CCXLV. CHOLINE-ESTERASE. AN ENZYME PRESENT IN THE BLOOD-SERUM OF THE HORSE.

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The question of the hydrolysis of acetylcholine by enzymes has been brought into prominence in connection with the investigations on the mechanism of nervous action which Loewi has carried out during the past decade. In his original paper on this subject Loewi [1921], working with isolated frogs' hearts, showed that stimulation of the vagus caused the formation or liberation in the heart of a substance which itself produces the effect previously considered to be due to the action of the nerve. It was recognised by Loewi [1922] that this so-called vagus-substance was a choline derivative, while Loewi and Navratil [1926, 1] later advanced evidence which indicated that it was an ester of choline, possibly acetylcholine, although Witanowski [1925], working in Loewi's laboratory, had previously stated that it was more stable than the latter substance. Not only did Loewi and Navratil find that the physiological activities of the vagus-substance and acetylcholine were indistinguishable, but they discovered that both substances were destroyed by aqueous extracts of frog's heart. The destructive agent present in such extracts was thermolabile and possessed other properties characteristic of enzymes; it was therefore considered to be an esterase. Following this work, Galehr and Plattner [1927, 1, 2] demonstrated that acetylcholine was similarly destroyed by defibrinated blood from various species. They were nevertheless of the opinion that no enzyme was involved but that the destruction was brought about by a process of adsorption catalysis, a view which was elaborated in subsequent papers by Plattner and his colleagues. It is unnecessary, however, to consider the latter work in detail since Engelhart and Loewi [1930] have now conclusively demonstrated the enzymic nature of the destructive agent; results of a similar nature have, moreover, been obtained by Matthes [1930].

That acetylcholine should be destroyed by esterases is not surprising. These enzymes, which are fairly widely distributed in the animal organism, are known to hydrolyse a variety of esters, and there seemed no reason to suppose that acetylcholine would be exceptionally resistant to their action. It has long been known, for example, that the blood-serum from many species contains an enzyme which attacks tributyrin, and it appeared logical to attribute the hydrolysis of acetylcholine by blood or serum to the action of this enzyme, and

to assume, in fact, that all esterases, and possibly lipases, would produce the same effect. Nevertheless, as pointed out by Stedman and Stedman [1931], the possibility that the destruction of acetylcholine was effected by a specific enzyme remained. No information was available on this point, for, in the work outlined above, pharmacological methods were employed to follow the destruction of the drug. Owing to the extraordinary activity of this substance, such methods can be and, in fact, were employed with minute amounts of material, the experiments of the above-mentioned authors being carried out with a fraction of a milligram of the drug. While these experiments served to demonstrate the destruction of acetylcholine, it was clear that they could give no information regarding the specificity or otherwise of the enzyme involved; much larger amounts of material would be required for this purpose. Stedman and Stedman therefore attempted to solve this problem indirectly by utilising an observation, made by Loewi and Navratil [1926, 2], that physostigmine, which potentiates the action of the vagus, does so in virtue of the fact that it inhibits the activity of the enzyme which causes the destruction of both acetylcholine and the vagus-substance. Assuming that physostigmine acted specifically on the latter enzyme, as seemed to be indicated by Matthes's results [1930], it appeared that the problem could be readily solved by examining the action of the alkaloid on an authentic esterase. Stedman and Stedman therefore examined the action on liver-esterase, using both methyl butyrate and tributyrin as substrates, of a group of urethanes which they had previously prepared and which, like physostigmine, could be classed as parasympathetic stimulants. In every case the activity of the esterase was inhibited by minute concentrations of the urethanes, a result which afforded strong evidence for the view that the enzyme which destroys acetylcholine is an esterase such as is present in the liver. This view, moreover, received additional support from the subsequent work by the same authors [1932], in which it was shown that whereas the activity of kidney-phosphatase towards glycerophosphoric acid was uninfluenced by the urethanes in question, that of serum-esterase from the guinea-pig towards tributyrin was inhibited to much the same extent as liver-esterase. Further, pancreatic lipase from the pig, although inhibited by relatively large concentrations of the urethanes when methyl butyrate was the substrate, was unaffected when the substrate was olive oil. These results certainly demonstrated that the inhibitory action of urethanes of the type concerned was specific to the extent that it was directed towards true esterases. Indirectly, they seemed to show that the destruction of acetylcholine by blood-serum and tissue extracts was caused by an esterase similar to that present in the liver and not by one of a specific nature.

Action of esterases and lipases towards acetylcholine.

Despite these results it appeared desirable to extend the work and to examine the activity of the various enzymes towards acetylcholine, using chemical methods of estimation. For the first experiments of this kind we

again selected liver-esterase, since preparations of this enzyme containing relatively little protein can be readily obtained by the methods developed by Willstätter and his co-workers [for details, see Stedman and Stedman, 1931]. When, however, the activity towards acetylcholine of such preparations was examined by the procedure described below, none was found. Even with a concentration of enzyme five times as great as that necessary to follow the hydrolysis of methyl butyrate under otherwise identical conditions (p_H 7·5), no hydrolysis of the acetylcholine beyond that due to the hydroxyl ions could be detected. The liver-esterases from two species, namely the pig and the cat, were tested with identical results. Pancreatic lipase from the pig [for preparation, see Stedman and Stedman, 1932] was similarly found to be inactive.

Method of estimation.

In view of these negative results we next turned our attention to the esterase present in horse-serum, this particular species being chosen partly because it had been employed in some of the pharmacological experiments mentioned above and partly because the serum could be readily obtained in quantity. For the estimation, the method of continuous titration, developed by Willstätter, Kuhn, Lind and Memmen [1927] in connection with liver-esterase, and subsequently used by many workers, was utilised under the following conditions. To 100 cc. of water, warmed to 30°, were added a measured volume of a solution of acetylcholine chloride of known concentration, 5 drops of a solution of bromothymol blue, and sufficient 0·0225 N alkali to bring the p_H to about 7·4. A measured volume, usually 1 cc., of the serum or solution to be tested was then added, the flask placed in the thermostat, and, after again adjusting the p_H of the solution, 0·0225 N sodium hydroxide was run into the flask, drop by drop, as required to maintain the p_H of the solution at the above value. Burette readings were recorded every 5 mins. for a period of 20 mins.

While the above method served to demonstrate the hydrolysis of acetylcholine by horse-serum, the results obtained were not as regular as was desired. Two factors were mainly responsible for this: the buffering action of the serum-proteins and the yellow pigment present in the serum. The latter modified the colour of the indicator to such an extent that it became difficult, if not impossible, to maintain the solution at a definite p_H. It was obvious that some purification of the enzyme would have to be effected if its properties were to be examined, and it thus became desirable to devise an improved method of estimation in order to facilitate the process of purification. Two modifications were therefore made in the above procedure. The first depended upon an observation which we made that butyrylcholine is hydrolysed more rapidly by the serum-enzyme than is acetylcholine. The former ester was therefore employed as substrate, a change which possessed a twofold advantage. Not only was there, for a given amount of enzyme, an increased liberation of

acid, but the correction which it was necessary to apply was smaller owing to the fact that butyrylcholine is much less susceptible than acetylcholine to the hydrolytic action of hydroxyl ions. The latter factor was not, it is true, of great importance when the experiments were carried out at p_H 7·4, for at this acidity the correction is almost negligible with either substrate; its advantage lay in the fact that it rendered possible the second modification, which consisted in carrying out the experiments at p_H not less than 8·6, using *o*-cresolphthalein as indicator. At this acidity the buffering action of the serum-proteins was much diminished, and hence the titrations were considerably sharper. Nevertheless, it cannot be claimed that accurate results were obtained with this modified procedure; it was used because it appeared to be the best available for the purpose of tracing the course of purification of the enzyme. When some success had been achieved in this direction, it was possible to revert to the method first described. It should be mentioned that, in order to avoid the possible inhibitory action of alcohol, aqueous solutions of the sodium salts of the indicators, prepared according to Clark [1925], were used throughout the work.

Choline-esterase activity of horse-serum.

It has been shown above that whereas liver-esterase and pancreatic lipase are without action on acetylcholine, an enzyme which is present in the blood-serum from the horse is able to hydrolyse both this substance and butyrylcholine. Although all the solutions of this enzyme which we have hitherto prepared have also attacked both methyl butyrate and tributyrin, their activities towards the latter substrates have been smaller than towards esters of choline. It is possible that different enzymes are involved. Assuming, however, that this is not the case and that the same enzyme has been responsible for the hydrolysis of these different types of esters, it is clear that the enzyme in horse-serum differs from liver-esterase in a manner analogous to that in which the latter differs from pancreatic lipase. It has been shown, for example, by Willstätter and Memmen [1924] that liver-esterase and pancreatic lipase resemble one another in hydrolysing both simple esters and fats, but differ enormously in the relative rates at which they attack these different types of esters, liver-esterase showing a decided preference for the former and pancreatic lipase for the latter type. Similarly, the horse-serum-enzyme, on the above assumption, resembles liver-esterase in hydrolysing methyl butyrate and tributyrin but differs from it in the fact that it also attacks esters of choline, and does so, moreover, more rapidly than it hydrolyses methyl butyrate. In order to emphasise this difference it is proposed to term the enzyme which hydrolyses esters of choline choline-esterase.

The choline-esterase activity of the serum from individual horses varies considerably. This is illustrated by the following experiments. Using, in each case, 0·1 g. of acetylcholine as substrate the acid liberated in 20 mins. at p_H about 7·5 by the enzyme present in 1 cc. of serum was equivalent, with three different sera, to 4·2, 3·6, and 2·45 cc. respectively of 0·0225 N alkali. Two

further specimens were tested at p_H about 8·8 with 0·1 g. of butyrylcholine as substrate; the corresponding figures were 7·2 and 4·65 cc. While, for the reasons explained above, no great accuracy is claimed for these figures, it is evident that wide variations in the choline-esterase content occur.

It is interesting to note that serum, if kept sterile, retains its choline-esterase activity practically unimpaired for months. The procedure we have usually adopted is to saturate the serum with chloroform and then to store it in the refrigerator.

Purification of choline-esterase.

In attempting to purify choline-esterase we have avoided devising a unit of activity for which any permanent meaning could be claimed. We have, however, used two magnitudes in order to give numerical expression to the yield of enzyme obtained and to the degree of purification effected in any operation. These magnitudes may, for convenience, be termed the volume-number and weight-number respectively. The former represents the volume, calculated from a determination of the activity of the preparation, of 0·0225N alkali which would be required to neutralise the acid liberated in 20 mins. by the enzyme obtained from 100 cc. of serum; the latter is the volume required to neutralise the acid similarly liberated by that contained in 1 g. of the dry material. In calculating the values of these numbers the assumption has been made that the activity is proportional to the enzyme content; this will, of course, only hold strictly under uniform conditions of estimation. During the progress of this work these conditions have been modified; for example, in the earlier experiments only 0·1 g. of acetyl- or butyryl-choline was used as substrate, whereas it was later found advantageous to employ 0·25 g. The values of the volume-numbers and weight-numbers obtained in different series of experiments will not therefore be comparable. Within any single series, however, where the conditions of estimation have been maintained as constant as possible, the values of these expressions give at least an approximate indication of the yield of enzyme which has been obtained and of its degree of purity. Typical experiments, carried out during the attempted purification of the enzyme, are briefly described below.

Dialysis. Collodion membranes were employed throughout these experiments, dialysis being effected under pressure in order to avoid great dilution.

100 cc. of serum were dialysed until salt-free. After removing a precipitate of euglobulin, 130 cc. of solution were obtained. Estimation: p_H, 8·8; substrate, 0·1 g. butyrylcholine chloride; 1·3 cc. required 4·8 cc. alkali in 20 mins. Volume-number, 480; volume-number for original serum, 465. No loss of activity occurs on dialysis.

Precipitation with ammonium sulphate. Preliminary experiments in which attempts were made to remove the enzyme from the dialysed solution by adsorbing it on alumina and kaolin having failed, precipitation with ammonium sulphate was examined. The following experiments give the results obtained with increasing concentrations of the salt.

20 g. of ammonium sulphate were dissolved in 100 cc. of serum. After 2 hours the solution was centrifuged and the centrifugate and precipitate dialysed separately. The precipitate, which readily dissolved in a small volume of water, gave 29 cc. of an almost colourless solution. Estimation: p_H, 8·8; substrate, 0·1 g. butyrylcholine chloride; 1 cc. required 0·85 cc. alkali in 20 mins. Volume-number, 25. The centrifugate yielded 117 cc. of solution containing 3·5 g. of solid. Estimation: 1 cc. required 4·1 cc. alkali in 20 mins. Volume-number, 480; weight-number, 117. Volume and weight numbers for serum, 465, 55.

In another similar experiment the precipitate from 200 cc. of serum was washed with a small volume of ammonium sulphate solution (20 g. salt; 100 cc. water) and the combined centrifugate and washings were dialysed. There were thus obtained 252 cc. of a yellow solution containing 3·6 g. solid per 100 cc. Estimation: p_H, 8·8; substrate, 0·25 g. butyrylcholine chloride; 10 cc. of the enzyme solution were diluted to 15 cc., when 2 cc. of this solution required 7·85 cc. alkali in 20 mins. Volume-number, 742; weight-number, 164. Corresponding numbers for serum, 830, 99.

The above results are not considered to be sufficiently accurate to warrant applying corrections for the hydrolysis of the substrate by hydroxyl ions. It is nevertheless clear that the protein precipitated by 20 g. of ammonium sulphate per 100 cc. of serum contains little if any enzyme. By removing this protein a considerable purification is therefore effected. Calculated on the dry-weight, the activity is approximately doubled.

105 g. of ammonium sulphate were dissolved in 300 cc. of serum and, after standing for some hours, the solution was centrifuged. The liquid was rejected. The precipitate was dissolved in water and the solution dialysed. Some protein which separated during dialysis was removed in the centrifuge, when 168 cc. of solution were obtained. Estimation: p_H, 8·8; substrate, 0·1 g. butyrylcholine chloride; 1 cc. required 7·1 cc. alkali in 20 mins. Volume-number, 398 (serum, 465). A large proportion of the enzyme is thus precipitated under these conditions. In view of the fact that a slight loss of material occurred in this experiment owing to a leak in one of the collodion membranes, the following experiment was carried out.

The precipitate obtained by dissolving 40 g. of ammonium sulphate in 200 cc. of serum was removed and washed as described above. The ammonium sulphate content of the combined centrifugate and washings was increased to 35 g. per 100 cc., the precipitate removed in the centrifuge, dissolved in water and dialysed. 83 cc. of solution were obtained containing 11 g. of solid per 100 cc. Estimation: p_H, 8·8; substrate, 0·1 g. butyrylcholine chloride; 1 cc. required 9·5 cc. alkali in 20 mins. Volume-number, 369; weight-number, 67. Corresponding numbers for serum, 465, 55. It is clear that some enzyme escaped precipitation and that little purification is effected under these conditions.

A further 200 cc. of serum were treated with 40 g. of ammonium sulphate

and the precipitate was washed as described above. The ammonium sulphate content of the combined centrifugate and washings was increased to 38 g. per 100 cc. and sufficient acetic acid was added to render the solution slightly acid to litmus. The precipitate was removed, dissolved in water, and the solution dialysed. There were thus obtained 80 cc. of solution containing 8·4 g. of solid per 100 cc. Estimation: p_H, 8·8; substrate, 0·1 g. butyrylcholine chloride; 1 cc. required 9·7 cc. alkali in 20 mins. Volume-number, 388; weight-number, 116. Corresponding numbers for serum, 465, 55.

40 g. of ammonium sulphate were dissolved in 100 cc. of serum, the precipitate dissolved in water and the solution so obtained dialysed. The salt-free solution measured 65 cc. and contained 8 g. of solid. Estimation: p_H, 8·8; substrate, 0·1 g. butyrylcholine chloride; 1 cc. required 7·2 cc. alkali in 20 mins. Volume-number, 468; weight-number, 58. Corresponding numbers for serum, 465, 55.

The above experiments demonstrate that the choline-esterase is precipitated with the more soluble proteins. Beyond the purification effected by the removal of the virtually inactive precipitate obtained with 20 g. of ammonium sulphate per 100 cc. of serum, simple fractional precipitation with this salt is evidently useless as a method for increasing the purity of the enzyme.

Glycerol extraction. The 65 cc. of solution obtained in the last experiment of the preceding section were evaporated to dryness at a temperature not exceeding 30°. The solid residue (8 g.) obtained was ground to a fine powder and extracted for 1½ hours at 30° with 90 % glycerol. Undissolved solid was removed in the centrifuge and the solution poured into a collodion membrane and dialysed. The residue was dissolved in water and similarly dialysed. The solution obtained from the extract measured 27 cc. and contained 0·11 g. of solid per cc. Estimation: p_H, 8·8; substrate, 0·1 g. butyrylcholine chloride; 1 cc. required 6·55 cc. alkali in 20 mins. Weight-number, 60; serum weight-number, 55. The residue yielded 24 cc. of solution containing 0·086 g. solid per cc. Estimation: 1 cc. required 5·7 cc. alkali in 20 mins. Weight-number, 66.

Extraction with ammonium sulphate. A quantity of serum was treated with 20 g. of ammonium sulphate per 100 cc., and, after removal of the inactive precipitate, dialysed. The solution so obtained was evaporated to dryness at a low temperature and the residue ground to a fine powder. 2 g. of the latter were extracted with 10 cc. of ammonium sulphate solution (20 g. salt; 100 cc. water). After centrifuging, the solution and residue were dialysed separately. The former gave 20 cc. of solution containing 6·4 g. solid per 100 cc. Estimation: p_H, 8·8; substrate, 0·25 g. butyrylcholine chloride; 1 cc. required 9·0 cc. alkali in 20 mins. Weight-number, 140; serum weight-number, 99. The solution obtained from the precipitate measured 9 cc. and had little activity: 1 cc. required 1·35 cc. alkali in 20 mins.

A further 2 g. of the solid were extracted for 1 hour with 10 cc. of ammonium sulphate solution (35 g. salt; 100 cc. water). The mixture was then centrifuged and the solution and residue were dialysed separately. The solution yielded

17·5 cc. of liquid containing 1·9 g. solid per 100 cc. Estimation: p_H, 8·8; substrate, 0·25 g. butyrylcholine chloride; 1 cc. required 5·35 cc. alkali in 20 mins. Weight-number, 282; serum weight-number, 99. The solution from the precipitate measured 19 cc. and contained 7·4 g. solid per 100 cc. Estimation: 1 cc. required 8·0 cc. alkali in 20 mins. Weight-number, 108.

Final method of purification.

The best preparation of choline-esterase which we have hitherto obtained has been prepared by a method based upon the preceding experiments. In this method we have utilised the observations that the protein precipitated with 20 g. of ammonium sulphate per 100 cc. contains little or no enzyme, and that extraction with a solution of 35 g. of ammonium sulphate in 100 cc. of water of the residue obtained by evaporation of the dialysed centrifugate gives a solution in which the ratio of enzyme to protein, as reflected by the weight-number of the preparation, is much increased. Unfortunately, however, the process of evaporation, even when carried out under extremely mild conditions, is accompanied by some destruction of the enzyme. We have therefore attempted to avoid evaporation by the following procedure, which also possesses the advantage that dialysis of large volumes of solution is avoided. Since this procedure has provided us with our best preparation of choline-esterase and has given consistent results with different specimens of serum, a typical experiment is described in detail.

Ammonium sulphate (20 g.) was dissolved in 100 cc. of serum. The precipitate was removed in the centrifuge, washed with a small volume of a solution of ammonium sulphate (20 g. to 100 cc. water) and the mixture again centrifuged. The concentration of ammonium sulphate in the combined centrifugates, which measured 115 cc., was increased to 40 g. per 100 cc. by the addition of 23 g. of the solid salt, and the solution rendered faintly acid to litmus by the addition of 8 cc. of $N/2$ acetic acid. After about an hour, the precipitate which formed was centrifuged off, the liquid being rejected. The precipitate was then stirred in the centrifuge-glasses with about an equal volume of a solution of 35 g. of ammonium sulphate in 100 cc. of water. After standing overnight, the suspension was again centrifuged and the clear liquid poured into a flask. In the course of a few hours a crystalline precipitate, probably of albumin, separated. This was removed, and the concentration of ammonium sulphate in the liquid, which measured 34 cc., was increased to 40 g. per 100 cc. by the addition of 1·7 g. of the solid salt; 0·5 cc. of $N/2$ acetic acid was also added to render the solution faintly acid. A precipitate separated; after a few hours this was removed, dissolved in a small volume of water, and the solution so obtained was dialysed. A small quantity of protein separated during dialysis. This was centrifuged off and rejected, leaving a clear solution with a faint brown colour. Estimation: p_H, 8·8; substrate, butyrylcholine chloride (0·25 g.); 1 cc. of solution, which contained 1·996 % of solid, required 12·85 cc. of alkali in 20 mins. Weight-number, 644; serum weight-number, 99.

When a correction is applied for the hydrolysis of the substrate by hydroxyl ions, these figures become 581 and 82 respectively. This indicates that, based on the dry-weight, the purified preparation was seven times as active as the original serum. The purification actually effected was, however, probably greater than this, for, as previously explained, some uncertainty exists regarding the accuracy of the estimations made with unpurified serum. The exact degree of purification is, however, unimportant. The advantage of the process lies in the fact that the contents of protein and of yellow pigment in the preparation have been reduced to such an extent that the activity of the enzyme can be followed with great accuracy.

Action of choline-esterase on various substrates.

The activity towards various substrates of the preparation of choline-esterase described above was examined at two different acidities, with the results shown in Table I. 1 cc. of the enzyme solution was used in each case.

Table I.

Substrate	p_H	cc. 0·0225 *N* NaOH used in 5 min. periods	Total	Non-enzymic hydrolysis	Total (corr.)
Butyrylcholine	8·8	3·25, 3·2, 3·2, 3·2	12·85	1·35	11·5
,,	7·3	3·25, 3·3, 3·0, 3·2	12·75	0·1	12·65
Acetylcholine	8·8	2·2, 2·05, 2·1, 2·05	8·4	2·4	6·0
,,	7·3	1·4, 1·5, 1·35, 1·3	5·55	0·2	5·35
Methyl butyrate	7·3	0·15, 0·2, 0·2, 0·15	0·7	0·1	0·6

As substrates, 0·25 g. of the chlorides of the choline esters and 0·25 cc. of methyl butyrate were employed. The indicators used were *o*-cresolphthalein and bromothymol blue. Corrections for the non-enzymic hydrolysis of the substrates were determined under conditions identical with those used in the enzyme experiments. In addition to these experiments, the action of 1 cc. of the enzyme preparation on tributyrin was examined stalagmometrically under the conditions (p_H, 7·9; T., 20°) described by Stedman and Stedman [1931]. The diminution in the drop number in 40 mins. was 22.

The results with another preparation are given in Table II.

Table II.

Substrate	p_H	cc. 0·0225 *N* NaOH used in 5 min. periods	Total	Non-enzymic hydrolysis	Total (corr.)
Butyrylcholine	8·6	1·0, 1·05, 1·1, 1·1	4·35	0·65	3·7
,,	7·4	1·15, 1·15, 1·15, 1·15	4·6	0·1	4·5
Acetylcholine	8·6	1·25, 1·25, 1·1, 1·2	4·8	1·2	3·6
,,	7·4	0·8, 0·85, 0·8, 0·8	3·25	0·15	3·1

In these experiments the enzyme preparation was diluted in order to reduce the rate of hydrolysis of the substrates, which was somewhat too great in the experiments of Table I. Owing to the slower rate at which it was necessary to add the alkali, this permitted a much more accurate control of p_H.

For comparison with the results of Tables I and II, the experiments carried out with 1 cc. of the serum from which the preparations used above were made are recorded in Table III.

Table III.

Substrate	p_H	cc. 0·0225 *N* NaOH used in 5 min. periods	Total	Non-enzymic hydrolysis	Total (corr.)
Butyrylcholine	8·8	2·3, 1·9, 2·1, 2·0	8·3	1·35	6·95
Acetylcholine	8·8	1·7, 1·7, 1·6, 1·45	6·4	2·4	4·0
Methyl butyrate	8·8	0·6, 0·55, 0·45, 0·6	2·2	0·2	2·0

Using tributyrin as substrate with 1 cc. of the same specimen of serum, the diminution in the drop number (p_H, 7·9; T., 20°) in 40 mins. was 23. While great accuracy cannot be claimed for the results in Table III, they are reproduced because they show that the relative activities of the enzyme preparation towards the three types of substrate employed, namely, esters of choline, methyl butyrate and tributyrin, have changed considerably during the process of purification. In particular, the activities towards methyl butyrate and tributyrin are much depressed in the purified preparation. This may indicate that different enzymes are involved, but the data at present available are insufficient to permit of a definite decision on this point.

Summary and discussion.

The preceding experiments have demonstrated that there is present in the blood-serum from the horse an enzyme, for which the name choline-esterase is suggested, which hydrolyses both acetyl- and butyryl-choline, the latter substrate being attacked more rapidly than the former. This enzyme is clearly different from the liver-esterases from the pig and the cat, since the latter are without action on esters of choline. Methods for the purification of choline-esterase are described.

The question of the distribution of the new enzyme has not yet been examined. It must not, however, be concluded from the above results that it is necessarily absent from the liver. The liver-esterase which we have employed has been obtained by extracting desiccated liver powder with dilute ammonia, desiccation of the liver having been effected by the use of acetone and ether. It is probable that such treatment would destroy choline-esterase. Thus, in some preliminary experiments not recorded above an attempt was made to purify the enzyme in horse-serum by precipitating it together with the proteins with acetone and then drying the precipitate by successive treatment with this solvent and ether. The dry preparation so obtained dissolved completely in water, but the resulting solution was without action on acetylcholine.

The presence in horse-serum of an enzyme which hydrolyses tributyrin has long been known. As far as we are aware, however, its action towards simple esters such as methyl butyrate has not hitherto been investigated. The above results now show that this ester, in addition to tributyrin and esters of

choline, is hydrolysed by an enzyme present in the serum. Whether one enzyme is responsible for the hydrolysis of all three types of substrate, or whether different enzymes are involved, cannot yet be decided. That the relative activities of purified preparations of the enzyme towards the three substrates should prove to be different from that of serum must, however, be regarded as evidence in favour of the latter view.

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REFERENCES.

Clark (1925). The determination of hydrogen ions. (Baltimore.)
Engelhart and Loewi (1930). *Arch. exp. Path. Pharm.* **150**, 1.
Galehr and Plattner (1927, 1). *Pflüger's Arch.* **218**, 488.
—— —— (1927, 2). *Pflüger's Arch.* **218**, 506.
Loewi (1921). *Pflüger's Arch.* **189**, 230.
—— (1922). *Pflüger's Arch.* **193**, 201.
—— and Navratil (1926, 1). *Pflüger's Arch.* **214**, 678.
—— —— (1926, 2). *Pflüger's Arch.* **214**, 689.
Matthes (1930). *J. Physiol.* **70**, 338.
Stedman and Stedman (1931). *Biochem. J.* **25**, 1147.
—— —— (1932). *Biochem. J.* **26**, 1214.
Willstätter and Memmen (1924). *Z. physiol. Chem.* **138**, 216.
—— Kuhn, Lind and Memmen (1927). *Z. physiol. Chem.* **167**, 303.
Witanowski (1925). *Pflüger's Arch.* **208**, 694.