CCXXXVII. CHOLINE ESTER FORMATION IN, AND CHOLINE ESTERASE ACTIVITIES OF, TISSUES *IN VITRO*.

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(Received 24 July 1936.)

THE physiological importance of acetylcholine in the nervous system has made it very desirable to secure further knowledge of the details of acetylcholine metabolism in the body. Little is known as yet of the mode of production of this choline ester in the tissues. Beznak [1934] has reported that the ester is formed in minced frog heart muscle when sufficient eserine is present to prevent its destruction by choline esterase. Chang [1935] states that it is formed *in vitro* from placenta. Much more is known [Plattner & Galehr, 1928; Ammon & Voss, 1935; Stedman & Stedman, 1935, 1] of the breakdown of acetylcholine by an esterase which has recently been isolated and purified by Stedman & Stedman [1935, 2]. The reader is referred to summaries of recent literature on the subject in articles by Gaddum [1935] and by Ammon [1935].

Experiments have been undertaken by the authors to determine whether acetylcholine is formed as a normal product of metabolism of tissues examined *in vitro* by the Warburg manometric technique, and the following paper is concerned with a description of the results which have been found so far.

Technique. Thin slices of tissue were cut from the organs freshly dissected from the animal and placed in a manometric vessel containing Ringer solution or a phosphate saline solution. The vessel was filled with oxygen, and determinations of the oxygen consumption of the tissue were made, using the well-known manometric methods of Warburg. The experimental period varied from 1 to 3 hours at 37° or at 38·4°, at the termination of which the solution in the manometric vessel was tested for its acetylcholine activity, and the tissue slices were removed, washed, dried and weighed.

Media. Solutions were all made up so as to have an osmotic pressure approximately equal to 0·16 *M* NaCl. The final volume of the medium was made up to 3·0 ml. with 0·16 *M* NaCl after all other desired substances had been added. The hydrogen ion concentration was maintained at pH 7·4 in the experiments to be reported here.

Tissue slices. The thickness of the slices varied from one experiment to another but usually lay between 0·2 and 0·4 mm. Several slices were employed, the total dry weight in each vessel varying from 10 to 20 mg.

Neutralization of acids. All acids were neutralized before use, solutions of sodium salts being prepared.

Estimation of choline ester. The choline ester was estimated by measuring the contraction of the eserinizd longitudinal muscle from the dorsum of a leech. The muscle, after dissection, was immersed at room temperature in an oxygenated saline medium of the following composition: NaCl=0·71 %, KCl=0·032 %, CaCl₂=0·018 %, NaHCO₃=0·012 %, glucose=0·077 %. The final volume of

medium in which the muscle was immersed was 7 ml. after the necessary additions had been made to it. 0.1–0.5 ml. of the solution from the manometric vessel was added to the medium in which the muscle was suspended, the amount added varying according to the activity of the solution in producing a contraction. Mixing was secured by the steady bubbling of oxygen through the medium. A number of contractions due to varying quantities of the experimental solution was recorded on a smoked drum by a lever to which the leech muscle was attached. These contractions were compared with those made by the addition of known quantities of acetylcholine chloride. A process of bracketing was employed to obtain the concentration of acetylcholine which on addition to the leech muscle produced a contraction most closely resembling that brought about by the experimental solution. Each contraction was allowed to take place for 3 min., after which the muscle was well washed.

Before commencing an estimation of choline ester, the leech muscle was allowed to stand, slightly stretched, in the oxygenated saline medium for 3–4 hours. Eserine (0.01 mg.) was added to it and the muscle was again allowed to stand for 15–20 min., after which it was washed with the saline medium. Its activity was then tested with known quantities of acetylcholine (e.g. 0.05–0.025 γ),¹ and its contractions were recorded, the muscle being well washed after each contraction and allowed to resume its initial length before the next contraction was obtained. Known quantities of the experimental solution were then added, the muscle being washed as before, and allowed fully to recover, after each contraction. Finally, known quantities of acetylcholine were again added and the contractions measured.

In this way it was possible to determine with fair accuracy the activity of the experimental solution in terms of acetylcholine chloride. The experimental error may be taken to be of the order of 10%.

Notation. In accordance with the usual notation the respiration Q_{O_2} is defined as the number of μ l. of oxygen (reduced to N.T.P.) absorbed per mg. dry weight of tissue per hour.

The symbol R_{ChE} is introduced to denote the rate of formation of choline ester. It is defined as the amount of choline ester produced in 1 hour by 100 mg. dry weight of tissue and is expressed in equivalents of γ (10^{-3} mg.) of acetylcholine chloride.²

The following is an example of the manner in which the R_{ChE} of a tissue was estimated. 0.25 ml. of the experimental solution produced a contraction of the eserinated leech muscle, the dimensions of which lay midway between those of the contractions given by 0.04 γ and 0.05 γ acetylcholine chloride. This figure could be reproduced at different times in the same leech muscle. Hence 0.25 ml. of the experimental solution contained choline ester equivalent to approximately 0.045 γ acetylcholine chloride. Therefore the total quantity present in the manometric vessel was equivalent to $\frac{0.045 \times 3}{0.25} \gamma = 0.54 \gamma$ acetylcholine chloride, since the total volume of experimental solution was 3 ml. This was formed in 3 hours by 13.6 mg. dry weight tissue. Hence $R_{ChE} = \frac{0.54 \times 100}{3 \times 13.6} = 1.3$.

¹ A stock acetylcholine preparation (obtained as acetylcholine chloride from Messrs Hoffmann La Roche, Ltd.) was prepared in 5% NaH_2PO_4 solution. From this stock solution all the high dilutions were made. To the final dilutions of acetylcholine was added eserine sulphate (1/7000), to correspond with the concentration of eserine in the experimental solutions.

² To obtain R_{ChE} in terms of equivalents of the base acetylcholine, the recorded values should be multiplied by the factor 0.80.

Formation of a substance resembling acetylcholine by brain cortex slices in vitro. When rat or guinea-pig brain cortex slices are immersed in an oxygenated medium containing glucose (0.16%), phosphate ($M/30$) and saline, and allowed to take up oxygen at 37° for 3 hours, the resultant solution, after removal of the slices, has no effect whatever on the eserized leech muscle preparation. If, however, there is added to the medium at the commencement of the experiment eserine sulphate at a concentration of about 1 in 7000, the resultant solution is strongly active and causes a large contraction of the leech muscle preparation.

The eserine appears not to affect the respiration of the brain slices. Thus in one experiment with guinea-pig brain it was found that in the absence of eserine $R_{\text{ChE}}=0$, $Q_{\text{O}_2}=9.0$ (average over 3 hours) and that in the presence of eserine $R_{\text{ChE}}=1.3$, $Q_{\text{O}_2}=9.0$ (average over 3 hours). Under no circumstances yet has it been found that a contraction of the eserized leech muscle takes place on addition of the solution, in which metabolism of tissue slices has been allowed to occur, unless eserine was added to the solution at the beginning or near the beginning of the experiment. The conclusion would be that the presence of eserine is necessary for the appearance of the active substance in question. This fact, in itself, makes it likely, as Chang & Gaddum [1933] point out, that the active substance is a choline ester whose normal destruction by an esterase in the tissue is inhibited by the eserine. Moreover, the active substance is most unstable in alkaline solution. Thus in one experiment with rat brain cortex in a phosphate-glucose-eserine-saline medium the activity of the medium, after 3 hours at 38.4° , was such that 0.1 ml. produced a contraction equal to that given by 0.035γ acetylcholine chloride ($R_{\text{ChE}}=3.0$). The remaining part of the medium (i.e. 2.9 ml.) was treated with 1 ml. N NaOH for 30 min. at room temperature. It was neutralized with 1 ml. N HCl and its activity tested on the leech muscle preparation. 0.5 ml. of the solution then gave no measurable contraction of the leech muscle. It was clear that the mild treatment with dilute alkali was sufficient to remove the entire activity of the medium.

The action of the substance on the eserized leech muscle preparation has been found, like that of acetylcholine, to be unaffected by atropine. Its action, like that of acetylcholine, is diminished or abolished by the presence of morphine.

If 0.5 ml. 1/1000 morphine tartrate solution be placed in the medium in which the eserized leech muscle is suspended, it is found that no contraction occurs on the addition of 0.05γ acetylcholine. The addition of a further 0.05γ acetylcholine brings about a slight contraction, which is much increased by still further additions of the acetylcholine. On washing the leech muscle preparation, the addition of 0.05γ acetylcholine brings about its normal effect. A precisely similar phenomenon occurs when the experimental solution containing the active substance is substituted for the acetylcholine. The presence of 0.5 ml. 1/1000 morphine tartrate solution prevents the contractions due to an amount of the active substance corresponding to 0.05γ acetylcholine, the addition of further quantities of the substance causing a contraction to appear. Reversibility is secured by washing the muscle.¹

Although the facts stated make it probable that the active substance in the medium is an ester of choline, there is no evidence to indicate that the ester is acetylcholine. We propose therefore not to commit ourselves further than to refer to the active substance as a choline ester. The final proof that the substance in question is, in truth, a choline ester must await its isolation.

¹ This effect of morphine will be described at length elsewhere by J. H. Q. and M. T.

Choline ester formation by brain slices. The first problem was to decide whether the choline ester found in the medium, after brain slices had been shaken in it for 3 hours at 37° was due simply to washing out of preformed ester from the tissue or whether it was formed in the course of metabolism of the tissue *in vitro*.

It has been known for some time that brain and other tissues contain an acetylcholine-like substance, and the amounts in extracts of these tissues have been estimated. Chang & Gaddum [1933] give a value of 0.4 γ of acetylcholine equivalent in 1 g. of dog brain. Kwiatkowski [1935] gives the following values: 1.5–3.8 γ in 1 g. dog brain, 0.4–2 γ in 1 g. cat brain, 0.6–2.3 γ in 1 g. guinea-pig brain. Dikshit [1934] gives 0.2–0.3 γ in 1 g. basal ganglia of the cat and 0.04–1.0 γ in 1 g. basal ganglia of the rabbit. Results of a similar order are given by Barsoum [1935].

The amount to be anticipated, therefore, as being due to complete removal from, say, 15 mg. dry weight of guinea-pig brain, should be (taking Kwiatkowski's figures) of the order $(0.6-2.3) \times \frac{15}{200} \gamma = 0.045$ to 0.17 γ (taking 200 mg. as being approximately the dry weight of 1 g. guinea-pig brain). Now the average amount of choline ester (i.e. acetylcholine equivalent) found in a medium, after metabolism in it of guinea-pig brain cortex (15 mg. dry weight) under optimal conditions, has been about 0.7 γ and in the case of rat brain cortex about 1.2 γ . Taking into account the fact that slices of brain cortex have been used, these figures appear to be too high to be accounted for by simple diffusion of preformed ester into the medium.

A more conclusive answer to the problem is provided by an experiment making use of the known fact that choline esterase exists in relatively large quantity in the brain [Stedman & Stedman, 1935, 1; Ammon & Voss, 1935]. This fact we have confirmed and it will be reported upon later in this paper.

The amount of choline esterase in brain cortex is far more than sufficient to decompose the preformed choline ester in the tissue in 30 min. at 37°, the assumption being made that the tissue choline ester is attacked by choline esterase at a rate comparable with the rate of hydrolysis of acetylcholine. Making a calculation from the rate of breakdown of acetylcholine chloride by cat brain cortex found by Stedman & Stedman [1935, 1], it appears that 15 mg. dry weight of the tissue will remove 525 γ acetylcholine chloride in 15 min. at 30°. We have found comparable figures for rat and guinea-pig brain cortex (see Table XIII). Since the total quantity of choline ester likely to exist preformed in the brain is of the order of 0.2 γ (for 15 mg. dry weight), it is obvious that there is sufficient esterase present to bring about its destruction at 37° in a few seconds.

Accordingly experiments were carried out in which eserine was added to the tissue in a phosphate-saline-glucose medium 30 min. after respiration in the Warburg manometric apparatus had commenced. The eserine was poured into the main vessel, in which the tissue was being shaken, from the side tube of the vessel. All experiments showed that the rate of choline ester formation, after this late addition of eserine, was of the same order as, but usually a little less than, when eserine was present in the medium from the start. A typical result is as follows:

Exp.		R_{ChE}
1 (a)	Rat brain. O ₂ . Phosphate-saline-glucose. Eserine	3.0
1 (b)	Rat brain. O ₂ . Phosphate-saline-glucose. Eserine added 30 min. after start of experiment	2.5

A number of estimations of R_{ChE} obtained with tissues to which eserine was added 30 min. after the commencement of each experiment will be recorded below.

Finally it should be pointed out that if experiments are carried out anaerobically (in nitrogen) the choline ester formation falls to a very low value. Thus in one experiment rat brain cortex slices in a phosphate-glucose medium gave a value of $R_{\text{ChE}}=4.2$ in the presence of oxygen, and a value of $R_{\text{ChE}}=0.4$ in the presence of nitrogen, this result being typical of those of a number of experiments. If the entire choline ester were preformed and simply diffused from the slices into the surrounding medium, it would be difficult to understand why the change from aerobic to anaerobic conditions should cause such a diminution in the R_{ChE} . Keeping brain slices under anaerobic conditions might, on the contrary, be expected to increase R_{ChE} by an increase in cell permeabilities due to autolytic changes.

The facts, taken as a whole, indicate that choline ester formation in tissue slices takes place as a result of metabolic changes in the tissues, and not more than a small fraction of the choline ester found can be attributed to preformed choline ester in the tissues.

We turn, now, to a more detailed consideration of the rate of choline ester formation in brain cortex slices.

Variation of R_{ChE} with time. When estimations are made of the rate of choline ester formation in brain slices for various periods of time at 37° or 38.4° it is found, in the case of the rat, that the greatest rate occurs in the first hour. The rate falls off appreciably after this time. Calculations of R_{ChE} are shown in Table I. The values of R_{ChE} are averages over the experimental periods stated. Such a decrease in rate may be expected if a part of the choline ester accumulating in the first hour is due to diffusion of preformed ester from the tissue slices into the medium. The same phenomenon occurs, however, if eserine is added 30 min. after the commencement of the experiment (see Exp. 5, Table I). The conclusion must be drawn therefore that the rate of choline ester formation, in rat brain cortex slices does not remain constant during the whole course of the experiment, but is most rapid at its commencement.

Table I. R_{ChE} . *Brain cortex slices.*

Variation with time. Eserine sulphate = 1/7000.

Exp.	Animal	Temp. ° C.		Time from commencement of exp.		
				1 hour	2 hours	3 hours
1	Guinea-pig	37.0	Ringer-bicarbonate* solution. 95% O ₂ : 5% CO ₂ . Eserine	0.9	1.0	—
2	Rat	38.4	Phosphate-saline-glucose.† O ₂ . Eserine	5.5	2.9	3.2
3	Rat	38.4	Phosphate-saline-glucose.† O ₂ . Eserine	4.5	2.8	2.2
4	Rat	38.4	Phosphate-saline-glucose.† O ₂ . Eserine	5.3	3.5	3.5
5	Rat	38.4	Phosphate-saline-glucose.† O ₂ . Eserine added 30 min. after start of experiment	4.3	2.8	2.7

* Ringer-bicarbonate solution: NaHCO₃=0.025 M; KCl=0.005 M; CaCl₂=0.0025 M; KH₂PO₄=0.001 M; MgSO₄=0.0012 M; NaCl=0.11 M.

† This medium=sodium phosphate buffer (pH 7.4) 0.03 M; KCl=0.002 M; CaCl₂=0.001 M; NaCl=0.13 M; glucose=0.02 M.

Dependence of choline ester formation on aerobic conditions. It has been already mentioned that the rate of choline ester formation in brain cortex slices is greatly dependent on aerobic conditions. This is shown clearly by the results given in Table II. Anaerobic conditions were secured by passing nitrogen through the manometric vessel in the inner tube of which was a small stick of damp yellow phosphorus. Potash (0.2 ml. 6%) was placed in a side tube of the vessel. During the course of the experiment no movement of the levels of the manometric fluid occurred, indicating no absorption of gas. In a bicarbonate-glucose-Ringer medium in an atmosphere of 95% N₂ + 5% CO₂ there was also little or no formation of choline ester. When eserine was added to the medium under anaerobic conditions (by pouring in from a side tube of the manometer vessel) 30 min. after commencement of the experiment, not more than the slightest trace of choline ester could be detected. This was scarcely measurable after 2½ or 3 hours' shaking of the cortex slices at 38.4° (see Exps. 4 and 5, Table II). It seems reasonable to suppose that the small value of R_{ChE} which is found with rat brain cortex slices under anaerobic conditions when eserine is present in the medium from the start of the experiment is due to washing out of preformed choline ester from the slices. The amount found is of the same order as that to be expected from the values, already quoted, of choline ester in brain extracts.

Table II. R_{ChE} of rat brain cortex.

Phosphate 0.03 M. NaCl 0.13 M. Eserine = 1/7000. Glucose 0.02 M.

Exp.	Medium	Time hours	Temp. ° C.	O ₂	N ₂
1	Phosphate-saline*-eserine-glucose	3	37	1.8	0.4
2	Phosphate-saline-eserine-glucose	2.5	38.4	4.2	0.4
3	Phosphate-saline-eserine-glucose	2.5	38.4	3.0	0.9
4	Phosphate-saline-glucose. Eserine added 30 min. after commencement of exp.	2.5	38.4	2.5	0.0
5	Phosphate-saline-glucose. Eserine added 30 min. after commencement of exp.	2.5	38.4	3.0	0.0

* K⁺ and Ca⁺⁺ were absent from the medium in this experiment. In the others K⁺ = 0.002 M, Ca⁺⁺ = 0.001 M.

Influence of the ionic environment on R_{ChE} . The R_{ChE} of rat brain cortex slices examined in a phosphate-sodium chloride medium is usually about the same value as that in a bicarbonate-sodium chloride medium,¹ organic metabolites being absent from these media; a few experiments have shown that a similar result applies to guinea-pig brain cortex. The effects of the addition of K and Ca ions on the two media are, however, different. Results with rat brain cortex are given in Table III. It will be seen that the low value of R_{ChE} found in a phosphate-sodium chloride medium is scarcely affected by the addition of K and Ca ions (Exp. 4). In bicarbonate media where the R_{ChE} is a little higher there is a definite increase in the rate of choline ester formation when K, or a mixture of K and Ca, ions are added (Exps. 2, 3, 4). Another difference between the rates of choline ester formation, depending on whether phosphate or bicarbonate media are used, will be noted when we turn to the consideration of the effects of glucose.

¹ In bicarbonate (0.025 M) media the atmosphere in the manometer is composed of a mixture of 95% O₂ and 5% CO₂; in media containing no bicarbonate pure oxygen is present and the CO₂ formed during metabolism is absorbed by potash (on filter-paper) in the central tube of the manometer vessel.

Table III. *Rat brain cortex.*Eserine = 1/7000. 38.4°. NaCl 0.13 *M*.

Exp.	Medium	R_{ChE}
1. (a)	Phosphate buffer 0.03 <i>M</i> . NaCl. K^+ and Ca^{++} absent	0.6
(b)	Ringer-bicarbonate solution*	1.2
2. (a)	0.025 <i>M</i> NaHCO_3 . NaCl. KCl absent	0.9
(b)	0.025 <i>M</i> NaHCO_3 . NaCl. KCl 0.005 <i>M</i>	1.3
(c)	0.025 <i>M</i> NaHCO_3 . NaCl. KCl 0.005 <i>M</i> . CaCl_2 0.0025 <i>M</i>	1.1
3. (a)	0.025 <i>M</i> NaHCO_3 . NaCl	1.1
(b)	0.025 <i>M</i> NaHCO_3 . NaCl. 0.002 <i>M</i> KCl. 0.001 <i>M</i> CaCl_2	1.7
(c)	0.025 <i>M</i> NaHCO_3 . NaCl. 0.002 <i>M</i> KCl. 0.001 <i>M</i> CaCl_2 . 0.001 <i>M</i> MgSO_4	1.9
4. (a)	Phosphate buffer 0.03 <i>M</i> . NaCl	0.8
(b)	Phosphate buffer 0.03 <i>M</i> . NaCl. 0.002 <i>M</i> KCl + 0.001 <i>M</i> CaCl_2	Q_{O_2} (3 hr.) = 4.0 0.7
5. (a)	0.025 <i>M</i> NaHCO_3 . NaCl	Q_{O_2} (3 hr.) = 3.2 1.0
(b)	0.025 <i>M</i> NaHCO_3 . NaCl. 0.002 <i>M</i> KCl. 0.001 <i>M</i> CaCl_2	1.4
(c)	0.025 <i>M</i> NaHCO_3 . NaCl. 0.004 <i>M</i> KCl. 0.002 <i>M</i> CaCl_2	1.4

* Composition given under Table I.

The effects of glucose on choline ester formation in brain slices. In phosphate media the presence of glucose accelerates greatly the rate of choline ester formation in brain cortex slices. This is shown by a few typical results shown in Table IV. The effect of the glucose with rat brain is to increase R_{ChE} four- or five-fold, and

Table IV. *Rat brain cortex.*Eserine = 1/7000. Phosphate = 0.03 *M*. NaCl = 0.13 *M*.

Exp.	Medium	Time hours	Temp. ° C.	R_{ChE}
1. (a)	Phosphate-NaCl	3	37	0.7
(b)	Phosphate-NaCl-glucose (0.01 <i>M</i>)	3	37	2.8
2. (a)	Phosphate-NaCl-KCl (0.002 <i>M</i>)- CaCl_2 (0.001 <i>M</i>)	3	38.4	0.7
(b)	Phosphate-NaCl-KCl (0.002 <i>M</i>)- CaCl_2 (0.001 <i>M</i>)- glucose (0.01 <i>M</i>)	3	38.4	2.9
3. (a)	Phosphate-NaCl-KCl (0.002 <i>M</i>)- CaCl_2 (0.001 <i>M</i>)	3	38.4	1.1
				Q_{O_2} (av. 3 hr.) = 4.1
(b)	Phosphate-NaCl-KCl (0.002 <i>M</i>)- CaCl_2 (0.001 <i>M</i>)- glucose (0.02 <i>M</i>)	3	38.4	3.2
				Q_{O_2} (av. 3 hr.) = 11.6
4. (a)	Phosphate-NaCl-KCl (0.002 <i>M</i>)- CaCl_2 (0.001 <i>M</i>)	3	38.4	0.7
				Q_{O_2} (av. 3 hr.) = 3.2
(b)	Phosphate-NaCl-KCl (0.002 <i>M</i>)- CaCl_2 (0.001 <i>M</i>)- glucose (0.02 <i>M</i>)	3	38.4	3.6
				Q_{O_2} (av. 3 hr.) = 10.8

its effect is still large in the absence of added potassium and calcium ions (see Exp. 1). The effect of glucose is equally great when eserine is added to the medium 30 min. after the commencement of the experiments. When we turn however to bicarbonate media we note the following phenomena.

The addition of glucose to a bicarbonate medium containing no added K or Ca ions results in a large acceleration in the rate of choline ester formation. The R_{ChE} may rise from 0.4 to 2.2 (see Exp. 1, Table V). The further addition of K and Ca ions to a bicarbonate-glucose medium lowers the R_{ChE} (Exps. 2 and 3,

Table V). The addition of glucose to a bicarbonate-Ringer medium has but a small effect on the rate of choline ester formation (Exps. 1 and 4, Table V). With guinea-pig brain cortex we have noticed that the presence of glucose usually diminishes the R_{ChE} found in a bicarbonate-Ringer medium.

Table V. *Rat brain cortex.*

Time, 3 hours. Temp. 38.4°. Eserine 1/7000. 95% O ₂ + 5% CO ₂ .		
Exp.	Medium	R_{ChE}
1. (a)	0.025 <i>M</i> NaHCO ₃ . NaCl	0.4
(b)	0.025 <i>M</i> NaHCO ₃ . NaCl-glucose (0.01 <i>M</i>)	2.2
(c)	Ringer*-bicarbonate	1.3
(d)	Ringer*-bicarbonate-glucose (0.01 <i>M</i>)	1.6
2. (a)	0.025 <i>M</i> NaHCO ₃ . 0.13 <i>M</i> NaCl. 0.02 <i>M</i> glucose	3.7
(b)	0.025 <i>M</i> NaHCO ₃ . 0.13 <i>M</i> NaCl. 0.002 <i>M</i> KCl. 0.001 <i>M</i> CaCl ₂ . 0.02 <i>M</i> glucose	2.2
3. (a)	0.025 <i>M</i> NaHCO ₃ . 0.13 <i>M</i> NaCl. 0.02 <i>M</i> glucose	2.3
(b)	0.025 <i>M</i> NaHCO ₃ . 0.13 <i>M</i> NaCl. 0.002 <i>M</i> KCl. 0.001 <i>M</i> CaCl ₂ . 0.02 <i>M</i> glucose	1.4
(c)	0.025 <i>M</i> NaHCO ₃ . 0.13 <i>M</i> NaCl. 0.004 <i>M</i> KCl. 0.002 <i>M</i> CaCl ₂	1.4
4. (a)	Ringer*-bicarbonate	1.5
(b)	Ringer*-bicarbonate-glucose (0.16%)	1.3

* Composition given under Table I.

The reasons for these phenomena are as yet obscure, but attention should be drawn to the fact that the aerobic glycolysis by brain slices in a bicarbonate-glucose medium is always much greater in the absence of potassium and calcium ions than in their presence [Dickens & Greville, 1935].

Turning once more to the consideration of phosphate-glucose media we find that the addition of potassium and calcium ions has the reverse effect to that happening in a bicarbonate-glucose medium, i.e. the R_{ChE} is increased. Typical results are shown in Table VI.

Table VI. *Rat brain cortex.*

Time 3 hours. Temp. 38.4°. Eserine 1/7000. O₂. Phosphate=0.03 *M*. NaCl=0.13 *M*.

Exp.	Medium	R_{ChE}	Q_{O_2}	
			First 30 min.	3 hr. (av.)
1. (a)	Phosphate-NaCl-glucose (0.02 <i>M</i>)	1.8	15.3	7.5
(b)	Phosphate-NaCl-glucose + KCl (0.002 <i>M</i>), CaCl ₂ (0.001 <i>M</i>)	3.6	12.8	10.8
2. (a)	Phosphate-NaCl-glucose (0.02 <i>M</i>)	1.9	16.0	8.5
(b)	Phosphate-NaCl-glucose + KCl (0.002 <i>M</i>), CaCl ₂ (0.001 <i>M</i>)	2.2	15.3	11.6
(c)	Phosphate-NaCl-glucose + KCl (0.008 <i>M</i>), CaCl ₂ (0.004 <i>M</i>)	2.6	14.6	12.6
3. (a)	Phosphate-NaCl-glucose (0.01 <i>M</i>)	2.6	15.8	9.7
(b)	Phosphate-NaCl-glucose (0.01 <i>M</i>) + KCl (0.002 <i>M</i>)	4.3	—	—
(c)	Phosphate-NaCl-glucose (0.01 <i>M</i>) + KCl (0.004 <i>M</i>)	4.0	14.0	12.1
4. (a)	Phosphate-NaCl-glucose (0.02 <i>M</i>)	3.0	—	—
(b)	Phosphate-NaCl-glucose (0.02 <i>M</i>) + KCl (0.002 <i>M</i>)	4.1	—	—
5.* (a)	Phosphate-NaCl-glucose (0.02 <i>M</i>)	2.0	—	—
(b)	Phosphate-NaCl-glucose (0.02 <i>M</i>) + KCl (0.002 <i>M</i>) + CaCl ₂ (0.001 <i>M</i>)	2.7	—	—

* In this case eserine was added 30 min. after commencement of the experiment.

The effects of addition of K and Ca ions are not very regular, but they are always in the same direction—that of an increase in the R_{ChE} . It should be noted that in a phosphate-glucose medium to which no K and Ca ions have been added

the Q_{O_2} falls rapidly during the experimental period. In the presence of K and Ca ions the Q_{O_2} remains comparatively steady over a long period (see Exps. 1, 2 and 3, Table VI). This phenomenon has been recently observed by Dickens & Greville [1935].

There is little doubt that the presence of K and Ca ions markedly influence the course of glucose metabolism in tissue slices. They also affect choline ester formation in brain cortex slices in glucose media in such a way that R_{ChE} is high when aerobic glycolysis is high (bicarbonate, absence of K and Ca) and when respiration is high (phosphate, presence of K and Ca). It is reasonable to suggest that there is a definite link between the two metabolic processes, one involving glucose breakdown and the other choline ester formation.

Values of R_{ChE} calculated as averages from the results of a number of experiments are given in Table VII. These were obtained with rat brain cortex slices in various media in the presence and absence of glucose.

Table VII. R_{ChE} (average values).

Eserine = 1/7000. 38.4°.		Glucose absent	Glucose present
Medium			
0.025 <i>M</i> NaHCO ₃ + 0.13 <i>M</i> NaCl		0.7	2.7
0.025 <i>M</i> NaHCO ₃ + 0.13 <i>M</i> NaCl + 0.002 <i>M</i> KCl + 0.001 <i>M</i> CaCl ₂		1.4	1.6
0.03 <i>M</i> phosphate + 0.13 <i>M</i> NaCl		0.8	2.0
0.03 <i>M</i> phosphate + 0.13 <i>M</i> NaCl + 0.002 <i>M</i> KCl + 0.001 <i>M</i> CaCl ₂		0.9	2.9

Effects of metabolites other than glucose on R_{ChE} . The addition of sodium lactate (*dl*) or of sodium pyruvate to a phosphate-saline medium increases R_{ChE} with rat brain cortex slices, but the rate of increase of choline ester formation is not usually as great as with glucose. Representative results are given in Table VIII. The increase occurs, as with glucose, when eserine is present at the start of the experiment or when it is added 30 min. after the commencement of the experiment. The increase occurs whether K and Ca ions have been added or not.

Table VIII. *Rat brain cortex.*

Variations of R_{ChE} with substrate. Eserine = 1/7000. Phosphate = 0.03 *M*. NaCl 0.13 *M*.

Exp.	Medium	Time hr.	Temp. ° C.	R_{ChE}	Q_{O_2} (av.) 3 hr.
1. (a)	Phosphate-NaCl	3	37	0.7	3.5
(b)	Phosphate-NaCl-glucose (0.01 <i>M</i>)	3	37	2.6	8.6
(c)	Phosphate-NaCl-lactate (0.026 <i>M</i>)	3	37	1.8	9.4
(d)	Phosphate-NaCl-succinate (0.016 <i>M</i>)	3	37	0.7	6.5
2. (a)	Phosphate-NaCl-0.002 <i>M</i> KCl-0.001 <i>M</i> CaCl ₂	3	38.4	1.1	4.1
(b)	Phosphate-NaCl-0.002 <i>M</i> KCl-0.001 <i>M</i> CaCl ₂ -glucose (0.02 <i>M</i>)	3	38.4	3.2	11.6
(c)	Phosphate-NaCl-0.002 <i>M</i> KCl-0.001 <i>M</i> CaCl ₂ -lactate (0.02 <i>M</i>)	3	38.4	3.2	9.9
(d)	Phosphate-NaCl-0.002 <i>M</i> KCl-0.001 <i>M</i> CaCl ₂ -pyruvate (0.02 <i>M</i>)	3	38.4	2.0	9.7
3. (a)	Phosphate-NaCl-0.002 <i>M</i> KCl-0.001 <i>M</i> CaCl ₂ -glucose (0.02 <i>M</i>)	3	38.4	3.0	10.6
(b)	Phosphate-NaCl-0.002 <i>M</i> KCl-0.001 <i>M</i> CaCl ₂ -succinate (0.02 <i>M</i>)	3	38.4	0.6	6.8
4. (a)	Phosphate-NaCl-0.002 <i>M</i> KCl-0.001 <i>M</i> CaCl ₂ -glucose (0.02 <i>M</i>)	3	38.4	3.4	12.0
(b)	Phosphate-NaCl-0.002 <i>M</i> KCl-0.001 <i>M</i> CaCl ₂ -lactate (0.02 <i>M</i>)	3	38.4	2.4	9.9
5.* (a)	Phosphate-NaCl-0.002 <i>M</i> KCl-0.001 <i>M</i> CaCl ₂	2½	38.4	0.6	—
(b)	Phosphate-NaCl-0.002 <i>M</i> KCl-0.001 <i>M</i> CaCl ₂ -glucose (0.02 <i>M</i>)	2½	38.4	2.4	—
(c)	Phosphate-NaCl-0.002 <i>M</i> KCl-0.001 <i>M</i> CaCl ₂ -lactate (0.02 <i>M</i>)	2½	38.4	1.7	—
(d)	Phosphate-NaCl-0.002 <i>M</i> KCl-0.001 <i>M</i> CaCl ₂ -pyruvate (0.02 <i>M</i>)	2½	38.4	1.9	—
6.* (a)	Phosphate-NaCl-0.002 <i>M</i> KCl-0.001 <i>M</i> CaCl ₂	2½	38.4	0.7	—
(b)	Phosphate-NaCl-0.002 <i>M</i> KCl-0.001 <i>M</i> CaCl ₂ -glucose (0.02 <i>M</i>)	2½	38.4	2.5	—
(c)	Phosphate-NaCl-0.002 <i>M</i> KCl-0.001 <i>M</i> CaCl ₂ -fructose (0.02 <i>M</i>)	2½	38.4	2.3	—
(d)	Phosphate-NaCl-0.002 <i>M</i> KCl-0.001 <i>M</i> CaCl ₂ -galactose (0.02 <i>M</i>)	2½	38.4	1.8	—

* Eserine added 30 min. after the commencement of the experiment.

The addition of sodium succinate, however, does not result in any increased R_{chE} (see Exps. 1 and 3, Table VIII). This fact is of considerable interest for it shows that choline ester formation does not necessarily depend on an increased respiration of the brain cortex slices. Succinate increases markedly the respiration of brain cortex, though the increase is not so great as with glucose or lactate for equivalent concentrations; these results are noted in Table VIII.

Turning to sugars other than glucose we find that fructose is as effective as glucose but that galactose is less efficient. In Exps. 5 and 6 recorded in Table VIII eserine was added 30 min. after the start of the experiments.

Two experiments have shown that the presence of sodium glutamate (0.04 *M*) results in an increase of R_{chE} ; with rat brain cortex in phosphate-saline media R_{chE} was 1.7 (3 hours 38.4°). The increase is not as great as with glucose.

Effects of substituting α -glycerophosphate for phosphate on R_{chE} . The substitution of 0.03 *M* sodium α -glycerophosphate (pH 7.4) for phosphate buffer results in an increased value of R_{chE} for rat brain cortex. Exps. 1 and 2, Table IX, show that the value of R_{chE} is doubled. As is well known the presence of α -glycerophosphate increases the respiration of brain. The addition of glucose to the α -glycerophosphate medium increases the R_{chE} , the increase being of the same order as that which occurs when glucose is added to a phosphate medium.

Table IX. *Effect of α -glycerophosphate on R_{chE} . Rat brain cortex.*

Time, 3 hours. Temp. 38.4°. O_2 . Eserine 1/7000.

Exp.	Medium	R_{chE}	Q_{O_2} (av. 3 hr.)
1. (a)	0.03 <i>M</i> phosphate-NaCl-0.002 <i>M</i> KCl-0.001 <i>M</i> CaCl_2	0.7	3.0
(b)	0.03 <i>M</i> phosphate-NaCl-0.002 <i>M</i> KCl-0.001 <i>M</i> CaCl_2 + glucose (0.02 <i>M</i>)	2.9	11.8
(c)	0.03 <i>M</i> α -glycerophosphate-0.002 <i>M</i> KCl-0.001 <i>M</i> CaCl_2	1.4	8.4
(d)	0.03 <i>M</i> α -glycerophosphate-0.002 <i>M</i> KCl-0.001 <i>M</i> CaCl_2 + glucose (0.02 <i>M</i>)	4.0	12.4
2. (a)	0.03 <i>M</i> phosphate-NaCl-0.002 <i>M</i> KCl-0.001 <i>M</i> CaCl_2	1.1	3.8
(b)	0.03 <i>M</i> α -glycerophosphate-0.002 <i>M</i> KCl-0.001 <i>M</i> CaCl_2	2.2	8.3
3. (a)	0.03 <i>M</i> α -glycerophosphate-NaCl-0.001 <i>M</i> CaCl_2	1.2	7.1
(b)	0.03 <i>M</i> α -glycerophosphate-NaCl-0.001 <i>M</i> CaCl_2 + glucose (0.02 <i>M</i>)	2.9	11.6
4. (a)	0.03 <i>M</i> α -glycerophosphate-NaCl-glucose (0.02 <i>M</i>)	3.0	10.8
(b)	0.03 <i>M</i> α -glycerophosphate-NaCl-glucose (0.02 <i>M</i>) + KCl 0.002 <i>M</i> + CaCl_2 0.001 <i>M</i>	3.0	14.2

A few experiments have indicated that the R_{chE} in a α -glycerophosphate-NaCl-glucose medium is not markedly affected by the inclusion of K and Ca ions. Exp. 4, Table IX, shows a typical result. As in a phosphate medium the presence of K and Ca ions decreases the rate of fall of Q_{O_2} .

Effects of addition of choline and of sodium acetate on R_{chE} . The addition of choline (as the hydrochloride) to bicarbonate or phosphate media, in concentrations sufficiently low not to affect the leech muscle preparation when suitable volumes are taken for testing purposes, has no definite effect on the R_{chE} . This applies to both guinea-pig and rat brain cortex slices. Typical results are shown in Table X. The amount of choline chloride added to each vessel was 100 γ or 200 γ . Now had only 0.5% choline ester synthesis occurred from the choline added with the production of, say, 0.5 or 1.0 γ choline ester, this amount would have been easily measurable. The results show that with a concentration of choline chloride of 33 γ /ml. or 66 γ /ml. no perceptible synthesis of choline ester occurs. This is the case whether glucose is present or not.

The addition of sodium acetate (0.02 *M*) seems to have a small accelerating effect on R_{chE} (Exps. 6 and 7, Table X) whether choline is present or not. It

Table X. *Brain cortex slices.*

Eserine 1/7000. Phosphate 0.03 M. NaCl 0.13 M.			Time	Temp.	R_{ChE}
Exp.	Animal	Medium	hr.	° C.	
1. (a)	Guinea-pig	Bicarbonate-Ringer*	3	37	1.7
(b)	"	Bicarbonate-Ringer* + choline (100 γ)	3	37	1.8
2. (a)	"	Bicarbonate-Ringer*	3	37	0.9
(b)	"	Bicarbonate-Ringer* + choline (100 γ)	3	37	1.0
3. (a)	"	Phosphate-NaCl-glucose	3	37	1.3
(b)	"	Phosphate-NaCl-glucose-choline (100 γ)	3	37	0.0†
(c)	"	Phosphate-NaCl-glucose-choline (100 γ)	3	37	1.4
4. (a)	Rat	Phosphate-NaCl-glucose	3	37	2.8
(b)	"	Phosphate-NaCl-glucose-choline (100 γ)	3	37	3.2
5.	"	Bicarbonate-Ringer-glucose-choline (100 γ)	3	37	1.0
6. (a)	"	Bicarbonate-Ringer-glucose	3	38.4	1.0
(b)	"	Bicarbonate-Ringer-glucose-choline (200 γ)	3	38.4	1.1
(c)	"	Bicarbonate-Ringer-glucose-acetate (0.02 M)	3	38.4	1.6
(d)	"	Bicarbonate-Ringer-glucose-acetate (0.02 M) + choline (200 γ)	3	38.4	1.7
7. (a)	"	Bicarbonate-Ringer-glucose	2	38.4	1.3
(b)	"	Bicarbonate-Ringer-glucose + acetate (0.02 M)	2	38.4	1.7

* Composition of Ringer is given under Table I.

† Too small to be measured; eserine omitted in this case.

seems scarcely likely that so small an effect can be taken as an indication of the synthesis in the tissue of acetylcholine from acetate ions and choline.

It would be expected that eserine which inhibits the activity of choline esterase will not only prevent hydrolysis of choline ester, but will also inhibit its synthesis by this enzyme. The fact that the additions of choline and of acetate do not bring about any marked production of acetylcholine in the tissue in the presence of eserine suggests that the choline ester which is formed in brain tissue slices in the presence of eserine does not arise from the products of its hydrolysis.

Action of poisons on R_{ChE} .

(a) *Cyanide.* The addition of potassium cyanide (0.06 %) to an oxygenated bicarbonate-Ringer-glucose-eserine medium reduces the R_{ChE} of rat brain cortex to the anaerobic level. A value of $R_{ChE}=1.5$ was reduced, by the presence of cyanide, under aerobic conditions to $R_{ChE}=0.5$. Similarly, in a phosphate-saline-glucose-eserine medium, a value of $R_{ChE}=1.9$ was reduced to $R_{ChE}=0.4$ by the presence of cyanide (0.06 %).

(b) *Sodium fluoride.* The addition of 0.01 M NaF does not appreciably affect the R_{ChE} found with rat brain cortex in a phosphate-saline-glucose-eserine medium. In one experiment the value in absence of NaF was $R_{ChE}=3.4$ and in the presence of 0.01 M NaF R_{ChE} was found equal to 4.3. Yet in this experiment the Q_{O_2} was reduced by the fluoride from a value of 12.0 to 4.6.

Evidently even in the presence of fluoride the presence of glucose will stimulate the formation of choline ester under the conditions of our experiments.

Choline ester formation in various animal tissues. Choline ester formation has been found to be greatest with brain tissue slices. Among the tissues examined—brain, kidney, liver, spleen, testis, diaphragm and defibrinated blood—not more than traces of choline ester have been found in tissues other than brain and diaphragm. Diaphragm of the rat only has been examined and this proves

Table XI. R_{chE} of various organs.

Animal	...	...	Guinea-pig	Rat
Medium	...	...	Bicarbonate-Ringer-eserine	Phosphate-saline-glucose-eserine (K, Ca present)
Organ				
Brain cortex			1.7	2.5
Kidney			<0.3	<0.3
Liver			<0.3	<0.3
Spleen			<0.3	<0.3
Testis			<0.3	<0.3
Diaphragm			—	0.8
Blood (defibrinated)			<0.3	—

to be one-third as active as rat brain cortex when examined in a phosphate-glucose medium. The results are noted in Table XI. There is little doubt that choline ester formation, so far as our experiments *in vitro* have gone, is restricted chiefly to the brain.

Acetylcholine hydrolysis by tissue slices. The hydrolysis was measured in the Warburg apparatus by pouring 0.2 ml. acetylcholine chloride solution from the side tube of the manometric vessel into the main vessel which contained tissue slices suspended in 2.8 ml. of a bicarbonate-saline solution and noting the rate of evolution of CO_2 . The acetylcholine was added after temperature equilibrium (37° or 38.4°) had been attained. Air in the apparatus had been displaced by a mixture of 95% N_2 and 5% CO_2 , and strict anaerobiosis was secured by placing small pieces of damp yellow phosphorus in the central tube of the manometer vessel. The solution in the main vessel consisted of 0.025 *M* NaHCO_3 and 0.13 *M* NaCl , together with 0.002 *M* KCl and 0.001 *M* CaCl_2 .

As the acetylcholine was hydrolysed CO_2 was evolved from the medium, the rate of evolution being a measure of the rate of hydrolysis. A similar manometric method for following acetylcholine hydrolysis has already been used by Stedman & Stedman [1935, 1] and Ammon & Voss [1935]. The rate of hydrolysis is given in the following units:

$$Q_{\text{CO}_2}^{\text{N}_2} (\text{acetylcholine}) = \mu\text{l. CO}_2 \text{ (reduced to N.T.P.) evolved per mg. dry weight of tissue per hour.}$$

0.2 ml. of a solution containing 100 mg. acetylcholine chloride in 5 ml. water was placed in the side tube of the vessel. After pouring the acetylcholine into the main vessel its initial concentration became 0.0073 *M*.

With this initial concentration the rate of evolution of CO_2 and therefore the rate of hydrolysis are approximately constant until the concentration of acetylcholine reaches about 0.003 *M*, when the rate of hydrolysis begins appreciably to diminish. Typical results are shown in Table XII.

Values of $Q_{\text{CO}_2}^{\text{N}_2}$ (*acetylcholine*) are shown in Table XIII where it will be seen (a) with rat tissues that spleen is the most active organ in decomposing the choline ester, (b) with both guinea-pig and rat, kidney has feeble choline esterase activity, whilst liver and testis have activities less than that of brain cortex. The values recorded in Table XIII have not been corrected for the small blanks obtained in the absence of acetylcholine or of tissue.

The results (Tables XI, XIII) show that there is no relationship between the power of an organ to break up acetylcholine and its power to form choline ester *in vitro*.

Table XII.

Time from addition of acetylcholine t (min.)	Vol. of CO ₂ evolved v (μ l.)	v/t
Rat brain.		
45	116.0	2.5
75	192.0	2.5
105	262.8	2.5
135	318.5	2.4
180	384.7	2.1
240	445.4	1.8
Guinea-pig blood.		
45	87.0	1.9
75	150.0	2.0
105	211.7	2.0
135	271.3	2.0
165	318.5	1.9
195	354.0	1.8
240	381.5	1.5

Table XIII. *Acetylcholine hydrolysis by various organs.*

Bicarbonate-saline. 95 % N₂ + 5 % CO₂. Phosphorus.
Initial concentration of acetylcholine = 0.0073 *M*.
Taken over period 0–60 min.

	$Q_{\text{CO}_2}^{\text{N}_2}$ (acetylcholine).	
	Rat (38.4°)	Guinea-pig (37°)
Brain cortex	17.8	17.3
Liver	10.1	3.1
Kidney	7.9	3.2
Spleen	26.2	6.0
Testis	12.4	2.1
Defibrinated blood	5.2	13.3

SUMMARY.

1. When brain cortex slices are allowed to respire in a medium containing eserine a substance is formed which produces a powerful contraction of an eserized leech muscle preparation. The substance is only formed in the presence of eserine, is unstable in dilute alkaline solution and its effect on the leech muscle is counteracted, like that of acetylcholine, by the presence of morphine. It is considered to be a choline ester. It is shown that the choline ester is formed as a result of metabolic processes in the tissue slices, only a small fraction of it being due to preformed ester within the tissue.

2. The rate of choline ester formation is expressed by the symbol R_{ChE} . This represents the amount of choline ester formed in 1 hour by 100 mg. dry weight tissue and is expressed in equivalents of acetylcholine chloride.

3. R_{ChE} varies during the period of an experiment, it being greatest in the first hour.

4. Under anaerobic conditions, or under aerobic conditions in the presence of cyanide, R_{ChE} falls to a very low level. Oxygen is necessary for maintenance of R_{ChE} at a high level.

5. The presence of glucose greatly increases R_{ChE} in phosphate or bicarbonate media when potassium and calcium ions are absent. The addition of K and Ca ions decreases R_{ChE} in a bicarbonate-glucose medium and increases R_{ChE} in a

phosphate-glucose medium. Attention is drawn to the effects of these ions on glucose metabolism in bicarbonate and phosphate media. It is suggested that there is a link between glucose metabolism and choline ester metabolism in tissue slices.

6. The presence of sodium lactate, pyruvate, α -glycerophosphate or glutamate increases R_{ChE} but their effects are not as great as those of glucose. The presence of sodium succinate does not increase R_{ChE} .

7. The addition of sodium fluoride does not diminish R_{ChE} of brain in a glucose medium.

8. The addition of choline does not measurably increase R_{ChE} . Nor does the presence of a large excess of acetate ions markedly increase R_{ChE} .

9. Kidney, liver, spleen or testis has no measurable activity in forming choline ester under conditions most favourable for its production from brain. Rat diaphragm has about one-third the activity of rat brain.

10. Choline esterase activities of brain, liver, kidney, spleen, testis and defibrinated blood are recorded. There is no correlation between the choline esterase of an organ and its power of producing choline ester *in vitro*.

Our thanks are due to the Medical Research Council and to the Rockefeller Foundation for assistance grants to two of us.

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