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

III. THE INHIBITORY ACTION OF CERTAIN SYNTHETIC URETHANES ON THE ACTIVITY OF LIVER ESTERASE.

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PARTS I and II [Stedman, 1926; 1929, 1] of this series of communications have dealt with a new relationship between chemical constitution and physiological action. It has been shown that certain synthetic urethanes possess in common with the alkaloid physostigmine, which is also a urethane, the power of producing a constriction of the pupil when instilled into the eye. As a result of this work, as well as of extensions thereof published elsewhere [Stedman and Stedman, 1929; 1931], it has been concluded that substances which can be classed as phenyl esters of carbamic acids and which possess a basic group will, in general, exhibit similar miotic properties. Constitutional factors which repress, or tend to repress, such properties have already been discussed. Until recently the pharmacological examination of the synthetic urethanes had not extended beyond their action on the eye and it was not therefore certain that they would resemble physostigmine in other physiological properties. One of the urethanes, namely, the methylurethane of α -*m*-hydroxyphenylethyldimethylamine, which had already been shown to possess marked miotic properties and had been named miotine [Stedman, 1929, 2] on that account, has, however, now been submitted to a complete, and three others to an extensive although less complete, pharmacological examination [White and Stedman, 1931]. Miotine has thus been shown to possess physiological properties which are qualitatively identical with those of physostigmine, and similar results have been obtained with the other urethanes as far as they have been examined. The above relationship between chemical constitution and physiological action is therefore not limited to miotic activity but embraces all the known physiological actions of physostigmine. Now, Loewi and Navratil [1926] have attributed the action of physostigmine on the heart to its power of inhibiting the destruction of acetylcholine by an agent which they have shown to be present in the heart and in aqueous extracts of this organ. A similar agent is present in the blood of certain species [Galehr and

Plattner, 1927]. Loewi and Navratil consider this agent to be an esterase, a view which has been disputed by Plattner and Galehr [1928]. The arguments of Plattner have, however, been disposed of in a recent paper by Engelhart and Loewi [1930], who have brought forward convincing evidence of the enzymic nature of the substance causing the destruction of acetylcholine. Evidence leading to the same conclusion has also been published by Matthes [1930]. In view of the similarity between the actions of miotine and physostigmine on the heart, and in particular of the fact that miotine, like physostigmine, sensitises the vagus and potentiates the action of acetylcholine, it might be expected that miotine would also inhibit the destruction of acetylcholine by the agent in question. That this is actually the case has been shown by Dr Matthes, who kindly compared the action of miotine with that of physostigmine during his investigation of the inhibition by the latter of the destruction of acetylcholine by serum. The problem thus arises as to whether urethanes of the type discussed above, which have been shown to possess similar physiological properties, also share the property of inhibiting the action of the serum-enzyme. Another problem is, however, involved. In the investigations both of Loewi and his co-workers and of Matthes, the destruction of acetylcholine by the solutions under examination has been measured by biological methods, and no experiments appear to be recorded which indicate whether the enzyme which is responsible for this destruction will hydrolyse simple esters, or whether its action is specific towards acetylcholine. If the enzyme is a true esterase it should be capable of hydrolysing simple esters, in which case its activity, as well as the inhibition thereof by drugs, could be followed by titration methods. It appeared to us that these problems could be most conveniently, although indirectly, solved by examining the influence of the urethanes on the activity of the enzyme present in the liver and which is known to hydrolyse simple esters more readily than glycerides. The results of such an investigation are recorded in the present communication.

MATERIALS.

Esterase preparations. All the esterase solutions employed in these experiments have been prepared from pigs' livers by the method of Willstätter and Memmen [1924, 2]. The liver, obtained direct from the slaughter house, was finely minced, desiccated by successive treatment with acetone and ether, and the dry preparation powdered and sieved. Solutions of the enzyme were prepared, as required, by extracting 2 g. of this fine powder with 100 cc. of 0.025 *N* ammonia for 1 hour and removing the insoluble material in the centrifuge. In the earlier experiments no further purification of the esterase was attempted. In the later work, however, it was found to be advantageous carefully to acidify the ammoniacal extract with acetic acid, and, after removing the precipitate so produced, to dialyse the clear solution in collodion membranes for 3-4 days, a procedure similar to that first described by

Willstätter, Bamann and Waldschmidt-Graser [1928] and subsequently employed by other workers.

Substances examined for inhibitory action. The urethanes with a pharmacological action similar to that of physostigmine which have been examined for inhibitory action comprise three series of isomeric compounds, namely, the methylurethanes of *o*-, *m*- and *p*-hydroxybenzyltrimethylamine, the *o*-, *m*- and *p*-dimethylaminophenyl esters of methylcarbamic acid, and the methylurethanes of the isomeric α -hydroxyphenylethyldimethylamines. The structures of the *m*-isomerides in these three series are represented in formulae I, II and III respectively. These compounds have been examined both in the

form of their hydrochlorides and methiodides, the preparation of which has been effected by the methods of Stedman [1926; 1929, 1] and Stedman and Stedman [1929]. In the case of the *o*-isomerides, the hydrochlorides of which are hygroscopic, the base was dissolved in the calculated quantity of hydrochloric acid immediately before use. It should be noted that the urethane represented by formula III is the substance which has been named miotine. As will be evident from the formula, miotine and its position isomerides contain an asymmetric carbon atom. Unfortunately attempts to resolve these compounds have not so far met with success; the examination of their inhibitory actions towards liver esterase has therefore necessarily been carried out with the racemic substances. It will be convenient in the following pages to refer to the isomeric compounds corresponding with formula III as *o*-, *m*- and *p*-miotine, and to those corresponding with formulae I and II as the methylurethanes of the benzyl and phenyl series respectively.

In addition to urethanes with a physostigmine-like action, the methylurethane of choline iodide, $\text{CH}_3\text{NH.CO.OCH}_2\text{CH}_2\text{N}(\text{CH}_3)_3\text{I}$ [Stedman, 1929, 1], has been examined as an example of an aliphatic urethane. Some tests have also been made with the following alkaloids: pilocarpine, arecoline, ricinine. The two former were employed as nitrate and hydrobromide respectively, these salts being obtained from commercial sources. The ricinine was prepared from castor-oil seeds; since this alkaloid is soluble in water it was employed in the form of the free base.

Pilocarpine and arecoline were examined because the pharmacological actions of these alkaloids are similar to that of physostigmine. Ricinine does not exhibit such similarities. It occurs, however, in the castor-oil seed associated with a lipase, and since physostigmine also occurs in a seed, the Calabar

bean, it was thought that if the two alkaloids were found to resemble one another with respect to their inhibition of the activities of enzymes, this might form the basis of an explanation of the functions of alkaloids in general. As will be shown below, ricinine does not inhibit the activity of liver esterase; its possible influence on the activity of ricinus lipase, the enzyme with which it is associated naturally, has not yet been investigated.

EXPERIMENTS WITH METHYL BUTYRATE AS SUBSTRATE.

Technique. The activity of the esterase preparations has been determined by a method essentially similar to that developed by Willstätter and Memmen [1924, 1] for pancreatic lipase and subsequently adapted by the same authors [1924, 2] for use with liver esterase. For convenience of measurement, 1 cc. of substrate (methyl butyrate) was used for each 100 cc. of reaction mixture, as recommended by Bamann [1929]. The general procedure was as follows: 20 cc. of ammonia-ammonium chloride buffer (1 part 2.5 *N* NH_3 : 2 parts 2.5 *N* NH_4Cl ; p_{H} 8.9) contained in a 100 cc. measuring flask were diluted with 65–70 cc. of water, the exact volume depending on the volume of enzyme solution subsequently added, and the mixture brought to 30° in a thermostat. 1 cc. of methyl butyrate was then added and the flask shaken vigorously until the ester had dissolved completely. To the clear solution a measured volume (5 or 10 cc.) of the enzyme preparation was added, the total volume of the mixture brought to 100 cc. by the addition of water, and the whole thoroughly mixed. 20 cc. of the mixture were immediately withdrawn, run into a mixture of 25 cc. of 0.2 *N* hydrochloric acid with 20 cc. of water, and titrated with approximately 0.2 *N* sodium hydroxide, using bromocresol purple as indicator. Similar titrations were made at intervals of 20 minutes. With this procedure and using a suitable quantity of enzyme the amount of hydrolysis was approximately proportional to the time.

The enzyme solution consisted, in the earlier experiments, when ammoniacal extracts of liver powder were employed, of 5 cc. of a mixture of 5 cc. of the extract with 1 cc. of water, the latter being replaced by 1 cc. of a solution of the substance under examination in the experiments designed to test the inhibitory action of this substance. In the later experiments, in which the acidified and dialysed esterase preparation was employed, 5 cc. of enzyme solution were mixed with 5 cc. of ammonia-ammonium chloride buffer (of above composition but diluted 1 in 10), 2 cc. of water or solution added, and 10 cc. of this mixture used for the hydrolysis.

Activity of esterase preparations. The activity of the esterase preparations made from different livers was fairly constant. The following figures, which represent the number of cc. of 0.194 *N* alkali required to titrate the acid liberated in 20 cc. of the reaction mixture in 20, 40 and 60 minutes respectively, using 4.17 cc. of an ammoniacal extract of liver powder in 100 cc. of reaction mixture, may be taken as typical: 2.3, 4.75, 7.25. This result was

obtained on May 19th, 1930, with a fresh extract of a liver powder (L.P. 2) prepared on April 23rd, 1930, and almost identical values were obtained on June 10th, 1930, with a similar extract from the same powder. These facts are recorded in detail because of the following remarkable phenomenon which was observed with an extract of this powder but which has not so far been encountered with other preparations. On June 23rd, 1930, a fresh ammoniacal extract of L.P. 2 was rendered just acid to litmus by the addition of 0.5 *N* acetic acid. The precipitate, which formed slowly, was centrifuged off and the clear solution tested for activity under precisely the same conditions as those employed for the above ammoniacal extract, when the following figures were obtained: 4.45, 8.25, 8.95. Owing to the unexpectedly high activity of this extract the hydrolysis of the substrate was virtually complete in the experimental period of 1 hour, with the result that the reaction did not take the usual linear course. It is therefore not possible accurately to compare the activities of the different extracts of L.P. 2, but it is clear from the figures quoted that the activity of the acidified extract was at least twice as great as that of the ammoniacal extracts previously prepared from the same weight of the same powder. Dialysis of the acidified extract for 4 days was accompanied by an apparent slight diminution in activity, as is shown by the following titration figures: 3.4, 6.7, 8.35; this, however, was mainly due to the dilution which occurred during dialysis. The increase in activity observed on acidification can doubtless be explained by the removal of an inhibitory substance; from the difficulty in reproducing this experiment it appears that a very critical adjustment of conditions, probably of p_H , is necessary for the precipitation of this inhibitor.

Influence of time of contact between inhibitor and enzyme on inhibitory action. Rona and Bach [1920], in their studies on the inhibitory action of atoxyl on various lipases, have shown that the maximum inhibitory effect of this substance is not developed unless it is left for a certain period of time in contact with the enzyme before the addition of the substrate. A number of preliminary experiments having demonstrated that urethanes of the miotine type exert a pronounced inhibitory action on the hydrolysis of methyl butyrate by liver esterase, the influence of this time of contact of the inhibitor with the enzyme was investigated. For this purpose, 1 cc. of a solution of miotine hydrochloride, containing 1.2 mg./cc., was added to each of 7 test-tubes containing 5 cc. of an ammoniacal extract of liver powder. After varying intervals of time 5 cc. of the mixture were employed for a hydrolysis experiment. The results are given in Table I. The figures indicate that, under the conditions employed, the maximum inhibitory action of miotine hydrochloride is not exerted unless it has been in contact with the esterase for 2 hours prior to the addition of the substrate. The difference between the maximum inhibitory action and that produced after contact for 1 hour is, however, very small and almost falls within the limits of experimental error. It has therefore been considered sufficient, when comparing the inhibitory activities of the various

Table I. *Influence on inhibitory action of time of contact of inhibitor with enzyme.*

Substrate: methyl butyrate. Inhibitor: miotine hydrochloride. T=30°.

Time of contact (mins.)	Titration figures (cc. of 0.194 N NaOH required to titrate acid liberated in 20, 40 and 60 mins.)			Percentage inhibition
Control (no inhibitor)	2.5	5.15	7.75	—
0.1	1.8	3.7	5.6	28
15	0.9	1.95	3.0	61
30	0.75	1.5	2.25	71
45	0.6	1.25	1.9	75
60	0.5	1.1	1.7	78
120	0.4	0.9	1.5	81
330	0.4	0.9	—	81

urethanes, to allow the inhibitor to stand in contact with the enzyme for 1 hour before use.

The figures in Table I give some further important information. Miotine is an ester of methylcarbamic acid and might therefore be expected to suffer, like other esters, a more or less rapid hydrolysis when in contact with liver esterase. In the above experiments, however, it is clear that no appreciable hydrolysis has occurred in a period of 5½ hours. In this connection it should be mentioned that, although the hydrolysis experiments were carried out at 30°, the mixtures of miotine and esterase were allowed to stand at room temperature before addition to the reaction flask.

Some experiments on the influence of the time of contact of enzyme and inhibitor on the inhibitory action of the latter were also made under somewhat different conditions. Although the procedure employed in these experiments was not adopted in much of the subsequent work, the results are recorded here since they are not entirely without interest. In the preceding experiments the enzyme and inhibitor were in contact under conditions of concentration widely different from those obtaining in the final reaction mixture. In order to render these conditions more nearly comparable the following modified series of experiments was carried out. A solution of 1.2 mg. of miotine hydrochloride in 1 cc. of water was added to each of a series of flasks containing the diluted buffer mixture and enzyme (ammoniacal extract) previously warmed to 30°. After varying intervals of time, 1 cc. of methyl butyrate was added and rapidly dissolved by shaking, the volume adjusted to 100 cc., and the titrations were performed as before. The results are expressed in Table II. Unfortunately a quantitative comparison cannot be made between the results of Tables I and II since not only were different enzyme preparations employed but the amounts of inhibitor used were slightly different. Nevertheless it is evident that the inhibitory action of miotine hydrochloride is of the same order of magnitude in the two sets of experiments. The most striking point of difference is the definite indication of a destruction of the miotine in the second set after 4 hours' contact with the enzyme. We are inclined, however, to attribute this destruction to the alkalinity of the

Table II. *Influence on inhibitory action of time of contact of inhibitor with enzyme.*Substrate: methyl butyrate. Inhibitor: miotine hydrochloride. $T=30^{\circ}$.

Time of contact (mins.)	Titration figures (cc. of 0.194 N NaOH required to titrate acid liberated in 20, 40 and 60 mins.)			Percentage inhibition
Control (no inhibitor)	1.95	4.00	6.1	—
15	0.7	1.5	2.35	61
30	0.6	1.25	1.7	72
45	0.4	1.0	1.4	77
60	0.4	0.85	1.3	79
120	0.45	1.0	1.6	74
240	0.8	1.65	2.45	60

solution, combined with the fact that the enzyme and inhibitor were maintained at 30° during the whole period of contact, rather than to the action of the esterase.

Inhibitory actions of various urethanes. A number of the urethanes exhibiting miotic activity were examined for inhibitory action under fairly wide ranges of concentration of inhibitor. The results are collected in Table III.

Table III. *Inhibitory action of various urethanes with miotic activity.*Substrate: methyl butyrate. $T=30^{\circ}$. Initial $p_H=8.9$.

In the three instances marked (a), 10 cc. of the extract were employed owing to the diminution in the activity of the liver powder.

The solution used in the experiment marked (b) was prepared by extracting only 1.5 g. of liver powder with 100 cc. of $N/40$ ammonia.

Inhibitor	Liver preparation	Final conc. of inhibitor (mg. per 100 cc.)	Titration figures			Percentage inhibition
<i>m</i> -Miotine HCl	L.P. 2	Control	2.55	5.15	7.8	—
		10	0.2	0.5	0.9	89
		1	0.45	1.0	1.45	81
		0.1	1.25	2.6	4.05	48
		0.01	2.1	4.3	6.55	16
		0.001	2.4	4.8	7.3	6
<i>o</i> -Miotine HCl	L.P. 5 (a)	Control	2.15	4.35	6.5	—
		10	0.55	1.0	1.45	78
		1	1.6	3.3	4.9	25
		0.1	1.8	3.75	5.9	11
<i>p</i> -Miotine HCl	L.P. 2	Control	2.35	4.75	7.1	—
		10	0.0	0.3	0.45	94
		1	0.25	0.55	0.85	88
		0.1	0.9	1.5	2.55	64
		0.01	1.65	3.3	5.0	30
		0.001	2.35	4.5	6.85	4
<i>m</i> -Miotine MeI	L.P. 2	Control	2.45	5.0	7.5	—
		10	0.25	0.5	0.85	90
		1	1.25	2.4	3.75	51
		0.1	2.15	4.4	6.75	11
		0.01	2.15	4.8	7.2	4
		0.001	2.3	4.8	7.3	—
<i>o</i> -Miotine MeI	L.P. 5 (a)	Control	2.0	4.2	6.4	—
		10	0.25	0.7	1.1	83
		1	1.2	2.5	3.6	44
		0.1	2.05	4.1	6.2	3

Table III (*contd.*).

Inhibitor	Liver preparation	Final conc. of inhibitor (mg. per 100 cc.)	Titration figures			Percentage inhibition
<i>p</i> -Miotine MeI	L.P. 5 (<i>b</i>)	Control	1.75	3.4	5.2	—
		10	0.15	0.35	0.65	86
		1	0.85	1.55	2.35	55
		0.1	1.4	3.0	4.5	14
		0.01	1.6	3.25	5.0	—
		0.001	1.45	3.3	5.0	—
HCl of <i>o</i> -urethane (phenyl series)	L.P. 6	Control	2.5	5.1	7.7	—
		10	0.2	0.6	1.0	87
		1	0.6	1.2	1.85	77
		0.1	1.5	3.05	4.75	38
		0.01	2.05	4.15	6.4	17
		0.001	2.2	4.55	7.05	8
HCl of <i>m</i> -urethane (phenyl series)	L.P. 2	Control	2.45	4.85	7.5	—
		10	0.0	0.1	0.3	96
		1	0.35	0.5	0.75	90
		0.1	1.15	2.1	3.25	57
		0.01	1.8	3.55	5.45	27
		0.001	2.1	4.6	6.8	9
HCl of <i>p</i> -urethane (phenyl series)	L.P. 2	Control	2.3	4.75	7.4	—
		10	0.25	0.5	0.85	89
		1	0.7	1.35	2.0	73
		0.1	1.7	2.25	3.95	33
		0.01	2.2	4.4	6.65	11
		0.001	2.2	4.55	7.0	6
MeI of <i>m</i> -urethane (phenyl series)	L.P. 2	Control	2.45	5.1	7.6	—
		10	0.75	1.5	2.4	69
		1	1.7	2.35	3.95	37
		0.1	2.15	4.55	6.9	11
		0.01	2.25	4.75	7.2	5
		0.001	2.35	4.8	7.3	—
MeI of <i>p</i> -urethane (phenyl series)	L.P. 5 (<i>a</i>)	Control	2.1	4.4	6.55	—
		10	0.6	1.15	1.7	74
		1	1.35	2.65	4.0	39
		0.1	2.2	4.3	6.45	—
MeI of <i>o</i> -urethane (benzyl series)	L.P. 2	Control	2.35	4.75	7.3	—
		10	0.2	0.45	0.75	90
		1	1.15	2.3	3.4	53
		0.1	2.3	4.6	7.0	4
		0.01	2.4	4.75	7.25	—

In all these experiments the general technique described earlier was utilised, ammoniacal extracts of desiccated liver powder being employed as esterase solution in each case. Since this work was carried out over an extended period of time, different liver preparations, as indicated in Table III, were necessarily employed owing to the moderately rapid deterioration which such preparations undergo. The results demonstrate clearly the marked inhibitory action which urethanes of the type examined exert upon the activity of liver esterase. A detailed comparison of the figures shows further that, in general, the inhibitory activity of the hydrochlorides of the tertiary bases is greater than that of the corresponding quaternary iodides. While this is not universally the case, particularly in the higher concentrations, the inhibitory activities of the methiodides consistently decrease, with increasing dilution of the inhibitor, at a much greater rate than do those of the hydrochlorides of the tertiary

bases, with the result that at low concentrations the latter are uniformly more active. This difference between the two types of salts is emphasised because it is probably related to the mechanism of the inhibitory action.

Examination of other types of compounds for inhibitory activity. The following compounds were tested for inhibitory activity under conditions similar to those used in the preceding experiments: methylurethane of choline iodide, hydrochloride of α -*m*-hydroxyphenylethyldimethylamine, pilocarpine nitrate, arecoline hydrobromide, atropine hydrochloride and ricinine. The titration figures need not be recorded in detail since in no case was any inhibitory action observed in the highest concentrations examined, namely, 10 mg. per 100 cc. of reaction mixture.

Influence of p_H on inhibitory action. The technique adopted for the experiments in which methyl butyrate was employed as substrate is not very suitable for the investigation of p_H effects. Even when relatively large amounts of buffer are employed, the acid liberated during the hydrolysis is sufficient to cause some change in p_H ; if the solution is thereby brought to the acid side of neutrality, some loss in the activity of the esterase may occur with the result that the hydrolysis of the substrate will no longer proceed at a constant rate. This effect was actually observed in a number of experiments. In the experiment quoted in Table IV, which was carried out at p_H 6.8, proportionality between the amount of hydrolysis and the time was maintained;

Table IV. *Influence of p_H on the inhibitory action of miotine hydrochloride.*

Substrate: methyl butyrate. $T=30^\circ$.

p_H	Esterase preparation	Conc. of inhibitor (mg. per 100 cc.)	Titration figures			Percentage inhibition
6.8 (phosphate buffer)	Acidified ammoniacal extract of L.P. 5	Control	1.65	3.4	5.0	—
		10	0.5	1.25	2.0	60
		1	1.45	2.9	4.35	13
		0.1	1.6	3.2	4.85	3
		0.01	1.75	3.4	4.95	—
		0.001	1.7	3.5	5.15	—
8.9 (ammonia buffer)	Ammoniacal extract of L.P. 5	Control	1.85	3.8	5.75	—
		12	0.05	0.25	0.45	92
		1.2	0.35	0.75	1.15	80
		0.12	1.0	2.25	3.5	39
		0.012	1.65	3.3	4.9	15
		0.0012	1.85	3.6	5.45	5

this experiment therefore serves to illustrate the general influence of p_H on the inhibitory action of miotine hydrochloride. For comparison, the results of an experiment at p_H 8.9 carried out under similar conditions are included in the same table. The technique described in connection with the experiments quoted in Table II was employed for both experiments. The phosphate buffer used consisted of a mixture of equal parts of $M/3$ Na_2HPO_4 and $M/3$ NaH_2PO_4 , 70 cc. being employed in each 100 cc. of reaction mixture. When this buffer was employed, phenolphthalein was used as indicator for the titrations.

Unfortunately the enzyme solutions employed in these experiments, although prepared from the same liver powder, were of different degrees of

purity; the results are not therefore strictly comparable. Nevertheless, two important conclusions can be drawn from these experiments: firstly, the inhibitory action of miotine hydrochloride is smaller on the acid than on the alkaline side of neutrality; secondly, the inhibitory action falls off with decreasing concentration of inhibitor much more rapidly in acid than in alkaline solution.

Comparison of the inhibitory activities of isomeric urethanes. The urethanes which have been shown above to inhibit the hydrolysis of methyl butyrate by liver esterase are members of three series of isomeric compounds, with their corresponding methiodides. The results do not, however, necessarily indicate the relative inhibitory activities of the position isomerides within a given series or even of members of different series, for, as previously mentioned, the inhibitory activities of the various compounds were examined on different esterase preparations, and since the latter were made from different liver powders at varying times after their preparation they possessed different activities and probably contained varying amounts of natural inhibitors. On the assumption that any natural inhibitors which may be present in the enzyme preparation enter into competition with the urethanes, it is clear that in experiments designed to compare the activities of different compounds it is not sufficient to employ the same esterase activity; the comparison can only be regarded as valid in those cases where the same esterase extract has been employed. Some experiments, recorded in Table V, have consequently been carried out in which a comparison has been made of the inhibitory activities of the members of each series of urethanes towards the same enzyme preparation. Finally, the hydrochlorides of the *m*-isomerides of the three series have been compared under the same conditions as a measure of the relative activities of the three series of compounds. In all cases the ammoniacal extracts of the liver powders were purified by acidification with acetic acid followed by dialysis.

From the magnitude of the titration figures it is evident that the percentage inhibition shown in the last column of Table V must be subject to an error of a few per cent. Bearing this in mind, the more significant results revealed by a study of this Table may be summarised as follows.

Miotine series. The inhibitory activities of the hydrochlorides of the *m*- and *p*-isomerides are identical, that of the *o*-isomeride being considerably smaller; the inhibition produced by the latter is, in fact, slightly smaller than that caused by the two former when present in one-tenth of its concentration. No very great difference can be observed between the activities of the methiodides, the *m*- and *p*-isomerides showing about equal activity and the *o*-compound exhibiting a slightly greater effect. It is noteworthy and exceptional that the inhibitory activity of the hydrochloride of the *o*-isomeride is somewhat smaller than that of its methiodide in the same molar concentration.

Phenyl series. The activities of the hydrochlorides are definitely in the order $m > p > o$. The methiodides follow the same order; the *m*- and *p*-compounds show, however, practically identical activities.

Table V. *Comparison of inhibitory activities of various urethanes.*Substrate: methyl butyrate. T=30°. Initial $p_H=8.9$.

Inhibitor (methy lurethane)	Esterase preparation	Final conc. of inhibitor (molar $\times 10^{-3}$)	Titration figures			Percentage inhibition
<i>Miotine series:</i>						
	L.P. 6	Control	1.75	3.5	5.3	—
<i>m</i> -HCl		1/30	0.25	0.65	1.05	80
		1/300	1.15	2.4	3.7	30
<i>p</i> -HCl		1/30	0.4	0.8	1.2	77
		1/300	1.25	2.45	3.85	27
<i>o</i> -HCl		1/3	0.75	1.55	2.3	57
		1/30	1.5	2.95	4.5	15
<i>m</i> -MeI		1/3	0.7	1.35	1.9	63
		1/30	1.65	3.2	4.9	8
<i>p</i> -MeI		1/3	0.75	1.15	1.85	65
		1/30	1.4	2.9	4.4	17
<i>o</i> -MeI		1/3	0.4	0.8	1.25	77
		1/30	1.45	2.9	4.3	19
<i>Phenyl series:</i>						
	L.P. 6	Control	1.6	3.35	5.1	—
<i>m</i> -HCl		1/300	0.15	0.45	0.7	86
<i>p</i> -HCl		"	0.8	1.65	2.6	49
<i>o</i> -HCl		"	1.0	2.05	3.2	37
<i>m</i> -MeI		1/30	0.9	1.75	2.55	52
<i>p</i> -MeI		"	0.75	1.6	2.55	50
<i>o</i> -MeI		"	1.3	2.6	4.0	22
<i>Benzyl series:</i>						
	L.P. 7	Control	2.4	4.75	6.85	—
<i>m</i> -HCl		1/300	0.8	1.75	2.55	63
<i>p</i> -HCl		"	1.35	2.75	3.95	42
<i>o</i> -HCl		"	1.85	3.6	5.7	17
<i>m</i> -MeI		1/30	1.45	2.8	4.3	37
<i>p</i> -MeI		"	0.75	1.5	2.25	67
<i>o</i> -MeI		"	1.25	2.55	3.85	44
<i>All series:</i>						
	L.P. 7	Control	2.15	4.55	6.75	—
<i>m</i> -Benzyl-HCl		1/3000	1.95	3.8	5.85	13
<i>m</i> -Phenyl-HCl		"	1.15	2.45	3.6	47
<i>m</i> -Miotine-HCl		"	1.5	3.1	4.7	30
	L.P. 7	Control	2.4	4.75	6.85	—
<i>m</i> -Benzyl-HCl		1/300	0.8	1.75	2.55	63
<i>m</i> -Phenyl-HCl		"	0.1	0.35	0.45	93
<i>m</i> -Miotine-HCl		"	0.35	0.6	0.8	88

Benzyl series. In this series the order for the hydrochlorides is $m > p > o$, and for the methiodides $p > o > m$.

Comparison of the three series. The inhibitory activities of the hydrochlorides of the *m*-isomerides in equimolar concentrations are definitely different, the order being phenyl > miotine > benzyl.

The results in Table V may be further utilised in order to direct attention to the varying activities of the urethanes with different esterase preparations. Thus, to take the most marked case, the concentrations of miotine hydrochloride required to inhibit the acidified and dialysed extracts of two esterase preparations (L.P. 6 and L.P. 7) to exactly the same extent (30 %) are in the ratio 1:10, the extract from L.P. 6 requiring a final concentration of 1/300,000 *M* and that from L.P. 7 one of 1/3,000,000 *M*. The different responses of these enzyme preparations are doubtless related to their histories, which are as follows: the liver powder L.P. 6 was prepared on January 9th,

1931. An ammoniacal extract of this was made on January 14th and its activity tested by the standard procedure used in this investigation, when the following titration figures were obtained: 2.3, 4.6, 7.0. Practically identical values were obtained on the same day following the acetic acid treatment. The acid solution was then dialysed for 5 days, the dialysed solution giving, on January 19th, titration figures of 2.05, 4.05, 6.05. The diminution in activity shown by these figures is only apparent and can be accounted for by the slight dilution which the extract had undergone during dialysis. On the following day, however, the titration figures for the dialysed extract had fallen to 1.75, 3.5, 5.3. Thus, in 24 hours the activity had diminished about 12 %, and it was this extract with lowered activity which was employed for the comparison of the inhibitory activities of the urethanes of the miotine series. We are of opinion that this loss in activity can be attributed to the acidity caused by the relatively long period of dialysis, and we have found that, with the highly permeable collodion bags which we employ, such losses consistently occur when the process of dialysis is extended beyond 3 or, at most, 4 days.

The preparation L.P. 7 was made on February 18th, 1931. An ammoniacal extract was prepared on February 20th, and this was acidified and dialysed until February 23rd. In view of the fact that this extract, which was more sensitive to miotine hydrochloride than the one referred to above, proved to be stable, it appears probable that the presence of inactivated esterase in the enzyme solutions diminishes the inhibitory activity of urethanes of the type under discussion.

EXPERIMENTS WITH TRIBUTYRIN AS SUBSTRATE.

Technique. In order to follow the hydrolysis of tributyrin by liver esterase the stalagmometric method devised by Rona and Michaelis [1911] and subsequently employed by Willstätter and Memmen [1923] in connection with pancreatic lipase has been utilised. A straight stalagmometer with a water value of 81 at 20° was employed throughout the experiments.

In using this method it is frequently considered sufficient to work at room temperature without any special temperature control. At the time our experiments were carried out, however, the temperature of our laboratory fluctuated considerably and tended to be somewhat low, falling at times to below 10°. At such temperatures the activity of the enzyme is naturally considerably diminished, and it was therefore considered advisable to use a thermostat. The reaction mixture, contained in a small beaker, was placed in a bath at 20° and the stalagmometer supported directly over the beaker by means of a suitable stand. By raising the beaker and applying gentle suction at the top of the stalagmometer the mixture could be drawn into the latter. The beaker was then again lowered and the drops from the stalagmometer allowed to fall into it. By this arrangement fluctuations in the temperature of the liquid actually in the stalagmometer could not be prevented, but the

bulk of the reaction mixture was maintained constantly at 20°, thereby permitting a uniform action of the esterase.

The remaining procedure was as follows: 50 cc. of a saturated solution of tributyrin were mixed with 5 cc. of $M/3$ phosphate buffer (p_H 7.9 or 6.8) and the mixture brought to 20°. 1 cc. of a mixture of 1 cc. of esterase solution with 1 cc. of water in the control experiment, or of 1 cc. of esterase solution with 1 cc. of a solution of the urethane in the experiments designed to measure the inhibitory action of the latter, was then added and the drop number immediately measured. Two further counts were made at intervals of 20 minutes. As in the experiments with methyl butyrate, the enzyme was left in contact with the urethane for 1 hour before mixture with the substrate.

All the esterase solutions employed were prepared from ammoniacal extracts of liver powders acidified with acetic acid; in some cases the extracts were dialysed. In each case, 1 to 5 cc. of the enzyme preparation, the actual volume depending on its activity, were mixed with 2 cc. of $M/3$ phosphate buffer of the same p_H as that employed in the hydrolysis experiments, and diluted to 10 cc. with water.

Willstätter and Memmen [1923] have pointed out that commercial preparations of tributyrin frequently contain an impurity, with a saponification number corresponding with that of dibutyrin, which renders them unsuitable for use, without extensive purification, in the stalagmometric estimation of lipolytic activity. As a criterion of purity they recommend the preparation of two saturated aqueous solutions of the sample, one by shaking 5 to 10 g. of the tributyrin with 200 cc. of water, and the other by shaking 3 drops with a similar volume of solvent. If the tributyrin is pure, the two solutions should give the same drop number. The experiments recorded in this communication have been made with one delivery of B.D.H. tributyrin. When this was submitted to the test described above, the two saturated aqueous solutions gave identical drop numbers. According to the above criterion the sample was therefore quite pure and was employed in our experiments without further treatment.

Influence of p_H on inhibitory action. The study of the inhibition of the hydrolysis of tributyrin by liver esterase by urethanes of the type considered in this series of communications has been carried out partly with the object of ascertaining if the inhibitory action of such urethanes is independent of the type of substrate employed and partly because, owing to the sensitivity of the stalagmometric method, only relatively small amounts of tributyrin need be employed as substrate. This renders the method eminently suitable for a study of the influence of p_H on the inhibitory process, since no difficulty is experienced in buffering the small amounts of acid which are liberated by the action of the enzyme. The inhibitory activities of the hydrochlorides and methiodides of each member of the three series of urethanes mentioned above have therefore been examined, using a range of concentrations of inhibitor, at two different acidities, namely, p_H 7.9 and 6.8. Table VI gives some typical

Table VI. *Influence of p_H on the inhibitory action of various urethanes.*Substrate: tributyrin. $T=20^\circ$.

Inhibitor	Final conc. of inhibitor (mg. per 56 cc.)	p_H 7.9 Decrease in drop number in		p_H 6.8 Decrease in drop number in	
		20 mins.	40 mins.	20 mins.	40 mins.
<i>m</i> -Miotine HCl	0	14	25	12	20
	10	0	1	1	1
	1	1	1	5	8
	0.1	0	1.5	9	16
	0.01	8	17	10	18
	0.001	13	24	—	—
<i>m</i> -Miotine MeI	0	9.5	19	9	18
	10	0	1	5.5	11
	1	2	4	8	16
	0.1	7.5	15.5	—	—
HCl of <i>p</i> -urethane (benzyl series)	0	10	20	11	20
	10	0	0	0	0
	1	0	0.5	0.5	1
	0.1	1.5	3	9	15
	0.01	8	15	9	17
MeI of <i>p</i> -urethane (benzyl series)	0	9.5	18.5	8.5	15.5
	10	0	0	0.5	1.5
	1	1	1.5	2	3.5
	0.1	5	10	8	16
HCl of <i>m</i> -urethane (phenyl series)	0	9.5	17.5	7	14.5
	1	1	1	1	1.5
	0.1	1	1.5	0	1
	0.01	0	1	1	2
	0.001	1	3	2	4
	0.0001	4	9	7	14
MeI of <i>m</i> -urethane (phenyl series)	0	9	19	8.5	15.5
	10	0	0	2	2
	1	0	0	5	9
	0.1	2	4	—	—
	0.01	7	14	—	—

results. Similar results were obtained with the isomerides of the urethanes mentioned in Table VI; since, however, the same enzyme preparation was not employed with all the compounds, these results do not necessarily indicate correctly the relative inhibitory activities of the various compounds and are not therefore quoted. It should be mentioned in connection with these results that the same enzyme preparation was always employed in the experiments with a given urethane, although, owing to the smaller activity of the esterase at p_H 6.8 a greater volume of the enzyme preparation was used in the more acid solution. Thus in the experiments with the hydrochloride of the methylurethane of *p*-hydroxybenzyltrimethylamine in which the enzyme showed the same activity, corresponding with a decrease of 20 in the drop number in 40 minutes, at the two acidities, 33 % more enzyme was employed at p_H 6.8 than at 7.9. In every instance quoted in Table VI the inhibitory activity of a given urethane is smaller at p_H 6.8, *i.e.* on the acid side of neutrality, than at p_H 7.9, and the same effect was consistently observed with the remaining urethanes. As an example, the results with the hydrochloride of the methylurethane of *p*-hydroxybenzyltrimethylamine, mentioned above, may be taken,

since identical esterase activities, as shown by the control experiments, were employed at the two acidities. In this case, the amounts of inhibitor required to diminish the activity of the enzyme by 25 %, *i.e.* to diminish the decrease in drop number from 20 to 15, were 0.01 and 0.1 mg. at p_H 7.9 and 6.8 respectively. The inhibitory activity of the urethane at p_H 6.8 was thus only one-tenth of that which it exhibited at p_H 7.9. Ratios of the same order of magnitude were obtained with the other urethanes. To what extent the partial inactivation of the enzyme which takes place in acid solution, and which necessitated the employment of larger volumes of the preparation at p_H 6.8, contributes to this result cannot at present be determined.

Attention should further be directed to the results obtained with the hydrochloride of the methylurethane of *m*-dimethylaminophenol. Reference to Table VI will show that 0.0001 mg. of this substance produced, at p_H 7.9, an inhibition of the activity of the liver esterase of approximately 50 %. Since, in the final reaction mixture, the inhibitor was contained in a volume of about 50 cc., the final concentration of this substance required to diminish the activity of the esterase by this amount was about 1:500,000,000. This is the greatest activity which has been observed with any of the urethanes so far examined. It agrees with other results obtained with the same urethane, which has, in fact, proved to be the most active inhibitor amongst the three series of compounds investigated.

Comparison of the inhibitory activities of isomeric urethanes. In order to obtain a direct comparison of the activities of the members of a given series of urethanes, it was necessary, for reasons stated in connection with the similar experiments carried out with methyl butyrate as substrate, to measure the inhibitory activities on the same enzyme preparation. The results are recorded in Table VII. Different esterase solutions were employed for the three series, so that the figures recorded do not necessarily give a comparison of the activities of members of different series. In the benzyl and phenyl series, an interval of some days elapsed between the experiments with the hydrochlorides and methiodides. The enzyme had meanwhile undergone slight deterioration, as shown by the figures for the second control.

The order of inhibitory activity of the various isomerides on liver esterase, using tributyrin as substrate, is, according to the results of Table VII as follows.

Miotine series. The hydrochlorides do not show large differences in activity. Nevertheless they appear to be definitely in the order $m > o > p$. With the methiodides, which are considerably less active, the *o*-compound exhibits the greatest activity; the *m*- and *p*-isomerides are, within the limits of experimental error, equally active.

Benzyl series. The hydrochlorides of this series show greater differences in activity, the order being quite definitely $o > m > p$. The methiodides again show little difference in activity; the order $p > o > m$ is indicated by the results.

Table VII. *Comparison of the inhibitory activities of isomeric urethanes.*

Substrate: tributyrin. T = 20°. $p_H = 7.9$.				
Inhibitor	Final conc. of inhibitor (molar $\times 10^{-7}$)	Decrease in drop number in		Percentage inhibition
		20 mins.	40 mins.	
<i>Miotine series:</i>				
	Control	11	22	—
<i>m</i> -HCl	40	0	2	91
"	4	5	10	56
<i>p</i> -HCl	40	3.5	7.5	68
"	4	6	12	47
<i>o</i> -HCl	40	1	3.5	86
"	4	5	10.5	52
<i>m</i> -MeI	400	2	3	87
<i>p</i> -MeI	400	2	4	83
<i>o</i> -MeI	400	0	1.5	91
<i>Benzyl series:</i>				
	Control	12	23	—
<i>m</i> -HCl	400	1	2.5	87
"	40	8	16	30
<i>p</i> -HCl	400	4	8.5	61
"	40	10	20	13
<i>o</i> -HCl	400	0	1	96
"	40	3	6	84
	Control	11.5	21	—
<i>m</i> -MeI	400	7	15	29
<i>p</i> -MeI	400	6	12	43
<i>o</i> -MeI	400	6.5	13.5	38
<i>Phenyl series:</i>				
	Control	13	27	—
<i>m</i> -HCl	4	1	2	90
<i>p</i> -HCl	4	5	10	63
<i>o</i> -HCl	4	5	10.5	59
	Control	11.5	21	—
<i>m</i> -MeI	40	8	16	24
<i>p</i> -MeI	40	8	16	24
<i>o</i> -MeI	40	3	7	67

Phenyl series. In this series the *m*-hydrochloride is considerably more active than the *o*- and *p*-compounds, which can scarcely be distinguished from one another in activity. Of the methiodides, the *o*-compound is the most active, the *m*- and *p*-isomerides possessing the same activity. It should be noted that the order of inhibitory activity of both the hydrochlorides and methiodides of this series is identical with that of the miotine series.

DISCUSSION.

The foregoing results have established definitely that urethanes of the type which have been shown in earlier papers to possess very specific physiological properties also possess the common property of inhibiting in low concentration the activity of liver esterase. Of the nine urethanes examined, all exhibit this property to a marked degree, and not only do they do so in the form of their hydrochlorides but their methiodides are similarly, although usually somewhat less, active. It appears legitimate to conclude that a relationship exists between chemical constitution and inhibitory activity towards liver esterase analogous to that between constitution and physiological action in the same group. In conformity with this conclusion, the methylurethane of choline

iodide, a substance containing many structural features in common with the active urethanes but lacking the essential phenyl group, is inactive. Similarly, the hydrochloride of α -*m*-hydroxyphenylethyldimethylamine, the phenol from which miotine is prepared and which contains the phenyl and basic but not the urethane group, produces no inhibition in relatively high concentration.

Whether these results hold for the enzyme, present in blood-sera from certain species and in tissue extracts, which is normally responsible for the destruction of acetylcholine, has not yet been determined. Nevertheless, the fact that urethanes of the type which possess physiological properties similar to those of physostigmine also inhibit, in minute concentration, the hydrolysis by liver esterase of a simple ester such as methyl butyrate, affords substantial support to the view that the above-mentioned serum-enzyme, the activity of which towards acetylcholine has been shown by Loewi and his co-workers and by Matthes to be inhibited by physostigmine, is a true esterase such as is present in the liver and is not one which acts specifically towards acetylcholine.

It is not proposed to discuss in detail the question, which now arises, as to whether the physiological activity of urethanes of the miotine type is an indirect result of the inhibitory action which they exert upon esterases, since this problem has already been considered from many aspects by White and Stedman [1931], who have pointed out that while much of the activity of miotine, in particular its toxicity, may probably be attributed to its inhibition of the destruction of acetylcholine, other mechanisms are possibly also involved. One fact, which supports the suggestion, originally made by Loewi and Navratil [1926], that the action of physostigmine on the heart is due to its inhibitory action on the acetylcholine-destroying enzyme, may, however, be mentioned. It has been shown above that arecoline, in relatively high concentration, does not inhibit the activity of liver esterase. Now this alkaloid belongs, pharmacologically, to the physostigmine group of drugs and might therefore be expected to resemble physostigmine in its behaviour towards esterases. That it does not do so is in complete accord with some unpublished pharmacological experiments by Dr A. C. White, to whom we are indebted for information regarding his results, according to which arecoline, unlike physostigmine, does not potentiate the action of acetylcholine on the vagus.

It is not to be expected, even on the assumption that the whole of the physiological activity of urethanes of the miotine type is attributable to the resulting accumulation of acetylcholine in the organism, that an exact parallelism will exist between the magnitude of their physiological activities and that of their inhibitory activities towards esterases, for the drugs will be subject to many influences in the organism which are eliminated in *in vitro* experiments with enzymes. Thus, a property of a drug, such as adsorbability, which may render it particularly efficient in inhibiting the activity of an esterase, may actually prevent it from reaching the enzyme in the organism. Similarly, stability will be of greater influence in *in vivo* than in *in vitro* experiments, since in the former case the drug is subjected to the action of

more destructive agents. That no such parallelism does, in fact, exist follows from our experiments; the order of the inhibitory activity of various urethanes towards liver esterase in no way corresponds with the previously published provisional order for their mitotic activities.

Perhaps the most interesting questions which call for consideration in connection with our experiments are those concerning the nature of the inhibitory action and its bearing on the mechanism of the normal action of esterases. Of a group of compounds possessing a common structural feature, every member which has been examined has been found to inhibit, in minute concentration, the activity of liver esterase. These inhibitory substances, like the normal substrate of the enzyme, are esters. Nevertheless they are either not attacked, or are only hydrolysed extremely slowly, by the esterase. The suggestion thus arises that the urethanes combine with the esterase in the same way and by means of the same mechanism as do simple esters, but that the affinity between urethane and enzyme is enormously greater than that between simple ester and enzyme. Hence the esterase-urethane compound is formed preferentially and, owing to the inability of the enzyme to decompose the urethane, the hydrolysis of the normal substrate is prevented. The latent period which occurs in the hydrolysis of ethyl mandelate by liver esterase when esters of certain keto-acids are present has been similarly explained by Willstätter, Kuhn, Lind and Memmen [1927], and further experiments in support of this explanation have been published by Bamann and Schmeller [1930]. On the above basis the relative inhibitory activities of the various urethanes will be a measure of their affinities for the esterase, and any regularity which is observed between inhibitory and other properties should give a clue as to the nature of the affinity between the urethanes and esterase, and hence of the nature of the forces normally responsible for the formation of enzyme-substrate compound. Now, the following regularities have, in fact, been noted in our experiments.

(1) In a given series of urethanes the isomerides show inhibitory activities which, although usually different, are of the same order of magnitude.

(2) Of the three series examined, the hydrochlorides of the phenyl series are outstanding with respect to their high inhibitory activities.

(3) The methiodides are, in general, considerably less active than the hydrochlorides of the corresponding tertiary bases.

(4) The inhibitory activity of a given urethane is considerably smaller at p_{H} 6.8 than in slightly alkaline medium.

We believe that these results can be best interpreted by assuming that the affinities in question are, in the first instance, of the nature of adsorptive forces. If we postulate (a) that the urethane group confers high adsorbabilities on the inhibitory substances, (b) that the adsorbability and therefore inhibitory activity is increased by any factor which tends to lower the solubility, (c) that the free bases and not the salts are the active inhibitors, our reasons for this belief will be at once clear. In accordance with postulates (b) and (c),

any factor which tends to convert the readily soluble salts into the sparingly soluble bases, thus diminishing the solubility and increasing the concentration of the active constituent, would be expected to assist adsorption and hence increase inhibitory activity. Increase in alkalinity is such a factor, and as shown above this increases the inhibitory activity. Similarly diminishing the basic strength of the compounds would constitute another such factor; corresponding with this the compounds which are the weakest bases, namely, the methylurethanes of the phenyl series, show the greatest inhibitory activity. Further, solubility is increased by converting the tertiary bases into their quaternary ammonium salts, the free bases of which are readily soluble in water. This should diminish adsorbability and hence inhibitory activity, as is actually the case.

Nevertheless, adsorption is clearly not the only process involved. The possession of surface activity of a high order will not necessarily confer upon a compound the marked inhibitory activity shown by the above urethanes. Moreover, it has been shown by Willstätter, Kuhn and Bamann [1928] that esters of *d*- and *l*-mandelic acids exhibit different affinities towards esterases, while Murray and King [1930] have demonstrated that certain enantiomorphous alcohols inhibit the activity of liver esterase to different degrees. Optical isomerides would be expected to possess identical adsorbabilities; it is therefore almost certain that some factor other than adsorption is also involved. We are of opinion that, following adsorption, the inhibitor enters into especial relation with the enzyme, the exact nature of which is not at present clear, and it is probably this second stage of the combination process which is the cause of the different inhibitory activities of isomeric urethanes. One fact which has been elicited in our experiments tends to support this view. In examining the inhibitory activities of the various urethanes towards liver esterase, it has been found that the general effect of those factors which we have interpreted above as exerting their influence by producing changes in adsorbability is the same whether the substrate is methyl butyrate or tributyrin. The relative activities of isomeric urethanes vary, however, with the nature of the substrate, a result which cannot be explained on the basis of adsorption.

SUMMARY.

1. Urethanes of the type which have previously been shown to possess physiological properties of the same kind as physostigmine have been found to possess the common property of inhibiting the activity of liver esterase. A relationship thus exists between chemical constitution and inhibitory action towards liver esterase analogous to that between constitution and physiological activity in the same group.

2. This inhibitory action is exerted in high dilution. Thus, the hydrochloride of the *m*-dimethylaminophenyl ester of methylcarbamic acid, which

is the most active urethane examined, produced an inhibition of 50 % in the hydrolysis of tributyrin by liver esterase when in a final concentration of about 1:500,000,000. The same urethane inhibited the hydrolysis of methyl butyrate to about the same extent when in a final concentration of $\frac{1}{3} \times 10^{-6} M$, which corresponds with one of about 1:13,000,000.

3. Three series of isomeric urethanes, which have been termed the methyl-urethanes of the phenyl, benzyl and miotine series respectively, have been examined for inhibitory activity in the forms both of their hydrochlorides and methiodides. The relative activities of the isomerides, when methyl butyrate is employed as substrate, are as follows.

Hydrochlorides. Phenyl series: $m > p > o$; benzyl series: $m > p > o$; miotine series: $m = p > o$.

Methiodides. Phenyl series: $m \geq p > o$; benzyl series: $p > o > m$; miotine series: $o > p \geq m$.

Using tributyrin as substrate the following orders, which differ from those above, are obtained:

Hydrochlorides. Phenyl series: $m > p \geq o$; benzyl series: $o > m > p$; miotine series: $m > o > p$.

Methiodides. Phenyl series: $o > p = m$; benzyl series: $p > o > m$; miotine series: $o > p = m$.

4. The inhibitory activity is, in every case, greater in slightly alkaline medium than at p_H 6.8.

5. In general, the methiodides exert a considerably smaller inhibitory effect than do the hydrochlorides.

6. The relative inhibitory activities of the various urethanes do not correspond with their relative miotic activities. It is pointed out that such correspondence is not to be expected even on the assumption that the whole of the physiological activity of the urethanes is due to their inhibitory action on esterases.

7. The mechanism of the inhibitory action is discussed.

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