal fluid, injected intravenously in rabbits and dogs is accompanied by a depressorpressor effect, the small pressor component being quantitatively equal to that of the isotonic solution and the over reaction of the animal to the depressor effects, while the depressor was due to the concentrated inorganic Na and K salts. He further concluded that the augmented depressor effect of cerebro-spinal fluid was due to a small amount of organic material in solution.

It was not the aim of my experiments to determine whether pressor substances are normally present in the cerebro-spinal fluid or not. From the table it will be seen that the haemodynamic activity of the cerebro-spinal fluid when tested in the same species, varies considerably. The effect/

effect produced by the normal cerebro-spinal fluid injection was therefore disregarded and attempts were made to see if any depressor substance was liberated in the cerebro-spinal fluid after central vagal stimulation. As has been pointed out before that in 6 out of 27 experiments there was an evidence of an increased depressor activity of the cerebrospinal fluid collected after vagus stimulation. The depressor effect of 3 samples of cerebro-spinal fluid out of these 6 was not like the depressor effect of acetvl choline. The effect was more (cf.Fig. 3a) like histamine than acetyl choline. / In only 3 remaining experiments therefore there appeared a substance resembling acetyl choline in its haemodynamic action on cat's blood pressure. The fall of blood pressure produced by intravenous injections of these three samples was quick and transient. The effect of atropine could not be studied on the depressor activity of these samples of cerebrospinal fluid because sufficient quantities of the fluid could not be collected. It would appear from these experiments therefore that only in 10 per cent. of cases depressor substance appears in the cerebrospinal fluid after central vagal stimulation.

(3) /

(3) Effect of Cerebro-Spinal Fluid collected before and after Central Vagal Stimulation on the Frog's Heart.

The effect of cerebro-spinal fluid collected before and after stimulation of the central end of the vagi in cats was studied on the frog's heart. Clark's method (1926) of recording isometric contraction of the ventricular strip of frog's heart was chiefly used for the purpose. The strip was either stimulated by break induction shocks or the pace-maker was left intact while preparing the strip so that the strip contracted at its normal The Ringer's fluid was prepared according rate. to the formula suggested by Clark (1926). Rana temporaria were used for the experiments, for it was found that the heart of R. esculenta did not respond to acetyl choline so readily as the heart of R. temporaria. Several applications of acetyl choline solution varying from 1:10<sup>10</sup> to 1:10<sup>6</sup> were made to the strip to test its response. As pointed out by Clark the sensitivity of the strip increased after such applications of acetyl choline solutions and a strip thus sensitised was used to test the samples/

samples of cerebro-spinal fluid.

The sensitivity of the heart when tested by this method was found to vary considerably. Some strips showed a definite response even in such high dilutions of acetyl choline as 1:10<sup>11</sup>. Strips of heart in some other experiments showed no response with a 1:10<sup>7</sup> solution of acetyl choline. As a rule however concentrations of acetyl choline more than 1:10<sup>9</sup> showed a definite depression of the heart.

The cerebro-spinal fluid was slightly diluted with distilled water to make it isotonic with the Ringer's solution. The dilutions were not made very accurately, but the two samples of cerebrospinal fluid were diluted exactly similarly, as only a comparative study was to be made. The procedure adopted was as follows.

The ventricular strip was tested with different concentrations of acetyl choline and the depression of the heart produced by these solutions of acetyl choline noted. The two samples of cerebro-spinal fluid collected before and after central vagal stimulation were then diluted with distilled water and applied to the strip. The order of application varied/ varied, sometimes the vagal sample was applied first and sometimes the normal was applied first. In some of those cases which showed a depression of the heart after application of the cerebro-spinal fluid, the nature of the depressor substance was tested by treating the strip with the same quantity of cerebro-spinal fluid after atropinisation with a 1:10<sup>6</sup> solution of atropine. The results obtained in one such experiment are shown in Fig. 4.

The response of a ventricular strip contracting to its pace-maker is shown in this figure. The strip was treated with four different concentrations of acetyl choline and the fifth application was a 1:10<sup>9</sup> solution of acetyl choline seen at the beginning of the tracing. It will be seen that there is a moderate depression of the heart after application of this strength of acetyl choline. The Ringer was then changed, the strip allowed to contract for some time and then a 1:5 solution of cerebro-spinal fluid obtained after stimulation of the central end of the vagus, applied to the ventricular strip. The cerebro-spinal fluid was, in this case, diluted with an equal volume of water and this diluted cerebro-spinal fluid was put in a volume/

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volume of Ringer 2.5 times as great, so that the final dilution of cerebro-spinal fluid in the bath was 1:5. The effect produced by this application of cerebro-spinal fluid is seen in the figure. The third application was that of the normal cerebrospinal fluid diluted exactly in the same way. Application of normal cerebro-spinal fluid produced a very slight depression of the heart as is seen in the figure. The strip was then soaked in a 1:10<sup>6</sup> solution of atropine for 3 minutes and after this interval the same quantity of cerebro-spinal fluid obtained after vagal stimulation as was used before atropinisation of the strip, was added to the bath. As will be seen from the figure there was no depression of the heart after this application of the cerebro-spinal fluid. The effect of normal cerebro-spinal fluid was similarly tested and the experiment terminated by application of a 1:109 and later 1:10<sup>6</sup> solution of acetyl choline. The latter two results are not shown in the figure, but there was no response to acetyl choline as the strip was properly atropinised. It will therefore be seen from the figure that a substance having a depressant action on the heart muscle which was antagonised by atropine, appeared in the cerebrospinal/

spinal fluid after stimulation of the central end of the vagus, when no such substance was present in the normal cerebro-spinal fluid. The quantity of this substance, diluted five times was roughly equal to a 1:10<sup>9</sup> dilution of acetyl choline. Quantitatively, therefore, the concentration of this substance in the cerebro-spinal fluid was about 1 in 200 million.

The appearance of such a substance having an acetyl-choline-like action is, however, not a constant effect. The following table shows that such a substance appears only rarely in the cerebrospinal fluid as a result of central vagal stimulation.

Table V. /

### Table V.

The effect of cerebro-spinal fluid, obtained before and after central vagal stimulation on the ventricular strip of frog's heart and the action of atropine in some experiments.

NO.	Effect of normal - cerebro- spinal	Effect of cerebro- spinal fluid after central vagal stimulation	Effect of vagal cerebro-spinal fluid after atropine.
100	fluid.	and mussions in the r	brasl eerobro-
lpl	mi Tiula, n	nd in One experiment	the quantity
2	ae?	nt sub?tance present	in the formal
3	bro?epinel	luid fos increased	ifter control
4	1 ?timilati	op. ?nres out of t	not done
5	s ?nerefore	suggo?t thet a cont	not done
		arated a substance 1	
		en hat a depressant	
		ar stiffps, The na	
		Ande ?as studied is	
		that Southing the v	
		solution of strop	
		etion. In less th	
		ere, tonte appeared :	
		al fidid after sent	
		a hed to seetyl-che	
	an Tragin a		-
17	?	?	?

+ = stimulation of ventricle.

- = depression of ventricle.

It will be seen from the table that out of 17 experiments, in 2 there appeared a substance in the cerebro-spinal fluid after central yagal stimulation having a depressant action on the ventricular strips of the frog's heart, when no such substance was present in the normal cerebrospinal fluid, and in one experiment the quantity of the depressant substance present in the normal cerebro-spinal fluid was increased after central vagal stimulation. Three out of these 17 experiments therefore suggest that a central vagal stimulation liberated a substance in the cerebrospinal fluid which had a depressant action on the frog's ventricular strips. The nature of this depressant substance was studied in two experiments, and it was found that soaking the ventricular strip in a 1:10<sup>6</sup> solution of atropine antagonised its depressant action. In less than 12 per cent. of cases, therefore, there appeared a substance in the cerebro-spinal fluid after central vagal stimulation which had an acetyl-choline-like action on the frog's heart.

(4) / 0 (8), so that only a small hole (8)

(4) Effect of Cerebro-Spinal Fluid, collected before and after Central Vagal Stimulation on the Rat's Intestines.

Besides the depressor action on the blood pressure and depressant action on the heart, acetyl choline has the important action of producing stimulation of the intestines. Experiments were therefore made to see if the cerebro-spinal fluid collected after central vagal stimulation had this property.

Rabbit's gut is ideal to test the presence of an acetyl-choline-like substance in a given solution, but it requires comparatively larger quantities of Ringer's solution to suspend a small piece of rabbit's gut in it. As only small quantities of cerebro-spinal fluid could be collected, it was found necessary to use a bath having a capacity not more than 2.0 c.c. It was therefore decided to use the rat's gut instead of rabbit's.

An apparatus prepared by Mr N.E. Condon and shown in Fig. 5 was used. A large sized glass tube (A) about 4 cm. long was attached to a bent glass tube (**B**), so that only a small hole (**B**) formed/ formed the continuity between the two. The open end of the tube (C) was connected to an air reservoir and the air bubbled through the small hole (B). The tube (A) which formed the bath, had a capacity of approximately 2.0 c.c. A piece of rat's gut about 2 cm. long was suspended in the bath by fixing its lower end to a hook on a bent wire (D) and attaching its upper end to a writing lever. The whole apparatus was immersed in a wide mouthed thermos flask and the temperature was thus maintained at 39°C. It was found that the temperature of the bath kept nearly 39°C. for about one hour and the variation in three hours was not more than 3°C. As the experiment was usually completed in less than an hour, it was found unnecessary to use more elaborate apparatus for maintaining the temperature constant. Solutions were changed and test solutions added by means of a pipette.

There was a very marked variation in the response of the rat's gut to acetyl choline. The most sensitive preparations responded to a strength of 1 in 500 million of acetyl choline, while some did not respons even with a 1 in 1 million concentration. Some pieces of gut which showed a sensitivity/ sensitivity to acetyl choline were used to test the presence of an acetyl-choline-like substance in the cerebro-spinal fluid, Fig. 6 shows one such experiment.

The upper tracing shows the results obtained before atropinisation of the gut and the lower after atropine. It will be seen that application of cerebro-spinal fluid (equal quantities of Ringer and cerebro-spinal fluid were used in this experiment) obtained after vagal stimulation produced a contraction of the gut slightly less than that produced by a 1 in 500 million concentration of acetyl choline, while the normal cerebro-spinal fluid did not produce any marked action. It will further be seen that atropine completely antagonised this action of the cerebro-spinal fluid obtained after vagal stimulation.

It is difficult to do any quantitative estimations from the results obtained by using the rat's gut, for the response was very variable and the same concentrations of known acetyl choline solutions did not always give comparable contractions of the gut. In this experiment the cerebro-spinal fluid was diluted 1:2 and the response/ response was approximately equal to a 1:500 million acetyl choline. It could be calculated therefore that the acetyl choline concentration in the cerebro-spinal fluid was approximately 1:250 million. The nature of this substance is suggested to be like acetyl choline by the fact that the contraction produced was antagonised by atropine.

# (5) Discussion.

It will be seen from the experiments on cat's blood pressure and frog's heart that only in a small proportion of experiments (approximately 10 per cent) a substance resembling acetyl choline in its action appeared in the cerebro-spinal fluid after central vagal stimulation. Experiments on rat's gut supported the observation that this substance, when it appeared in the cerebro-spinal fluid, had an acetyl-choline-like action. Tests on cat's blood pressure and frog's heart should detect the presence of about 1 in 200 million dilution of acetyl choline ( $0.005 \gamma$  in 1 c.c.). The general result of these experiments is to show that the acetyl choline content of the normal cerebro-spinal/

spinal fluid as well as that obtained after central vagal stimulation is less than 1 in 200 million. Occasionally, however, this concentration is reached or exceeded and could be detected by the biological tests.

The cerebro-spinal fluid used for these tests was obtained by cisternal puncture. The formation, circulation and absorption of cerebro-spinal fluid are still controversial problems, but many authors believe (Weed, 1922) that it is formed in the ventricles of the brain from the choroid plexuses. The cerebro-spinal fluid formed in the lateral ventricles passes into the third ventricle through the foramina of Monro and through the aqueduct of Sylvius into the fourth ventricle. The fluid then passes through the foramina of Magendie and Luschka into the cisternal dilatation of the subarachnoid space and then spreads over the surface of the brain and spinal cord.

Cushing (1925) pointed out that the normal flow of cerebro-spinal fluid in the perivascular channels is from the brain towards the subarachnoid space. There are therefore two sources of cerebrospinal/

spinal fluid: the choroid plexuses in the ventricles and the perivascular channels found in relation with brain vessels. The fluid is eventually absorbed through the arachnoid villi into the venous sinuses. The fluid which originates in the brain and flows through the perivascular channels should therefore contain the products of metabolism in the brain. If there is any acetyl choline liberated in the hypothalamic region as a result of central vagal stimulation, it should therefore appear in stronger concentration in the cerebro-spinal fluid circulating in these perivascular "lymphatics". The cerebro-spinal fluid in the cisterna magna chiefly consists of the fluid derived from the choroid plexuses in the ventricles, while only a small portion of it will consist of the fluid derived from the other source. If acetyl choline is liberated in the central nervous system as a result of central vagal stimulation, its quantity will naturally be very small and it will only partly appear in the cerebro-spinal fluid collected from the cisterna. It is thus possible to understand why in only a small proportion of cases it is possible to detect the acetyl-cholinelike substance in the cerebro-spinal fluid collected from the cisterna.magna.

Part IV. Occurrence of an Acetyl-Choline-Like

Substance in the Brain.

(1) Literature.

- (2) Preparation of brain extracts.
- (3) Physiological and physico-chemical properties of brain extracts.
- (4) Identification of active principle in brain extracts,
- (5) Concentration of acetyl choline in brain extracts.

substance deletly responsible for decreases effects

IV. OCCURRENCE OF ACETYL-CHOLINE-LIKE SUBSTANCE

IN THE BRAIN.

#### (1) Literature.

Since the observation of Schäfer and Moore (1896) that intravenous injections of brain extracts produce a fall in blood pressure, there has always been a controversy about the nature of the substance in these extracts. Mott and Halliburton (1899) thought it to be choline because both choline and brain extracts produced a temporary fall in blood pressure and a remarkable increase in the splenic waves. Their observation was strongly supported by the discovery of Gulewitsch (1899) who by chemical tests demonstrated choline in fresh ox brain extracts. More support was given to Mott and Halliburton's observation by Hunt (1900) who agreed that the substance chiefly responsible for depressor effects of brain extracts was choline.

Halliburton made a preliminary statement of his results at the Physiological Society meeting held on 17th February, 1900, and at the same meeting Osborne and Vincent read a paper on the same subject, agreeing/

which have the property of lowering blood pressure. He. however, produced convincing tracings which clearly demonstrated that atropine completely neutralised the depressor activity of the brain extracts and confirmed his original observation about the presence of choline. Dixon (1901) in a private communication agreed with Halliburton that the depressor effects were antagonised by atropine. Apart from physiological tests, Halliburton demonstrated choline by chemical methods also. A couple of years later Vincent and Sheen (1903) confirmed the original observations of Osborne and Vincent and denied that atropine antagonised the action of brain extract. They found that in their experiments, preparation of extracts by Halliburton's method and adjusting the doses as recommended by him did not confirm his results. They therefore concluded that choline was not the most active agent in the brain extracts. Both sides, however, seem to have agreed on the question of presence of choline in the brain, the point of discussion being whether the most active depressor agent was choline or not.

Miller and Miller (1911) explained the variance in/

in results obtained by previous workers regarding the nature of the depressor substances in tissue extracts by observing that the experimental conditions were different in different experiments. They observed that injections of tissue extracts prepared from one species, when injected into several different species, gave different results. They used dogs in their experiments for preparing the extracts and testing them, and found that alcoholic extracts of all nervous tissue contained a depressor substance. They further observed that prolonged heating in an autoclave destroyed the depressor substances present in the brain and anterior lobe of the hypophysis but did not materially affect the depressor substance in the posterior lobe.

The multiplicity of depressor substances in brain extracts was recognised by Halliburton as early as 1901. Many workers have confirmed that observation since then. Major and Weber (1929) using ox brain found that there are at least three substances present - histamine, choline and a third unidentified substance whose action is not affected by atropine. In a subsequent communication (1930) they/

they observed that the depressor activity of certain brain extracts was not due to histamine, choline or acetyl choline. It is significant to note in this connection that while preparing the extracts they had treated the filtrate with sodium hydroxide. Euler and Gaddum (1931) made a careful study of the vasodilator substances in the tissue extracts and pointed out that there may be as many as five different substances having a depressor effect on blood pressure. They pointed out that particularly in brain there occurs a substance which lowers the blood pressure in atropinised rabbits and also stimulates the tone and rhythm of atropinised rabbit's gut. Major, Nanninga and Weber (1932) studied the physiological activity of brain extracts and arrived at the conclusion that choline or acetyl choline were not the active agents producing depressor effects in those extracts. These results were published as a preliminary communication (1932 ) and subsequently to this Chang and Gaddum (1933) published a paper dealing with the depressor activity of brain extracts. They found that the acetyl choline equivalent of dog's brain was about/

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about 0.4  $\gamma$  per gram of brain tissue. It will thus be seen that the multiplicity of depressor substances in brain extracts may partly explain the divergence of results obtained by several workers, but there is some definite evidence pointing to the occurrence of acetyl-choline-like substance in the brain. The writer therefore undertook experiments to determine whether acetyl choline or an acetylcholine-like substance was normally present in the brain.

#### (2) Preparation of Brain Extracts.

Fresh brains of cats and rabbits were used for this purpose. In some experiments the whole brain was used for extraction, but in most cases different portions of the brain (cortex, basal ganglia and cerebellum ) were used. Extracts were prepared from these three different parts of the brain and their depressor activity compared. The extracts were prepared by macerating the brain tissue in an acetone-water-acid mixture, containing 75 per cent. acetone, 15 per cent. distilled water and 10 per cent. N/100 acetic acid. The tissue was ground in/ in a mortar with silver sand and made into an emulsion, using 2.0 c.c. of the mixture (acetoneacid) for every gram of fresh tissue. The emulsion was allowed to stand for 24 hours at 5°C., filtered, and the filtrate evaporated to dryness in vacuo at room temperature. The residue was dissolved in distilled water, filtered and used for injections as crude extract. No attempt was made to purify the extract, less acetyl choline if present might be destroyed during the process of purification. The material used was quite fresh. The brain was removed as quickly as possible, weighed and put in the acetone-acid mixture as soon as possible.

(3) Physiological and Physico-chemical Properties of the Brain Extract.

Some of the physiological and physico-chemical properties of the extract were studied.

(i) Action of brain extract on the circulatory system.

Small quantities of the extract were injected in cats. The animals were anaesthetised with paraldehyde and ether or urethane, and their blood pressure recorded by the usual method. Injections were/ were given through a cannula in the femoral vein and run in with about 1.0 c.c. of warm saline. Intravenous injections of brain extract produced a marked fall in blood pressure. The fall was abrupt and of short duration, and the recovery of the blood pressure was complete after such a fall. It was not uncommon to see the blood pressure going above the normal level after the recovery for a short time and come back to the original level again. The fall in blood pressure was accompanied by a slowing of the heart. In short, the nature of the fall of blood pressure was indistinguishable from that produced by an intravenous injection of acetyl choline. The similarity between intravenous injections of acetyl choline and brain extract is strikingly seen in Fig. 7.

The action of brain extract was studied on frog's heart also. Clark's method of isometric contraction of ventricular strip was used for the purpose. Some experiments were also done by perfusing the heart through the inferior vena cava. Application/ Application of small quantities of brain extract to such preparations invariably produced a depressant action, varying from a moderate depression to (Figs.12 and 15) complete cardiac standstill.  $\measuredangle$  On the frog's heart also the nature of depression was the same as that produced by acetyl choline. The action of brain extracts therefore was about the same as that of acetyl choline on the circulatory system.

(ii) Action of brain extract on respiratory system.

Intravenous injections of brain extract were given in cats to study the action on respiration. Small quantities introduced intravenously usually produced a temporary acceleration of respiration, as did small quantities of acetyl choline. It appears that in both cases the effect was mostly due to the marked effect on blood pressure.

The effects of intraventricular injections of the brain extracts were also studied in cats. The injections were given through a small hole trephined at the junction of the coronal and sagittal sutures as described in Part II. It was found that such injections produced a depressant action on respiration, varying from a slight depression to complete/ complete cessation of respiration. Fig. 8 shows the results obtained in one such experiment. The animal,weighing 3.5 kg. was anaesthetised with paraldehyde and ether and an injection of 0.1 c.c. of a basal ganglia extract of a rabbit was given at the arrows. It will be seen from the figure that such an injection produced a considerable depression of respiration and a slight rise in blood pressure. The depressant effect was temporary. A second injection of the same quantity of cortical extract of the same rabbit produced less marked but more prolonged depression of respiration. The animal completely recovered its normal respirations 8 min. after the 2nd injection in this experiment.

As has been pointed out in Part II, intraventricular injections of acetyl choline have the effect of producing a depression of respiration. The same effect is produced by injecting small quantities of the brain extract intraventricularly. It will be seen, therefore, that both acetyl choline and brain extract have the same action on respiration, whether introduced intravenously or intraventricularly.

(iii) /

(iii) Action of brain extract on the intestines.

The physiological action of brain extract was studied on the isolated pieces of intestines of rabbits and rats. For experiments on rabbit's isolated gut, an apparatus constructed on the same model as Dale's apparatus for perfusion of isolated organs, was used. The water outside the bath was kept at constant temperature by an electric thermoregulator. The Tyrode solution in the bath was oxygenated with air and a piece of gut about 4.0 cm. long was suspended in it. The lower end of the gut was fixed to a hook and the upper attached to a frontal lever.

The animal was killed by opening the carotid arteries, its abdomen opened and the whole length of the gut taken out in pieces about 1 foot long. The lumen of the gut was washed with tap water by inserting a small funnel at one end and allowing the tap water to run through it. These were then kept under Ringer's solution and put in an icechest. Small pieces about 4.0 cm. long were cut whenever required and suspended in the bath.

For studying the action of the brain extract on the rat's gut, the apparatus described in Part III was/ was used. The rats were stunned by a blow on the head, their carotids opened and intestines taken out. Pieces of intestine were suspended in the bath and their contractions recorded by a frontal lever.

Brain extracts were added to the bath by means of a pipette. The effect of brain extract (basal ganglia extract) on the movements of an isolated piece of rabbit's gut is shown in Fig. 9. It will be seen that the extract produced a marked increase in the tone of the muscle. The nature of the stimulant action produced on the rabbit's gut is about the same as that produced by a small dose of acetyl choline.

The same effect is observed by applying a solution of brain extract to isolated rat's gut. There is a marked contraction of the piece of gut and an increase both in the tone as well as the amplitude of contraction of the pendular movements.

(iv) Stability of the brain extract.

The physiological properties of these extracts are markedly lessened by storing at room temperature for 4 days. They lose their properties more slowly if stored at 5°C., the activity being approximately/ approximately reduced to half after storing for a week.

Heating the extract partly destroys its activity. The effect of heat on the basal ganglia extract is shown in Fig. 10.

Movements of a piece of an isolated rabbit's gut are shown in this experiment. The first application of 1 c.c. of basal ganglia extract of a rabbit produced the usual effect on the movements of the gut. The same quantity of the extract was then heated on a water bath for 5 minutes, the residue dissolved in water and applied to the same piece of gut. It will be seen from the figure that heating has only partly destroyed the activity of the extract.

Similar experiments were done on the blood pressure of cats and the results obtained on rabbit's gut confirmed. Heat therefore partly destroys the physiological activity of the extract.

The effect of alkali was studied on the physiological activity of the extracts. The alkali used was N. NaOH. Different quantities were added to the extracts and allowed to act for a varying length of time. The extracts thus treated were then/ then tested on rabbit's gut and cat's blood pressure. Fig. 7 shows the effect of NaOH on the depressor effect of the basal ganglia extract. One third the volume of N. NaOH was added to the extract in this experiment, and the alkali was allowed to act for 5 minutes. An equivalent solution of acetyl choline was also treated in the same way and used as a control. It will be seen from the figure that alkali considerably destroys the vaso-depressor activity of the brain extracts. The control experiment done with a known solution of acetyl choline also shows the same degree of destruction. It will be seen therefore that alkalies like NaOH destroy the activity of brain extract to the same extent as they do that of acetyl choline.

The combined effect of alkali and heat was tried in some experiments. Boiling the extracts with NaOH completely destroyed their physiological activity. There was no effect on blood pressure of cats or isolated pieces of rabbit's gut. Solutions of acetyl choline treated similarly behaved in the same way.

The general result of these experiments was to show that brain extracts have about the same physiological/ physiological activity as acetyl choline and closely resembled acetyl choline in some physicochemical properties as well. Different biological tests were therefore employed to identify the active principle in the extracts.

## (4) Identification of the Active Principle in Brain Extract.

As has been pointed out at the beginning of this part, the multiplicity of physiologically active substances in brain extracts has been generally recognised. As will be discussed later, the result of my experiments was also to show that there are substances, other than acetyl choline, present in the brain extracts. Demonstration of acetyl choline in the brain will go a long way in establishing the theory of humoral transmission, and therefore an attempt was made to see if such a substance occurs in the brain or not. The two most important tests employed for the identification of acetyl choline are the antagonism by atropine and enhancement of the action by physostigmine. These tests were therefore employed to test the presence of acetyl choline in the brain extracts.

(i) /

# (i) Action of atropine on the physiological activity of brain extracts.

The controversy regarding the antagonistic action of atropine on the vaso-depression produced by intravenous injection of brain extracts has been already referred to. I used cats and rabbits brain for preparing the extracts and these were tested on cat's blood pressure and rabbit's intestines. Extracts prepared from one species were therefore tested on two species, one belonging to the same as the extracts were prepared from, and the other a different one. I found that the reactions provoked by these extracts were about the same, and the change in species did not materially influence the reactions.

The effect of atropine on the cat's blood pressure was tested by the following method.

The animal was anaesthetised withurethane or parladehyde and ether and the blood pressure recorded by the usual method. A preliminary dose of acetyl choline was given intravenously to test the response. This produced the usual marked fall in blood pressure and the degree of vaso-depression thus produced noted. An intravenous injection of about/ about 0.5 to 1.0 c.c. of the extract prepared from the basal ganglia, cortex or the cerebellum, was then given, and the fall in blood pressure produced by such injection noted. An intravenous injection of atropine was then given in small repeated doses till a complete paralysis of the vagus was produced. This was tested by injecting acetyl choline intravenously and also by stimulating the vagus in the neck. After the animal was effectively atropinised a second injection of an equal quantity of brain extract as was given before atropinisation of the animal, was given. The response of this injection was noted and the effect of the two injections, before and after atropine compared.

The dose of atropine usually employed was about 1 mg. per kg. of the weight of the animal. This quantity satisfactorily paralysed the vagus as no depressor effects were observed either by vagus stimulation or an acetyl choline injection. It was not uncommon to find that such atropinisation of the animal lowered the level of blood pressure in many experiments. The level of blood pressure thus lowered was not, however, too low to test depressor/ depressor reactions. In some experiments, however, complete atropinisation could be effected without any marked changes in the level of blood pressure.

Intravenous injections of brain extracts before and after atropine did not give uniform results. The depressor effect was only lessened in some experiments and completely abolished in others. Fig. 11 shows the complete paralysis of the depressor effect of an intravenous injection of 0.5 c.c. of basal ganglia extract of a cat, on cat's blood pressure. The drum in this case was turned back to get superimposed tracings. It will be seen from the figure that the extract before administration of atropine produced a fall in blood pressure (A) while the same quantity after atropine did not (B). The level of blood pressure was the same in both cases.

In some other experiments, however, the antagonistic action of atropine was only partial. Administration of the brain extract in these cases did produce a fall in blood pressure after atropinisation of the animal, but the fall was less than that produced by the control injection. It was not uncommon, however, to get extracts whose depressor activity/ activity was completely abolished byatropine.

The antagonistic effect of atropine on the stimulant action of the brain extracts on rabbit's gut was also tested. The piece of gut was suspended in Tyrode's solution which was kept at 39°C. and oxygenated with air as described previously. A small quantity of acetyl choline was added to the bath to test the reaction. The solution was then changed, fresh Tyrode's solution put in and the reaction of the gut to a small quantity of the brain extract recorded. The gut was then soaked in a fresh Tyrode's solution containing atropine. The concentration of atropine varied from 1:1,000,000 to 1:10,000 and was allowed to act from 5-20 minutes. Atropine solution was then washed off and the reaction of the gut to acetyl choline and the brain extract again recorded. Fig. 9 shows the results obtained in one such experiment. A 1 in 20 million concentration of acetyl choline produced a contraction of the gut as seen in the figure. A small quantity (0.5 c.c.) of basal ganglia extract of a rabbit produced about the same result. The gut was then suspended in Tyrode's solution containing a/

a 1:50,000 concentration of atropine for 15 minutes. The same quantities of acetvl choline and basal ganglia extract were then applied, and as the figure shows, the results were totally different. Acetyl choline did not produce any action at all on the movements or the tonus of the gut. The extract first produced a lowering of the tone which was quickly regained and the amplitude of contraction of the pendular movements slightly increased. This increase in the amplitude of contraction was also seen when the extract was applied before atropine and was evidently due to some other substance present in the extract. This experiment, however, showed that there is some substance present in the brain extract whose activity is definitely antagonised by atropine.

As in the case of blood pressure, the results obtained with the rabbit's gut also were inconstant. The motor activity of some extracts was completely abolished by atropine, while that of others was abolished only partially. These results therefore confirmed those obtained with cat's blood pressure.

A few experiments were also done where the effect of atropine on the depressant action of brain/

brain extracts on the frog's heart was tested. Isometric contractions of the frog's ventricular muscle were recorded in these experiments as described before. Extracts of brain were then applied both before and after atropine, and the difference in the response noted. Fig. 12 shows the results obtained in one such experiment. The ventricular strip was stimulated in this experiment with electric shocks at the rate of 22 per minute. An extract prepared from the basal ganglia and medulla was first applied and as seen in the figure it produced a moderate amount of depression of the heart. A comparatively strong solution of atropine (1:100,000) was then allowed to act for about 5 minutes, washed, and the strip stimulated with shocks as before. Application of an equal quantity of the same extract this time failed to produce the effect showing that the original depression was due to a substance whose activity could be abolished by atropine.

All these experiments therefore show that atropine has a decided action on the physiological activity of the brain extracts.

(ii) /

(ii) Action of physostigmine on the physiological activity of the brain extracts.

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One of the most important tests that has been recently employed for identification of acetyl choline is the fact that acetyl choline produces a contraction of the frog's rectus muscle, which is considerably enhanced by soaking the muscle in physostigmine. This test was therefore used to identify the physiologically active substance in brain extracts.

The apparatus described in Part II. for recording movements of the isolated rat's gut, was used for this purpose. The muscle, rectus abdominis was obtained from R. temporaria and R. esculenta. A strip about 4 cm. long was suspended at room temperature in Ringer's solution oxygenated with air, and was stretched by weights attached to the recording lever. Solutions of acetyl choline and brain extract were then added to the bath and the slow tonic contraction of the strip recorded on a slowly moving drum. The strip was then soaked in a 1:100,000 solution of physostigmine for/

for 40 minutes and the effects of subsequent application of acetyl choline and brain extracts noted. Fig. 13 shows the results obtained in one such experiment. The basal ganglia extract was in this case prepared from cat's brain. Contractions of the rectus muscle obtained by application of the extract twice at 20 minutes interval are shown in the figure as "Normals". This showed that application of the same quantity of the extract produced the same degree of contraction. The muscle was then soaked in a 1:100,000 solution of physostigmine for 40 minutes and exactly an equal quantity of the extract was added, without washing physostigmine out. The contraction produced this time was considerably stronger and is shown in the figure as "Eserinised". The Ringer was then changed and the muscle soaked in a 1:100,000 solution of atropine for 15 minutes. A subsequent application of the extract produced a · contraction approximately equal to that produced by the first two applications.

The nature of contraction of the frog's rectus produced by different chemical substances is described by Chang and Gaddum in a recent publication. They pointed out that the contraction produced by acetyl/ acetyl choline starts quickly and describes a curve which is more convex upwards. The most important point, however, was the enhancement of acetyl choline action by physostigmine. Chang and Gaddum further pointed out that if the action of an extract was increased by physostigmine when tested on the frog's rectus muscle, that fact alone was sufficient to draw the conclusion that the effect was not due to any other substance except acetyl choline. The nature of the contraction seen in Fig. 13 and the marked increase in the contraction after physostigmine are therefore sufficient to conclude that acetyl choline was present in the brain extracts.

# (iii) Action of choline esterase on the physiological activity of brain extract.

Stedman, Stedman and Easson(1932) have shown that a specific enzyme occurs in the blood of animals which has a hydrolysing action on choline esters. They have also prepared this enzyme in a concentrated purified form and named it cholineesterase. This enzyme was therefore used to identify the physiologically active substance in brain extracts. Dr Stedman kindly supplied me with this enzyme/ enzyme and experiments were done on cat's blood pressure, rabbit's intestine and frog's heart with this esterase. The action of the enzyme was found to be very rapid, for it completely destroyed acetyl choline in high dilutions such as 1:1,000,000, in less than 5 minutes. In highly concentrated forms it destroyed acetyl choline in less than 2 minutes at room temperature. By itself the enzyme had no detectable pharmacological action on the blood pressure or respiration, and produced no alteration in the records of these so as to interfere with quantitative estimations. Another advantage was that injections of extracts and acetyl choline could be repeated in the same animal and the results thus could be properly controlled. I used the following method to test the effect of this esterase on brain extracts.

The animal was anaesthetised with urethane or paraldehyde and ether, and its blood pressure recorded by the usual method. A cannula was fixed in the femoral vein for giving intravenous injections. A control injection of acetyl choline was given intravenously to test the response. The extract to/ to be tested was then divided in three equal portions. The first was injected intravenously and the fall in blood pressure produced by this injection noted. The second was mixed with about half its volume of the enzyme which was allowed to act for about 15 minutes, and injected intravenously as before. The third portion was then injected to see if the response was the same as the first one. A dose of acetyl choline treated with the enzyme was then injected to test the activity of the enzyme and this was followed by a control injection of acetyl choline.

Out of the three injections of the brain extract, the first and the third showed exactly similar depression of blood pressure. The vasodepression produced by the second injection of the extract, i.e. one treated with the esterase, varied in different experiments. The activity of some extracts was completely and of others partially destroyed by the enzyme. These results showed that the acetyl choline content of different extracts and the relative proportion of vaso-depressor substances present in the extracts varied considerably. The/ The results therefore agreed with those done with atropine. Fig. 14 shows the effect of esterase on the basal ganglia extract of a cat. At A. an intravenous injection of 0.5 c.c. of the extract was given, and this produced the usual fall of blood pressure and recovery. At B. an equal quantity of the extract previously treated with the enzyme was given. It will be seen from the figure that the enzyme completely destroyed the vaso-depressor activity of the extract, showing that most of the depressor activity of this extract was due to acetyl choline.

The effect of the esterase was similarly tested on the isolated rabbit's gut and ventricular strip of the frog's heart. The effect on the heart is shown in Fig. 15.

The isometric contractions of frog's ventricle were recorded by Clark's method, using 20 shocks per minute to stimulate the muscle. A basal ganglia extract prepared from rabbit's brain was added at the arrow. The effect was a complete cessation of cardiac contractions. The Ringer's solution was then changed and the same quantity of the extract treated with esterase was applied at the second arrow/ arrow. It will be seen from the figure that this application produced depression of the heart which was considerably less than that produced by the first.

The result of this experiment was to show that the cardiac depression produced by the first application was due to more than one substance. The activity of one was destroyed by the esterase while that of the second was not. This second substance was responsible for the depression seen after the second application of the extract. The esterase in this case was quite active and used in sufficient quantity to destroy any acetyl choline present.

It will be seen from the foregoing experiments that the activity of the brain extracts is sometimes completely and sometimes partially destroyed by the choline-esterase. As the physiological activity of the brain extracts was antagonised by atropine, enhanced by physostigmine and destroyed by the specific enzyme, it could be concluded that acetyl choline was one of the active principles producing the physiological effects of the extracts.

(5)/

(5) Concentration of Acetyl Choline in Brain Extracts.

Leimdörfer (1930) first pointed out that the depressor activity of the extracts prepared from the basal ganglia was greater than that of those prepared from any other part of the brain. Experiments were done to study the relative depressor activity of extracts prepared from the basal ganglia, the cortex and the cerebellum.

The extracts were prepared according to the method described in the earlier part. The different portions of the brain were weighed, emulsified with acetone-acid mixture, allowed to stand for 24 hours in a refrigerator at 5°C., filtered, dried in vacuo and dissolved in water in such proportions that each c.c. of the solution represented an equal weight of the basal ganglia, cortex or cerebellum. These were then tested on cat's blood pressure and rabbit's intestines for vaso-depressor and intestinal motor activity. My results agreed with those of Leimdörfer. I found that the vaso-depressor activity of the basal ganglia was most marked and that of the cerebellum least, the activity of the cortex being slightly lower than that of the basal ganglia. Fig. 16 shows/

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shows the effect of intravenous injections of extracts prepared from the cortex and basal ganglia of a cat. Equal quantities (0.5) were injected in each case, and, as will be seen from the figure, the fall in blood pressure produced by the basal ganglia extract is slightly greater than that produced by the cortical. The relative activity of the basal ganglia and cerebellar extracts is shown in Fig. 17.

Quantitative estimations were made to determine the acetyl choline equivalent of extracts of basal ganglia prepared from the cat's and rabbit's brain. An intravenous injection of the extract representing a known weight of the basal ganglia was given in cats and the fall in blood pressure produced by such injection noted. Different quantities of acetyl choline were then injected in the same way and the amount required to produce the same degree of vaso-depression was taken to be the acetyl choline concentration present in that quantity of the extract. From this the acetyl choline equivalent per gram of the tissue was calculated. The following table represents the results obtained.

Table VI /

### Table VI.

The acetyl choline equivalent of basal ganglia extracts per gram of fresh tissue.

No.	Animal	Acetyl choline equivalent in $\gamma$ .
l	rabbit	0.10
2	П	0.40
3	cat	0.20
4	II	0.30
5	rabbit	0.04
6	tt	0.04
7	cat	0.50
8	II	0.25
9	н	1.30
10	u	0.30
11	n	0.25
12	n	0.40

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It will be seen from the table that the depressor activity of extracts of the basal ganglia varied a good deal. The acetyl choline equivalent of the rabbit's basal ganglia varied from 0.4 to 0.04  $\gamma$  per gm. of fresh tissue and the average of the 4 experiments gave a value of about 0.14  $\gamma$ .

In the case of the cat the values varied from 0.2 to 1.3  $\gamma$  per gm. of fresh tissue, and the average of 8 experiments gave a value of about 0.44  $\gamma$ . The value I obtained in the cat therefore agrees with that obtained by Chang and Gaddum in the dog.

-102-DISCUSSION. Part V. 

### V. DISCUSSION.

Evidence has been presented in the foregoing pages that there is a close similarity between central vagal stimulation and intraventricular acetyl choline on the respiratory movements of the Both these procedures produce an inhibition cat. of respiration, which is unaffected by administration of atropine or physostigmine given both intravenously and intraventricularly. In view of the theory of humoral transmission, built mainly on the experiments of Loewi, a hypothetical theory has been put forward to explain this similarity on similar grounds. It has been therefore assumed that central vagal stimulation inhibits respiration by liberating acetyl choline in the central nervous system. A strong proof in support of this theory has been the observation that the effects of central vagal stimulation may come on after cessation of the stimulus.

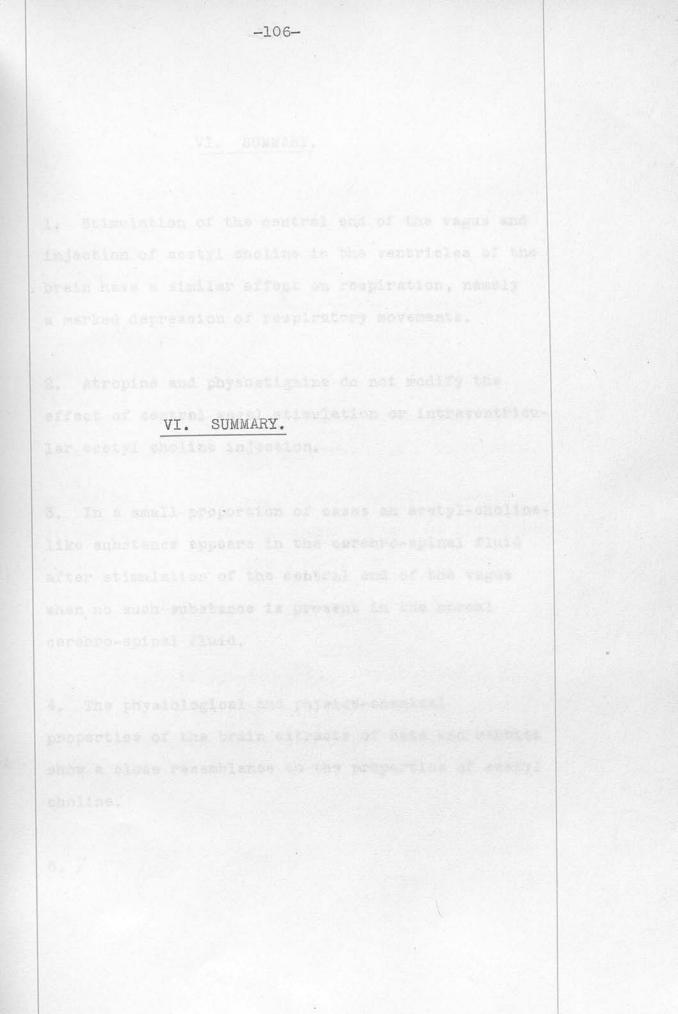
The perivascular channels form the "lymphatics" of the brain and the products of metabolism are drained through these into the cerebro-spinal fluid / fluid. Attempts to detect acetyl choline in the cerebro-spinal fluid after central vagal stimulation were not very successful, but in a certain proportion of experiments an acetyl-choline-like substance could be detected in the fluid after such stimulation.

Occurrence of acetyl choline in the brain is another argument in favour of humoral transmission. It has been shown by Chang and Gaddum that the acetyl choline concentration is high in those organs which are directly under control of the vagus, when compared with those which are not. The auricles have a greater acetyl choline concentration than the ventricles, and it is well known that the vagus has a more control over the auricles than the ventricles. Experiments described in Part IV showed that acetyl choline definitely occurred in the brain extracts. Experiments with atropine, physostigmine and the choline esterase, all pointed that acetyl choline is normally present in the brain. Another important point is the fact that the concentration of acetyl choline was higher in the basal ganglia than in any other part of the brain. Claims have/

have recently been made, with increasing insistence, that the diencephalic centres play an important role in the physiology of the animal. The list of diencephalic "centres" has been rapidly increasing and many functions of the cortex have now been credited to the diencephalon. Occurrence of acetyl choline in the diencephalon in higher concentrations than other parts of the brain therefore seems significant.

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The evidence so far collected in support of humoral transmission in the central nervous system has been indirect. The only direct evidence is the fact that acetyl choline occasionally occurred in the cerebro-spinal fluid after central vagal stimulation, when it was not present in the normal cerebro-spinal fluid, or that if it was present in the normal cerebro-spinal fluid, its concentration in it was increased after central vagal stimulation. These results were, however, obtained only in a limited number of experiments. The indirect evidence was, however, more convincing. It has thus been possible to present certain data which suggest that humoral transmission may occur in the central nervous system.



#### VI. SUMMARY.

5. Vertone piplovical Lests Applied to the brain

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1. Stimulation of the central end of the vagus and injection of acetyl choline in the ventricles of the brain have a similar effect on respiration, namely a marked depression of respiratory movements.

2. Atropine and physostigmine do not modify the effect of central vagal stimulation or intraventricular acetyl choline injection.

3. In a small proportion of cases an acetyl-cholinelike substance appears in the cerebro-spinal fluid after stimulation of the central end of the vagus when no such substance is present in the normal cerebro-spinal fluid.

4. The physiological and physico-chemical properties of the brain extracts of cats and rabbits show a close resemblance to the properties of acetyl choline.

5. /

5. Various biological tests applied to the brain extracts show that acetyl choline is normally present in the brain.

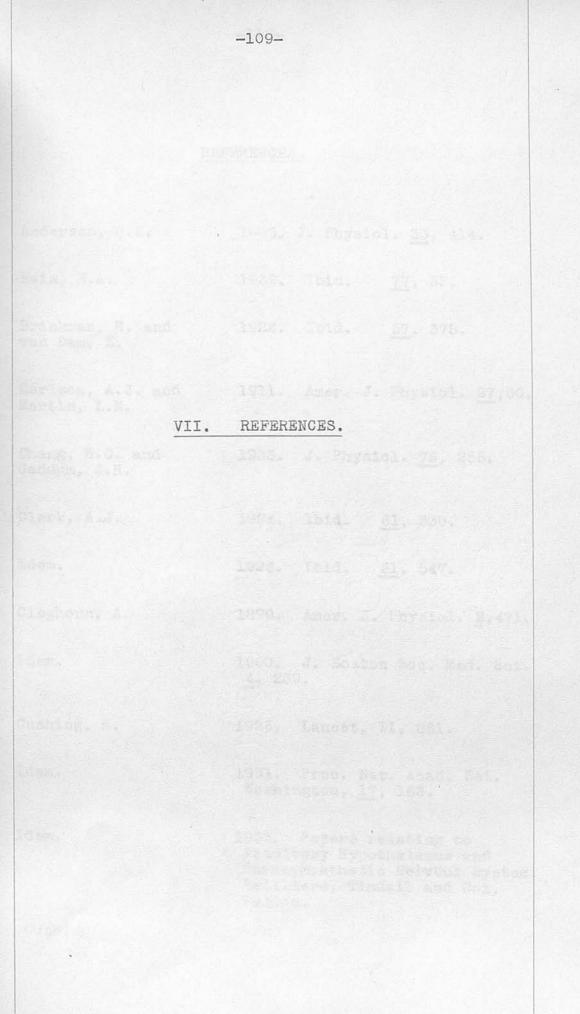
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6. The concentration of acetyl choline is highest in the basal ganglia, and lowest in the cerebellum; the concentration in the cortex is slightly lower than that in the basal ganglia.

7. The acetyl choline equivalent in the basal ganglia is about 0.44  $\gamma$  in cats and about 0.14  $\gamma$  in rabbits.

8. It is suggested that the effects of stimulation of the central end of the vagus are due to liberation of acetyl choline in the central nervous system.

I desire to acknowledge my indebtedness to Professor A.J. Clark for his suggestions and kindly criticism throughout the course of the work.



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Vincent/

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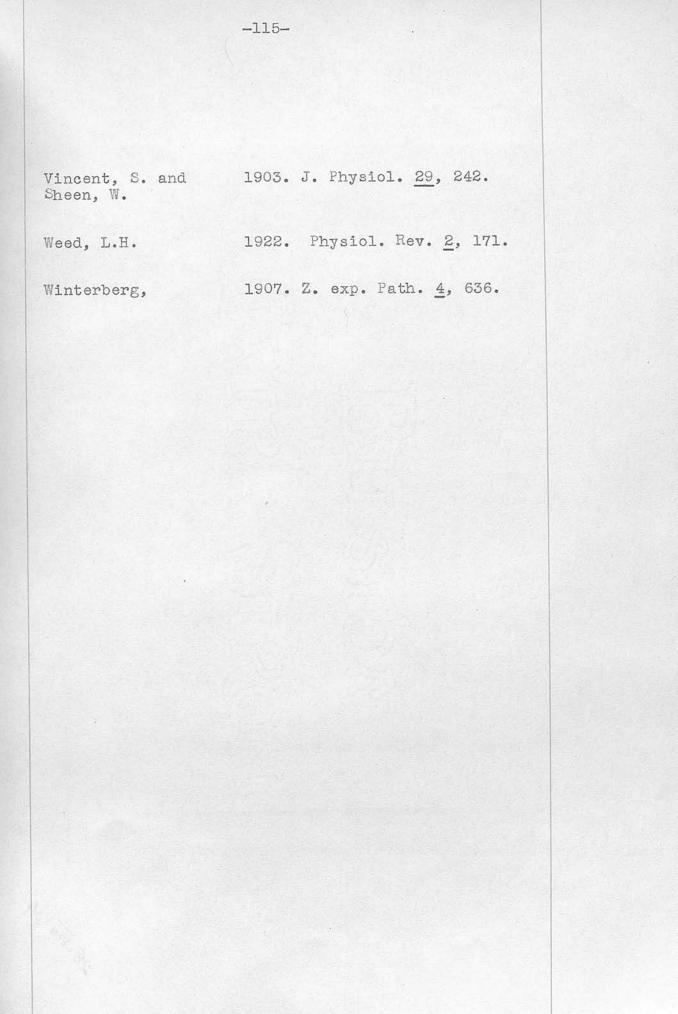


Fig. 1. Female cat, 2.9 kg. Paraldehyde and ether anaesthesia. R = respiration; B.P. = blood pressure. At A. 0.5  $\gamma$  of acetyl choline injected in the right lateral ventricle.

Note cessation of respiration followed by recovery and a slight fall in blood pressure.



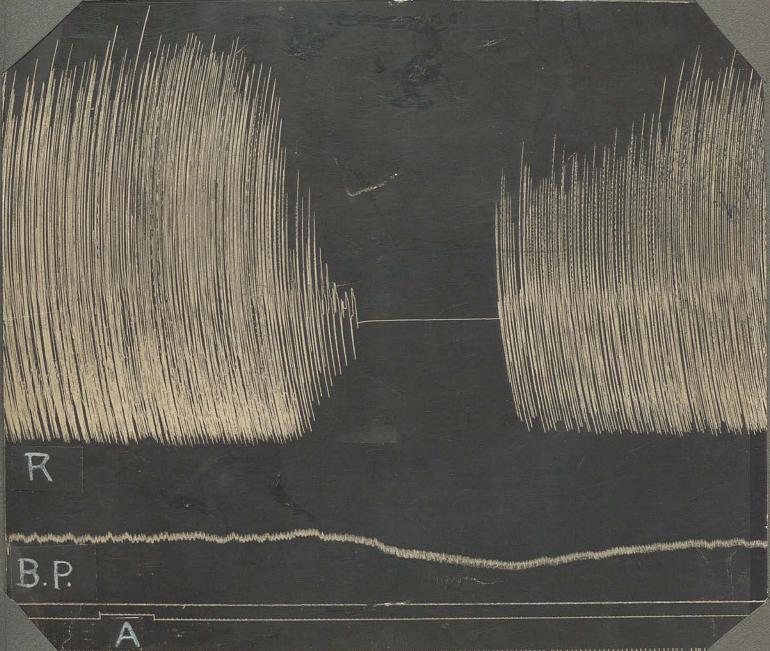


Fig. 2. Male cat, 2.1 kg. Paraldehyde and ether anaesthesia; artificial respiration.

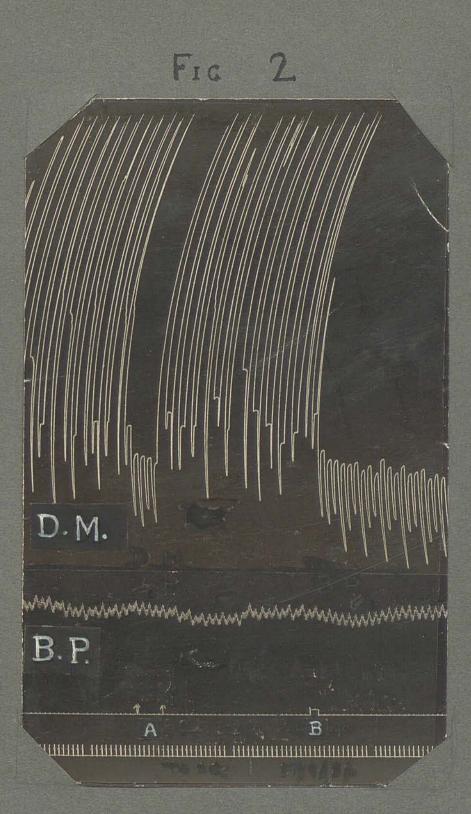
D.M. = Movements of the strip of diaphragm. B.P. = Blood pressure.

At A stimulation of the central end of the left vagus for 10 sec.

Note the complete cessation of diaphragm movements during stimulation and no appreciable change in blood pressure. The small movements of the diaphragm strip during the period of stimulation are due to mechanical effects of artificial respiration.

At B an injection of 0.1  $\Upsilon$  of acetyl choline given in the third ventricle.

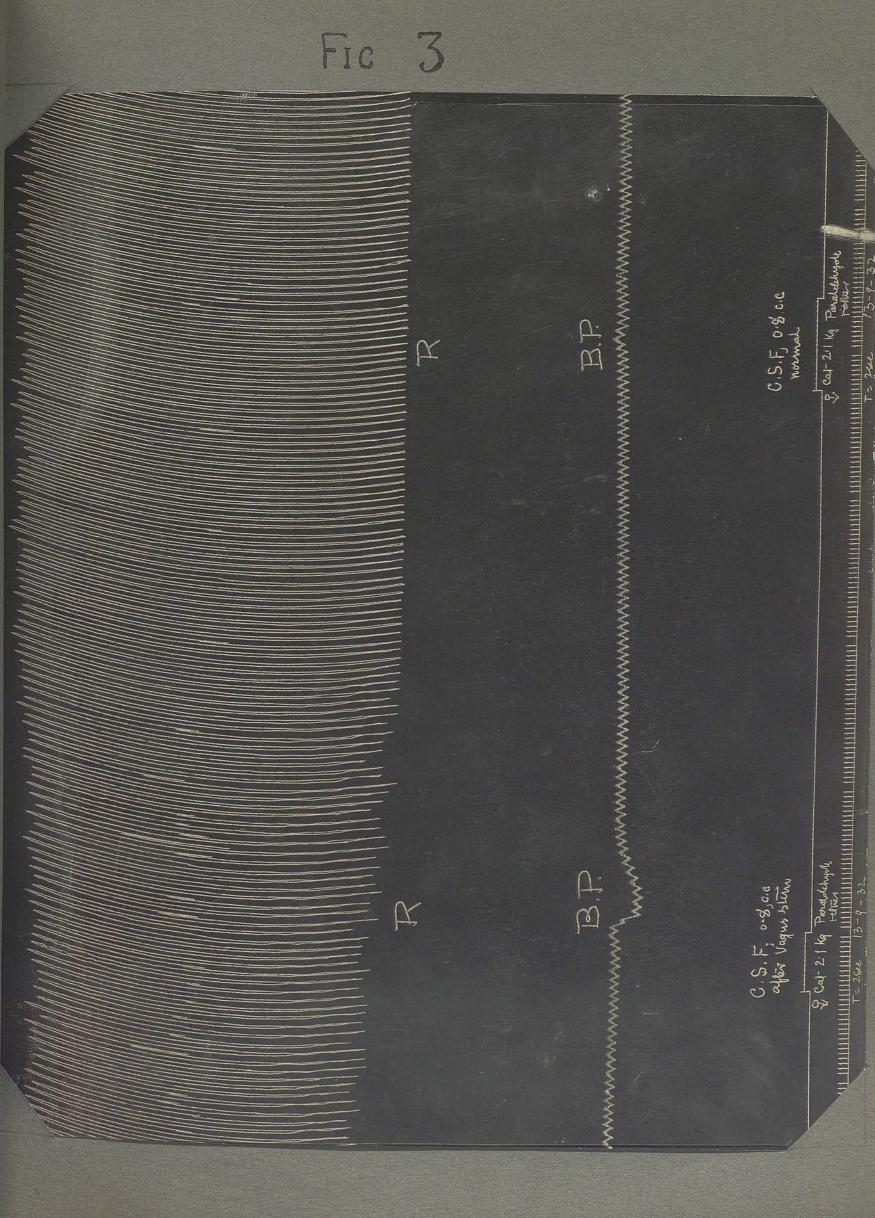
Note complete cessation of diaphragm movements after the injection.



ether anaesthesia. Paraldehyde and Male cat, 2.1 kg. 3. Fig.

R = Respiration; B.P. = Blood Pressure.

signal the same quantity of the normal cerebro-spinal fluid injected At the first signal 0.8 c.c. of cerebro-spinal fluid obtained after At the second central vague stimulation, injected intravenously. intravenously. Note that the vagal sample produces a slight vaso-depression while the normal sample has no appreciable effect on blood pressure. There is a slight stimulation of respiration after the first injection.



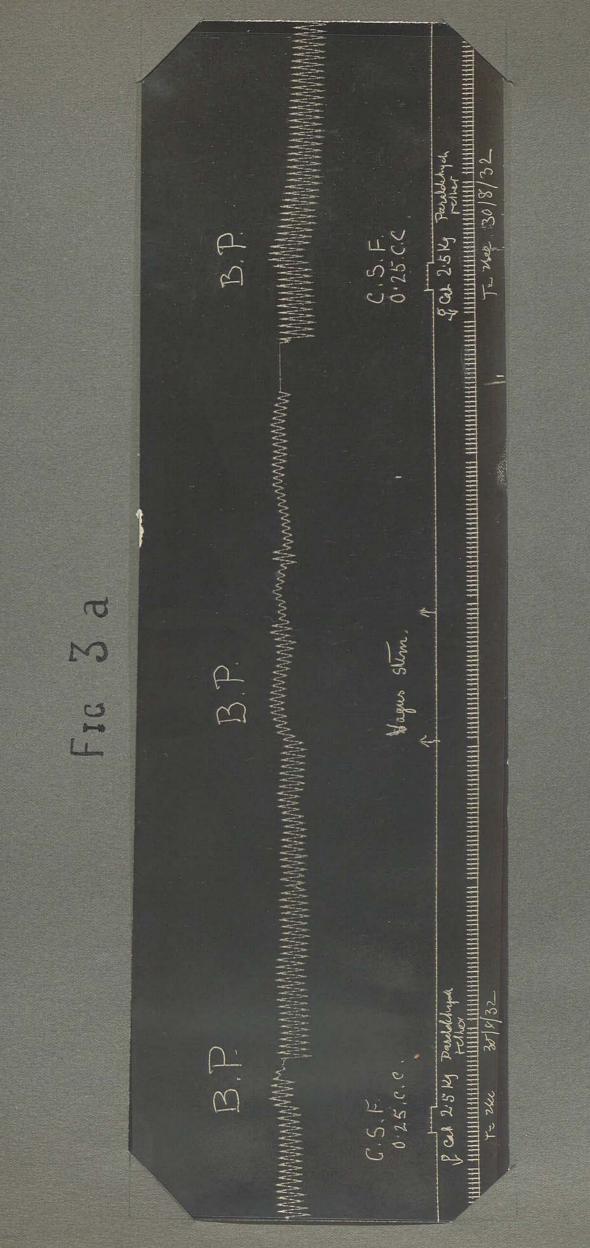
Paraldehyde and ether anaesthesia; Fig. 3a. Male cat, 2.5 kg. artificial respiration.

B.P. = Blood Pressure.

Star No

intravenously. This produced no appreciable change in blood pressure. The central end of the left vagus was then stimulated for about 1 min. (vagus stim.) between the arrows and a sample of cerebro-spinal fluid collected during stimulation. This was injected (0.25 c.c.) at the At the first signal 0.25 c.c. of normal cerebro-spinal fluid injected second signal.

Note the slight continued fall in blood pressure which is not like acetyl choline effect.

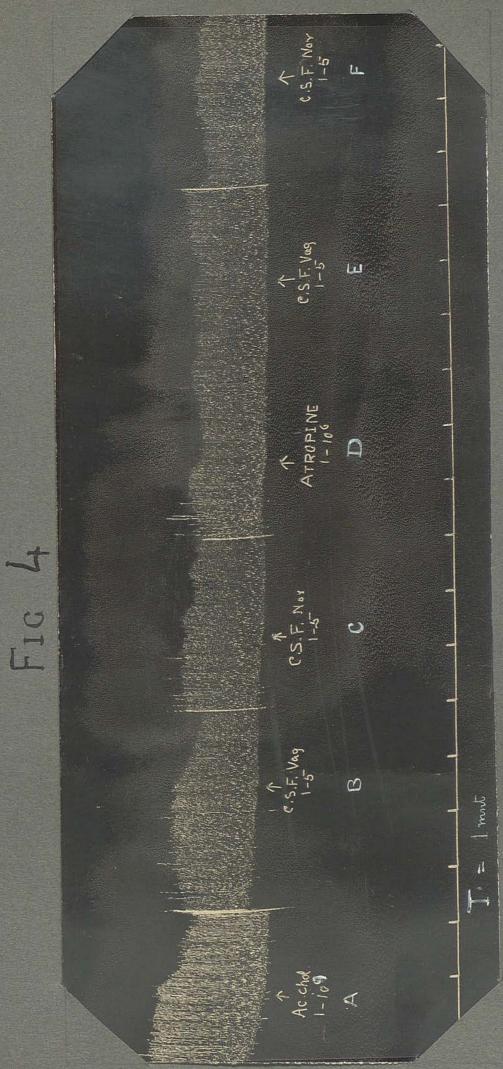


Isometric contractions of frog's ventricular strip. F18. 4.

Note depression. solution of acetyl choline. 1:109 A.

- dilution of cerebro-spinal fluid obtained after vagal stimulation. Note the depression. 1:5 B.
- Note very slight dilution of cerebro-spinal fluid normal. depression. 1:5 :0
- D. Atropine, 1:10<sup>6</sup>.
- Note there is no dilution of vagal cerebro-spinal fluid . depression. 1:5 ·1
- no Note there is dilution of normal cerebro-spinal fluid. effect. 1:5 · E

Time. 60 sec.



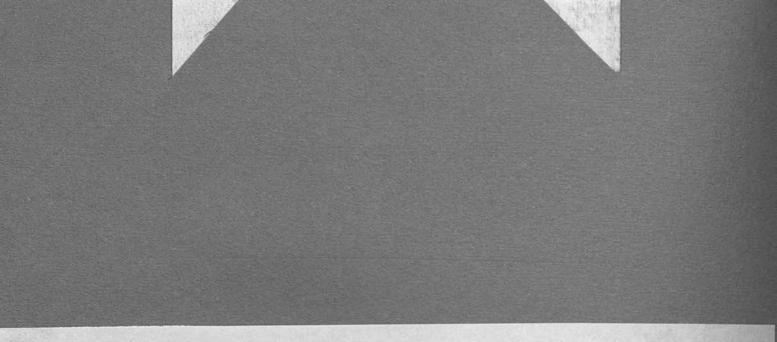


Fig. 5. Diagram of the apparatus used for experiments on rat's gut and frog's rectus abdominis muscle.

- A = Bath containing solution.
- B = Small hole connecting A and C.
- C = Bent glass tube connected with air reservoir.
- D = Bent wire carrying a hook at its end.

For recording movements of the rat's gut, the whole apparatus is immersed in a thermos flask containing water at 39°C.

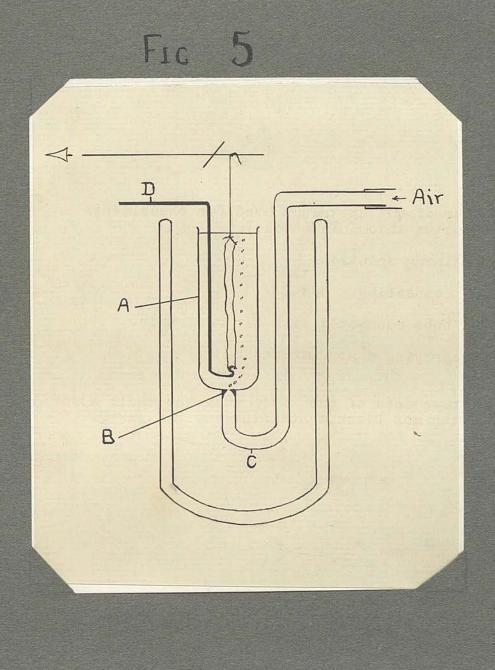
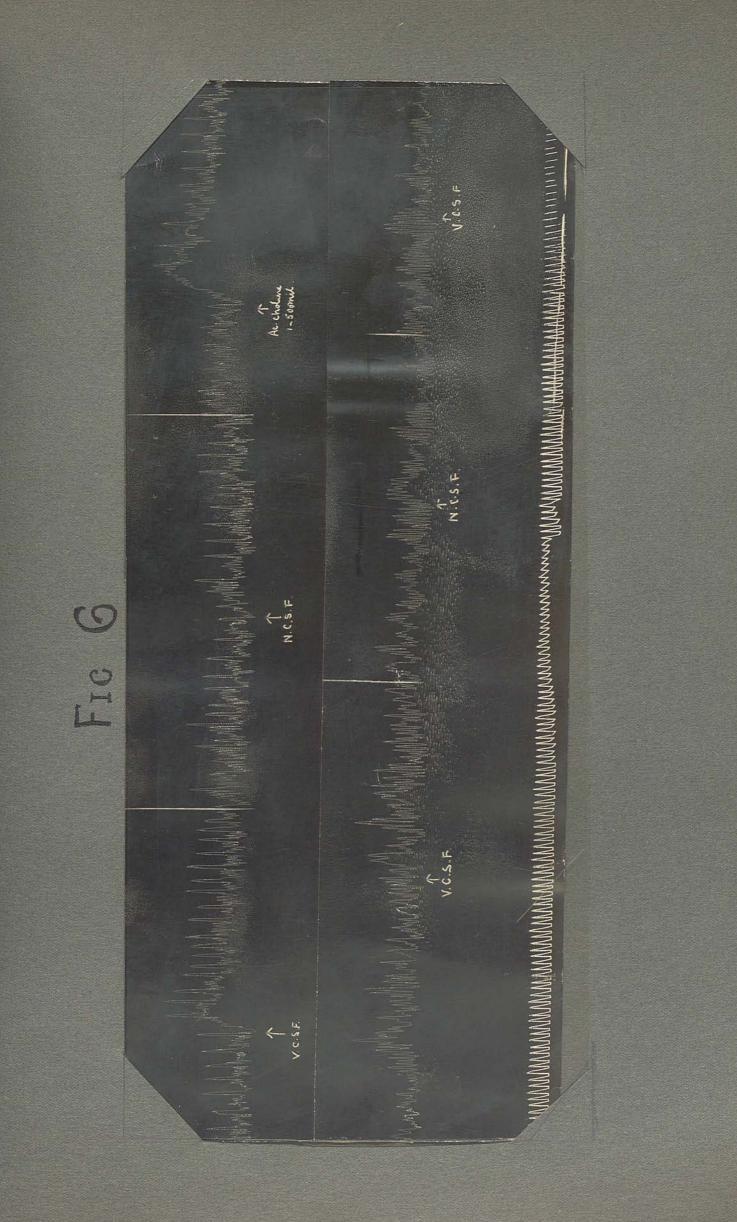


Fig. 6. Contractions of an isolated piece of rat's intestine suspended Upper tracing taken before atropine, lower tracing after atropine. in Tyrode's solution.

Note that V.C.S.F., N.C.S.F. and V.C.S.F. have no effect. (N.C.S.F.) had no appreciable action and a 1:500 million acetyl choline produced about the same effect as vagal cerebro-spinal fluid. The gut was then suspended in a 1:100,000 solution of atropine for 5 minutes, atropine washed, and vagal and normal In the upper tracing, vagal cerebro-spinal fluid (V.C.S.F. produced a contraction of the gut, normal cerebro-spinal fluid These are shown in the lower cerebro-spinal fluid repeated. tracing.



Paraldehyde and ether anaesthesia. Female cat, 3 kg. . 4 F18.

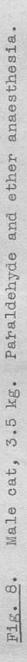
B.P. = Blood Pressure.

- N. 0.5 c.c. of basal ganglia extract of cat's brain treated with NaOH injected intravenously. 11 4
- The same quantity (0.5 c.c.) of the same extract injected intravenously. 11 m
- 0.1  $\gamma$  of acetyl choline treated with N. NaOH injected intravenously. 11 0

 $D = 0.1 \ Y$  of acetyl choline intravenously.

Note that alkalies destroy the depressor activity of acetyl choline and brain extracts. Also note the similarity between the vaso-depression produced by acetyl choline and brain extract.

		Aseliy & shadine 0.14.	16/6/33	Paralellingel 5 -
	A. A	A cetyl chalme. O'' y 2 adread (na.04.) C	T3 15 Stree	2 cal- 3 kg
hig (		Brann Errois, c.c.	10/6/333	Paradalundu & El Nin
	HA	Brain Ext 0.5, c, c. + alkaki. (NaoH) B	19/9 11111 11111111111	4 Cart 3kg P



R = Respiration; B.P. = Blood Pressure.

At A. 0.1 c.c. of the basal ganglia extract of rabbit given in the right lateral ventricle. Note depression of respiration and slight rise in blood pressure.

given intraventricularly. Note the prolonged depression of respiration. 0.1 c.c. of the cortex extract prepared from the same rabbit At B.



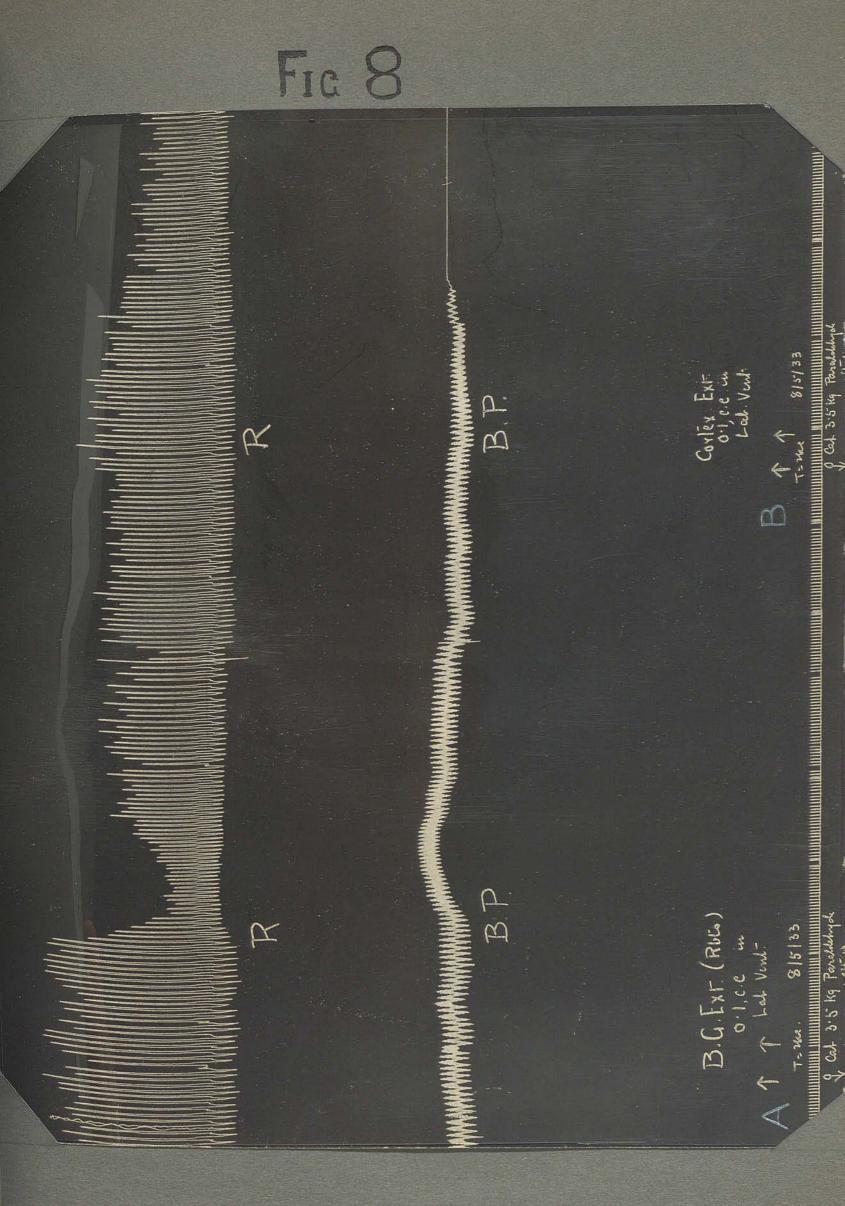
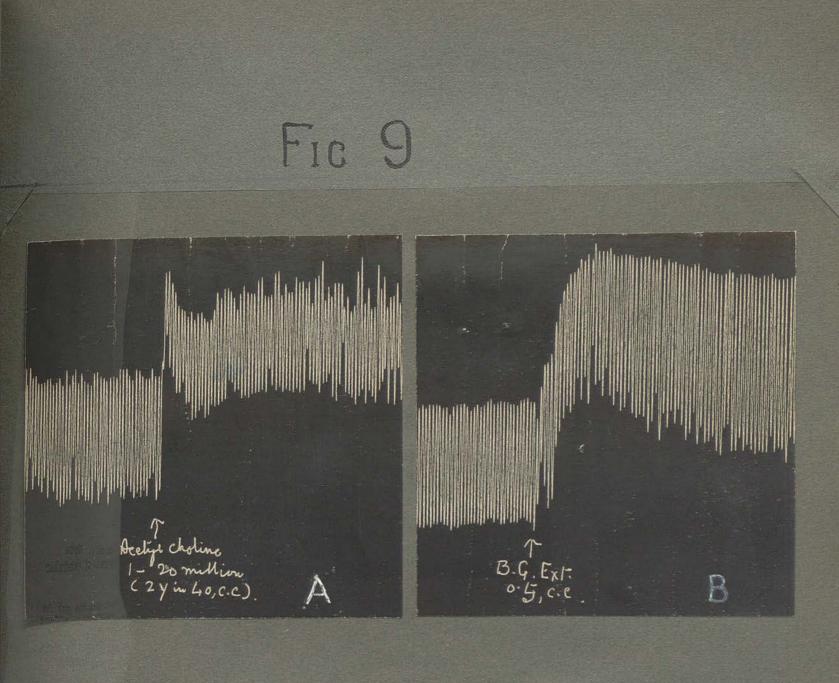


Fig. 9- Movements of the isolated piece of a rabbit's intestines suspended in Tyrode's solution.

- A = Effect of 1:20 million acetyl choline.
- B = Effect of 0.5 c.c. of basal ganglia extract (rabbit's). Note increase in tone and in the amplitude of contraction of pendular movements.
- C = Acetyl choline and basal ganglia extract after soaking the gut in 1:50,000 atropine for 15 min. Note acetyl choline has no effect while basal ganglia extract first produces a lowering of the tone which is quickly regained and an increase in the amplitude of contraction of pendular movements.



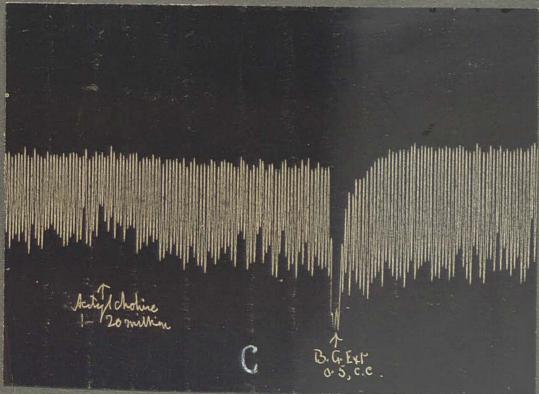
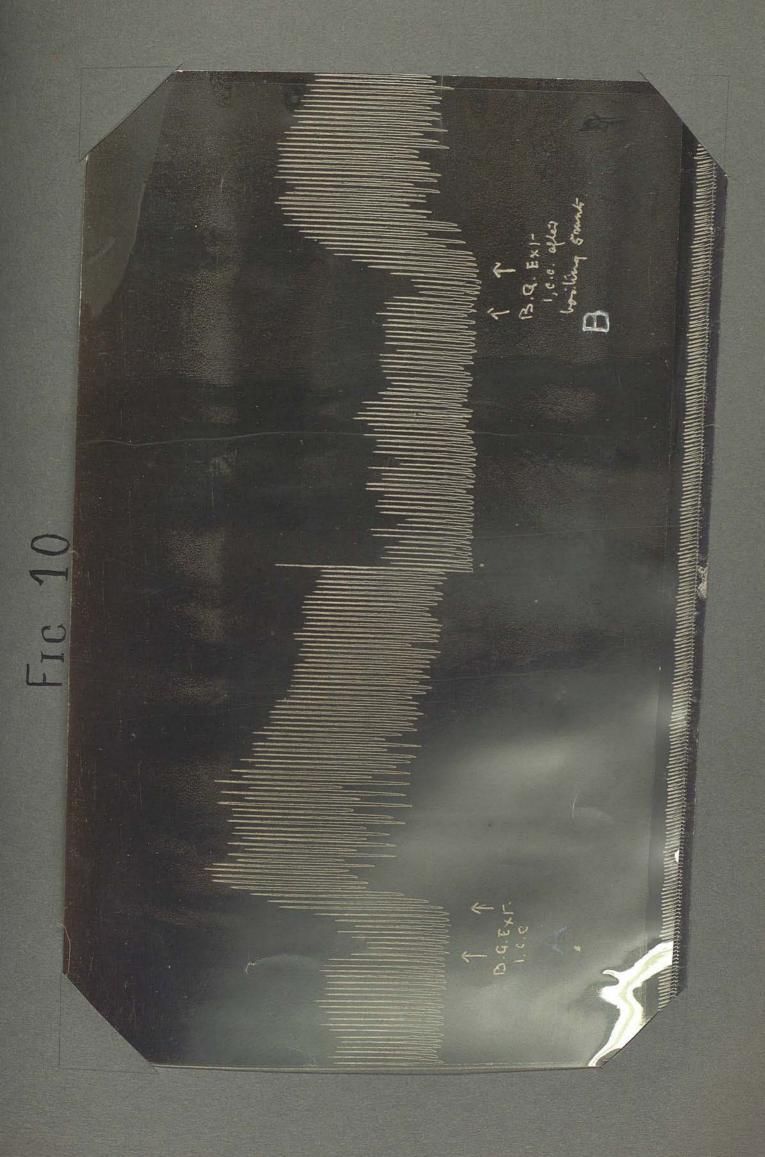


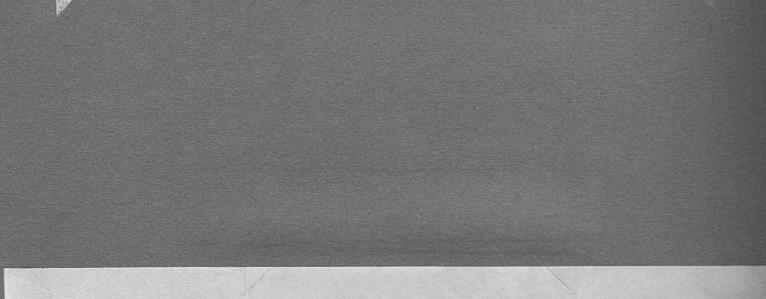
Fig. 10. Movements of an isolated piece of rabbit's intestine. 1 c.c. of basal ganglia extract added to the bath. At A,

The same quantity (1 c.c.) of the extract after boiling for 5 min. At B,

Note that boiling partly destroys the motor activity of the extract.

Time. 5 sec:



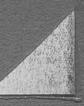


- Fig. 11. Female cat, 2.7 kg. Urethane anaesthesia. Blood pressure record. The drum turned back to get superimposed tracings. At the signal an injection of 0.5 c.c. of basal ganglia extract of a cat given intravenously twice.
  - A. Effect before atropinisation, and B. after atropinisation.

Note that atropine completely abolishes the depressor activity.

Time. 10 sec.





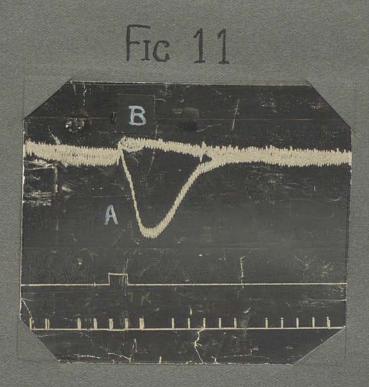


Fig. 12. Isometric contractions of the frog's ventricular strip. The strip driven at the rate of 22 shocks per min.

- At A. 1:10 dilution of an extract prepared from the medulla and basal ganglia of a rabbit. Note the depression.
- At B. The strip soaked in 1:100,000 solution of atropine.
- At C. 1:10 dilution of the extract.

Note that atropine abolishes the depressant action.

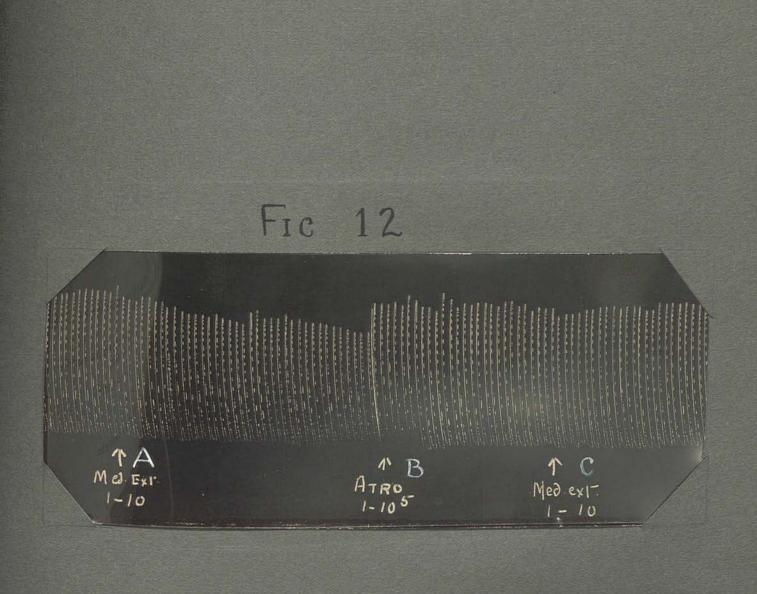


Fig. 13. Contractions of the rectus abdominis muscle of a frog.

Normals - show the contractions produced by 0.2 c.c. of basal ganglia extract of cat. Extracts added at arrows.

Eserinised - shows the effect of 0.2 c.c. of the same extract after 1:100,000 physostigmine.

Atropinised - shows the effect of 0.2 c.c. of the same extract after 1:100, 000 atropine.

Time. 60 sec.

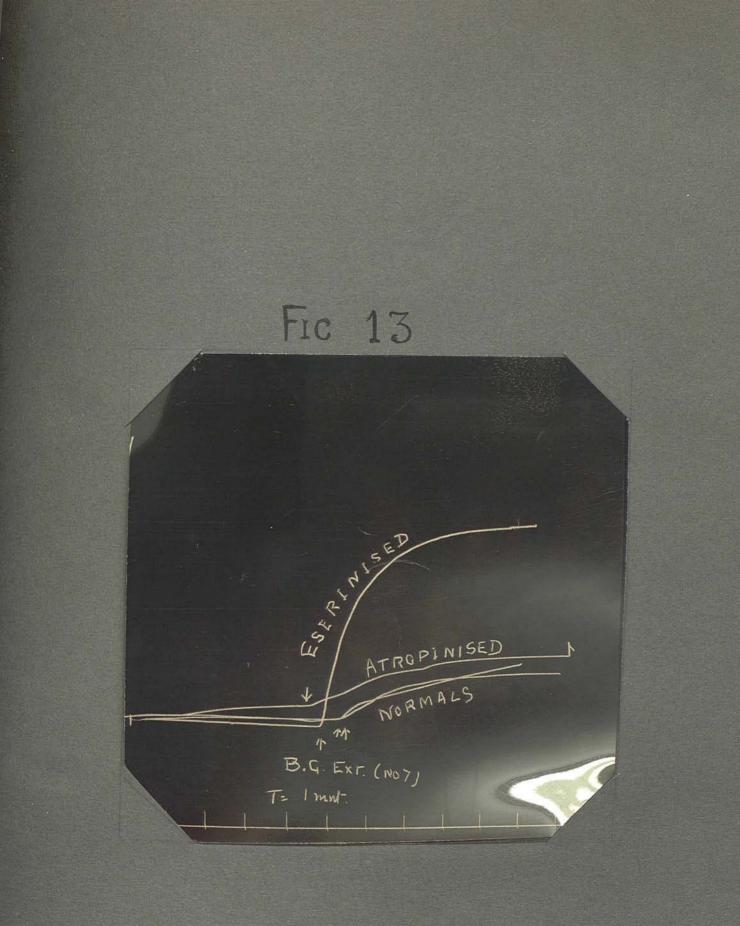


Fig. 14. Female cat, 2.8 kg. Urethane anaesthesia. B.P. = Blood Pressure. At A. 0.5 c.c. of basal ganglia extract of a cat. At B. 0.5 c.c. of the extract treated with the esterase.

Note that choline esterase completely abolishes the depressor activity of the extract.

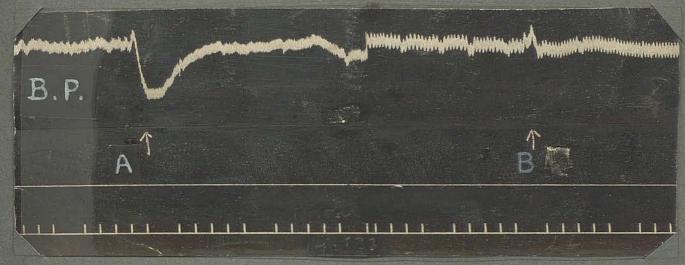
Time. 10 sec.

Fig. 15. Isometric contractions of the frog's ventricular strip driven at 20 shocks per minute.

At 1st arrow, basal ganglia extract (rabbit's) applied. Note marked effect on the heart.

At 2nd arrow, the same quantity of the extract treated with the esterase applied. Note that the degree of depression is considerably decreased.

## Fic 14



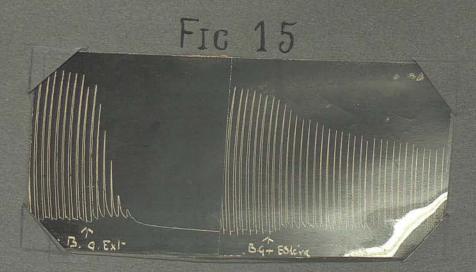
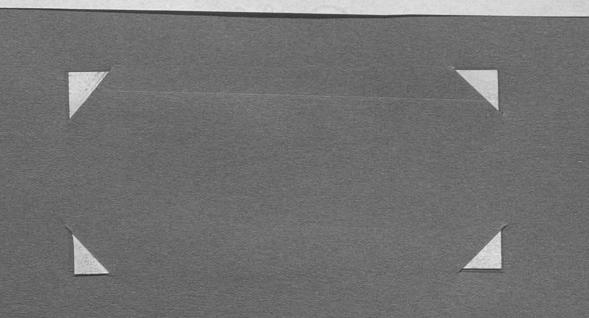


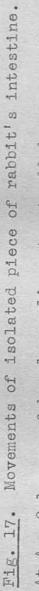
Fig. 16. Female cat, 3 kg. Urethane anaesthesia. B.P. = Blood Pressure.

At A. Cortex extract of a cat (0.5 c.c.) given intravenously. At B. Basal ganglia extract (0.5 c.c.) given intravenously. Note that vaso-depression is greater in the case of basal ganglia extract.

Time. 10 sec.



# Fic 16 ISABBARARARARARARARAR B.P. Coviex Ext 0.5 C.C. (acid acotone). & Cat- 3 Kg Vrethane B A (Ce TITLE TRACE REFERENCES TO THE TRACE PROVIDENT T= 10 see.



At A. 0.1 c.c. of basal ganglia extract added.

At B. 0.1 c.c. of cerebellar extract added.

Note that activity of cerebellar extract is considerably less.



