CXLIII. STUDIES ON THE RELATIONSHIP BETWEEN CHEMICAL CONSTITUTION AND PHYSIOLOGICAL ACTION.

IV. THE INHIBITORY ACTION OF CERTAIN SYNTHETIC URETHANES ON THE ACTIVITY OF ESTERASES.

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PART III [Stedman and Stedman, 1931] of this series of communications was concerned with an examination of the action towards liver esterase of the group of urethanes which had previously been shown to behave pharmacologically as "parasympathetic stimulants," and it was demonstrated that, while such urethanes possessed the power of inhibiting the action of this enzyme to a very high degree, other urethanes and bases did not produce a similar effect. It was therefore concluded that a relationship between constitution and inhibitory action towards liver esterase existed analogous to that between constitution and physiological action in the same group. This result afforded exceedingly strong, if not conclusive, evidence in favour of Loewi and Navratil's [1926] views regarding the mechanism of the action of physostigmine on the heart, and further appeared to indicate the nature of the enzyme which, according to Engelhart and Loewi [1930] and to Matthes [1930], brings about the destruction of acetylcholine and of the vagus substance. It is clear from Loewi's work, however, that the liver is not normally directly responsible for the destruction of the vagus substance, which, in his original experiments, was shown to be caused by aqueous extracts of the frog's heart. It therefore appeared to be desirable to examine the inhibitory action of the above group of urethanes towards serum esterase, particularly as Engelhart and Loewi, Matthes, and Plattner have shown that both whole blood and serum are capable of destroying acetylcholine. At the same time the actions of the urethanes on two other enzymes, namely pancreatic lipase and phosphatase, the normal substrates of which are esters, have been examined in order to determine the extent to which the inhibitory action of the urethanes is specific.

PANCREATIC LIPASE.

Enzyme preparation. The pancreatic lipase was prepared from pig's pancreas according to Willstätter and Waldschmidt-Leitz's method [1923]. The minced glands were desiccated by successive treatment with acetone and ether and the fibrous material was separated from the dry preparation by pounding it in a mortar and then shaking it through a fine sieve. The fine powder so obtained was extracted for 4 hours at 30° with 87 % glycerol (32 cc. for 2 g. of powder), centrifuged, and the extract stored in this form. Immediately before use, 15 cc. of this extract were diluted to 60 cc. with water, and again centrifuged to remove a fine precipitate which separated.

Hydrolysis of olive oil. The influence of miotine hydrochloride on the hydrolysis of olive oil by pancreatic lipase was first examined. Into each of two small stoppered bottles 2.5 g. of olive oil were weighed and 2 cc. of ammonia-ammonium chloride buffer (p_H 8.9) were then added. The contents of one bottle were now treated with 11 cc. of the above enzyme extract and those of the other with 11 cc. of the same extract containing 5 mg. of miotine hydrochloride which had been dissolved in it one hour previously. Each bottle was shaken for 3 minutes to emulsify the oil and then placed in a thermostat at 30° for one hour. The contents of each bottle were now washed with 100 cc. of rectified spirit into a flask, 20 cc. of ether added to dissolve the oil and the solutions titrated with 0.727*N* alcoholic potassium hydroxide, using thymolphthalein as indicator. In each case 6.8 cc. of the alkali were used. A control, using 11 cc. of water in place of the enzyme solution, required 2.4 cc. of the alkali. It is clear that miotine exerted no inhibitory action.

Hydrolysis of tributyrin. In order to follow the hydrolysis of tributyrin, the stalagmometric method described in Part III was employed. The above diluted extract (1 cc.) of pancreatic lipase was mixed with 2 cc. of phosphate buffer (p_H 7.9) and diluted to 10 cc. with water. One cc. of this preparation was treated with 12 mg. of miotine hydrochloride dissolved in 1 cc. of water and allowed to stand for one hour, when 1 cc. of the mixture was used in a hydrolysis experiment. For the control, 1 cc. of water was employed in place of the solution of miotine hydrochloride. The following figures represent the diminution in the drop number in successive periods of 20 minutes: control, 15, 23; in the presence of miotine, 14, 23. Miotine is thus without inhibitory action on the hydrolysis of tributyrin by pancreatic lipase.

Hydrolysis of methyl butyrate. The method employed was at first identical with that used in Part III with liver esterase, except that the reaction mixture contained a high percentage of glycerol which activates as well as stabilises the lipase. Into a 100 cc. graduated flask were introduced 50 cc. of a 50 % solution of glycerol and 20 cc. of buffer (1 part 2.5 *N* NH_3 :2 parts 2.5 *N* NH_4Cl ; p_H 8.9) and the mixture was then warmed to 30° in a thermostat. One cc. of methyl butyrate was dissolved as completely as possible in this by shaking, when 20 cc. of the diluted extract of pancreatic lipase were added and the volume was

made up to 100 cc. with water. The flask was again placed in the thermostat, 20 cc. of the mixture being immediately withdrawn, run into a mixture of 25 cc. of 0.2 *N* hydrochloric acid with 20 cc. of water and titrated with 0.2 *N* sodium hydroxide, using bromocresol purple as indicator. Similar volumes of the mixture were withdrawn at intervals of 20 minutes and titrated in the same way. In the experiment designed to test the inhibitory action of miotine, 12 mg. of the hydrochloride were dissolved in 25 cc. of the diluted extract of the enzyme and allowed to stand for one hour; 20 cc. of this solution were then used in a hydrolysis experiment. The following results were obtained, the figures representing the number of cc. of 0.2 *N* alkali required to titrate the acid liberated in 20 cc. of the reaction mixture in 20, 40 and 60 minutes respectively: control, 1.75, 2.7, 3.4; in the presence of miotine, 1.15, 1.85, 2.35. A small inhibitory effect is apparent, although it is much smaller in magnitude than with liver esterase.

Willstätter and Memmen [1924] have shown that calcium oleate exerts a marked activating action on the hydrolysis of methyl butyrate by pancreatic lipase. Another experiment was therefore carried out in the presence of this activator. The procedure was identical with that outlined above except that 2 cc. each of 2 % sodium oleate and calcium chloride solutions were added to the reaction mixture after the addition of the enzyme but before making up to volume. In view of the activation caused by this addition, only 5 cc. of the diluted glycerol extract of lipase were used. Nevertheless the same amount of miotine was employed. The following figures are typical of the results obtained: control, 2.25, 4.1, 5.05; in the presence of miotine, 1.7, 3.15, 4.25. Owing to the fact that the hydrolysis of methyl butyrate by pancreatic lipase does not take a linear course, possibly because of the changes which occur in the p_H of the solution, it is not possible to calculate in a simple manner the percentage inhibition produced by the miotine. Nevertheless it is clear from the figures that the inhibition caused by the miotine in this experiment is of the same order of magnitude as that produced by the same quantity in the absence of calcium oleate, although only one fifth of the amount of enzyme was required in the latter experiment.

An experiment similar to that last described was also carried out with the hydrochloride of the methylurethane of *m*-dimethylaminophenol, this particular urethane being chosen because it had proved to be the most active of the urethanes examined in inhibiting the hydrolysis of methyl butyrate by liver esterase. About 10 mg. of the urethane were employed, the remaining details being identical with those described above. The results are shown by the following figures: control, 2.05, 3.9, 4.75; in the presence of the urethane, 1.45, 2.9, 3.85. A small inhibitory effect is again apparent.

In view of the possibility that the glycerol, necessarily present in the above solutions, might diminish the inhibitory action of the urethanes, the influence of glycerol on the inhibitory action of miotine towards liver esterase was examined. An acidified and dialysed extract of liver powder, similar to those

described in Part III, was employed, the hydrolysis of methyl butyrate being followed under the conditions described in that paper with the modification that 50 cc. of water were replaced by 50 cc. of 50 % glycerol. As inhibitor, 0.01 mg. of miotine hydrochloride was employed. The following titration figures were obtained: control (without glycerol), 1.85, 3.8, 5.5; control (with glycerol), 1.85, 3.65, 5.45; in the presence of miotine (without glycerol), 0.55, 1.5, 2.2; in the presence of miotine and glycerol, 0.55, 1.4, 2.0. It is evident that the glycerol was without influence either on the activity of the esterase or on the inhibitory activity of miotine. The experiment further serves to illustrate the much greater sensitivity of liver esterase to miotine. With only a thousandth part of the amount used with pancreatic lipase a greater inhibition was produced.

KIDNEY PHOSPHATASE.

The preparation of kidney phosphatase employed was obtained by Erdtman's method [1927]. 600 g. of pig's kidneys were minced, suspended in 500 cc. of water to which much toluene had been added, and incubated for 2 days at 37°. The mixture was then filtered through a fine metal strainer, and the turbid filtrate treated with 1½ litres of rectified spirit. The precipitate so produced was filtered, stirred with 500 cc. of alcohol and again filtered, this process being repeated twice. It was then similarly treated with 500 cc. of ether, dried in the air and ground in a mortar. Extracts of the phosphatase were prepared by shaking 4 g. of this dry powder with 80 cc. of *N*/40 ammonia for 1½ hours and removing the solid material in the centrifuge.

The substrate employed was sodium glycerophosphate. To a mixture of 10 cc. of 5 % sodium glycerophosphate, 10 cc. of buffer (ammonia-ammonium chloride, p_H 8.9) and 60 cc. of water, previously warmed to 30° in a thermostat, were added 10 cc. of the above solution of phosphatase. The total volume was then made up to 100 cc. with water and the solution replaced in the thermostat. 20 cc. of the mixture were immediately withdrawn and run into 10 cc. of 10 % trichloroacetic acid. The free phosphate was then estimated in 25 cc. of the filtrate by precipitating it as phosphomolybdic acid, dissolving the latter after filtration in 10 cc. of 0.21*N* NaOH and titrating the excess alkali with 0.0995*N* nitric acid. Similar withdrawals and estimations were made at fixed intervals. A typical experiment will suffice to illustrate that miotine is without action on kidney phosphatase. 12 mg. of miotine hydrochloride were dissolved in 12 cc. of the enzyme solution and, after an interval of one hour, the phosphatase activity of 10 cc. of the solution was determined, a control being carried out simultaneously. The following figures represent the cc. of nitric acid required to neutralise the excess alkali in estimations on samples withdrawn immediately and after 80 minutes respectively: control, 18.2, 8.9; with miotine, 18.6, 8.9.

SERUM ESTERASE.

In order to examine the inhibitory action of the various urethanes towards serum esterase, the serum of the guinea-pig was chosen, since the blood of this

animal is known to contain a relatively high amount of the enzyme. 2-3 cc. of the serum, the exact volume depending upon the esterase activity of the sample employed, were treated with 2 cc. of phosphate buffer of p_H 7.9 and the mixture was diluted to 10 cc. with water. One cc. of this solution was mixed with 1 cc. of a solution of the urethane under examination and allowed to stand for about an hour, when the esterase activity of 1 cc. of the mixture was determined by the stalagmometric method described in Part III. In the control experiment, the solution of the urethane was replaced by an equal

Table I. *Inhibition of serum esterase by various urethanes.*Substrate: tributyrin. $T=20^\circ$. $p_H=7.9$.

Inhibitor*	Final conc. of inhibitor ($M \times 10^{-8}$)	Decrease in drop number in		Percentage inhibition
		20 mins.	40 mins.	
<i>Phenyl series:</i>				
Control	—	11	21	—
<i>m</i> -HCl	4	5	11	48
<i>p</i> -HCl	4	8	16	24
<i>o</i> -HCl	4	8	15	29
Control	—	13	25	—
<i>m</i> -MeI	400	5	9	64
<i>p</i> -MeI	400	1	4	84
<i>o</i> -MeI	400	1	3	88
<i>Miotine series:</i>				
Control	—	11	23	—
<i>m</i> -HCl	400	1	2	91
"	40	5	10	57
<i>p</i> -HCl	400	2	4	83
"	40	4	10	57
<i>o</i> -HCl	400	1	3	87
"	40	9	19	17
Control	—	14	27	—
<i>m</i> -MeI	4000	2	3	89
"	400	11	22	19
<i>p</i> -MeI	4000	1	3	89
"	400	13	23	15
<i>o</i> -MeI	4000	1	2	93
"	400	9	17	37
<i>Benzyl series:</i>				
Control	—	12	23	—
<i>m</i> -HCl	40	2	5	78
"	4	8	16	30
<i>p</i> -HCl	40	3	7	70
"	4	9	18	22
<i>o</i> -HCl	40	6	12	48
Control	—	14	26	—
<i>m</i> -MeI	400	8	17	35
<i>p</i> -MeI	400	6	14	46
<i>o</i> -MeI	400	7	15	42

* *Phenyl series*; methylurethanes of the isomeric dimethylaminophenols.*Miotine series*; methylurethanes of the isomeric α -hydroxyphenylethyldimethylamines.*Benzyl series*; methylurethanes of the isomeric hydroxybenzyl dimethylamines.

volume of water. The results obtained are given in Table I. The urethanes employed were identical with those used in Part III and are indicated by the same abbreviated names.

LIVER ESTERASE.

In view of the fact that serum from the guinea-pig was used in the above experiments it was thought that it would be of interest to examine the inhibitory actions of the various urethanes on the liver esterase from the same species. A preparation of this esterase was therefore made from a number of guinea-pig's livers using the same procedure as was employed in Part III in connection with liver esterase from the pig. The activity of the preparation towards methyl butyrate, however, proved to be much smaller than that from the pig and it was not, therefore, possible to follow the hydrolysis of simple esters by the technique employed in Part III. Despite its smaller activity towards methyl butyrate, this preparation nevertheless proved to be at least as active towards tributyrin as was the esterase from pig's liver. The experiments recorded in Table II were therefore carried out, using this substance as

Table II. *Inhibition of guinea-pig's liver esterase by various urethanes.*

Substrate: tributyrin. T=20°. $p_H=8.0$.				
Inhibitor	Final conc. of inhibitor ($M \times 10^{-8}$)	Decrease in drop number in		Percentage inhibition
		20 mins.	40 mins.	
<i>Phenyl series:</i>				
Control	—	14	26	—
<i>m</i> -HCl	40	0	0	100
<i>p</i> -HCl	40	9	18	31
<i>o</i> -HCl	40	7	16	38
Control	—	14	27	—
<i>m</i> -MeI	400	6	13	52
<i>p</i> -MeI	400	3	8	70
<i>o</i> -MeI	400	7	16	41
<i>Miotine series:</i>				
Control	—	11	22	—
<i>m</i> -HCl	40	4	8	64
<i>p</i> -HCl	40	6	14	36
<i>o</i> -HCl	40	4	8	64
Control	—	13	24.5	—
<i>m</i> -MeI	400	5	10.5	57
<i>p</i> -MeI	400	5	11	55
<i>o</i> -MeI	400	5	10.5	57
<i>Benzyl series:</i>				
Control	—	13	24	—
<i>m</i> -HCl	40	3	8	67
<i>p</i> -HCl	40	7	16	33
<i>o</i> -HCl	40	4	9	63
Control	—	14	27	—
<i>m</i> -MeI	400	4	9	67
<i>p</i> -MeI	400	5	12	56
<i>o</i> -MeI	400	6	13	52

substrate. The preparations of the esterase employed were obtained by extracting the desiccated liver powder with dilute ammonia, acidifying this extract with acid and, after removing the precipitate thus produced, dialysing for about 3 days in collodion membranes. In the various experiments recorded,

1.5–3 cc. of such an extract were mixed with 2 cc. of phosphate buffer of p_H 8.0 and the mixture was diluted to 10 cc. with water. One cc. of this solution was mixed with 1 cc. of water or of a solution of the urethane under examination, allowed to stand for about an hour, and 1 cc. of the mixture used in a hydrolysis experiment. The results obtained with the various urethanes are recorded in Table II.

SUMMARY AND DISCUSSION.

In so far as they show that the hydrolysis of tributyrin by the esterase from the liver of the guinea-pig is inhibited by small concentrations of the urethanes which have been shown to behave as parasympathetic stimulants, the above experiments constitute an extension to the liver esterase from a second species of the results obtained with the pig and reported in Part III of this series. While we have not carried out any extensive experiments with liver esterases from other species, we have nevertheless submitted those from a few to a preliminary examination and have found in each case that their activity is inhibited by miotine when present in concentrations of the same order of magnitude as employed in the above experiments. It would therefore appear legitimate to conclude that the urethanes of the type under consideration inhibit the activity of liver esterases in general and irrespective of the species from which they are derived.

Our experiments further demonstrate that the inhibitory action of the urethanes is not restricted to the liver enzyme but extends to the esterase which is present in the blood-serum of the guinea-pig and of certain other species. In view of the fact that it has been shown by the authors mentioned in the introduction that both whole blood and serum are capable of destroying small amounts of acetylcholine, a process which is inhibited by physostigmine, this result was, perhaps, to be expected. Nevertheless, it must be emphasised that the experiments here recorded have been carried out using tributyrin as substrate. While it would seem probable that the same enzyme is concerned in both processes, this cannot at present be regarded as definitely established. There exists, indeed, some evidence to the contrary. Thus, Takahashi [1930], has recently compared the lipolytic activities of the sera from a number of species, using tributyrin as substrate, and has found that they fall in the following order: rabbit > cat > horse > man > dog > cattle. This order is in general agreement with the results of earlier workers and also with some unpublished experiments which we have carried out. On the other hand, the order in which the defibrinated blood from a number of species destroys acetylcholine is, according to Galehr and Plattner [1927], man > pig $\geq$ cattle > dog > horse > rabbit > cat. The latter series is practically the reverse of that which holds for the hydrolysis of tributyrin and hence suggests either that different enzymes are concerned in the two processes or that some other factor, hitherto unrecognised, is involved. It is unlikely that a solution of this aspect of the problem will be possible until the blood-enzymes have been purified and

concentrated. Unfortunately such purification is attended with considerable difficulty on account of the quantity and ready solubility of the serum proteins. We are nevertheless at present engaged in an attempt to effect this purification.

Although the possibility thus exists that the hydrolysis of tributyrin and the destruction of acetylcholine are brought about by different serum enzymes, our results appear quite definitely to indicate that the enzymes, if specifically different, are of the same general nature. We have now established that the activities of both liver and serum esterases are inhibited by small concentrations of urethanes of the miotine type. Nevertheless, the above results show that the activity of pancreatic lipase, an enzyme which, although it resembles liver esterase in hydrolysing simple esters, differs from the latter enzyme in attacking fats relatively more rapidly, is not inhibited by miotine when its substrate is either a fat, in the form of olive oil, or tributyrin. When, however, its substrate is methyl butyrate, a simple ester, its action is inhibited by miotine, although a concentration of the drug which is high compared with that required with serum or liver esterase is necessary. Similarly, the hydrolysis of glycerophosphoric acid by kidney phosphatase, a process involving an ester of a different type from that attacked by liver esterase, is unaffected by miotine. It thus appears that the inhibitory action of the urethanes with which we are concerned is directed mainly towards true esterases, and it is therefore probable that the destruction of acetylcholine is brought about by an enzyme of the same nature.

Regarding the nature of the inhibitory action of the drugs in question, there appears little to be added to our comments in Part III. It was pointed out in that communication that the urethanes are esters and that combination between them and the enzyme probably involves the same mechanism as with the normal substrate, in which case the resulting inhibition would be due to the inability of the enzyme to hydrolyse the urethane. Some chemical evidence which supports this view may be mentioned. In Part I [1926] of this series it was mentioned that when aqueous solutions of certain of the urethanes are boiled, decomposition occurs with the production of methyl *isocyanate*. This decomposition was erroneously referred to as hydrolysis, but it is clear, as pointed out by Aeschlimann and Reinert [1931] in a paper concerned with the pharmacology of urethanes of the same type, that no hydrolytic process is involved. This can be seen from the following equation:

If, then, despite their structures as esters, these urethanes are not normally hydrolysed by ordinary chemical reagents but undergo a decomposition of a different nature, it is not difficult to understand the inability of esterases to bring about their destruction. The inhibitory action would be a direct consequence of this, provided the urethanes were endowed with high affinities for the enzyme.

The general relations between the nature of the urethane and its inhibitory activity towards liver esterase observed in Part III and discussed in that paper have again been encountered in the experiments described above and require no further comment. The order of activity of isomeric urethanes appears, however, to differ according to the source of the enzyme. While it would be in accordance with other observations that the enzymes from different species should exhibit differences in this respect, it is more difficult to understand why the esterases from the liver and serum of the same species should show similar differences. The obvious explanation, that the two latter enzymes are different substances, may be correct. We feel, however, that it would be wiser at the present time not to lay too much stress upon these small differences, which might conceivably be caused by impurities which are present in the enzyme solutions.

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