In comparisons with simple esters, it must be remembered that the alcohol groups are different, two being primary, and one secondary. The rates of hydrolysis of esters containing these different groups are undoubtedly different and would have to be considered in accurate comparisons. The use of monacetin or of the symmetrical diacetin in these cases would, however, remove this objection.

Summary.

Solutions of methyl alcohol, ethyl alcohol, and acetone exerted inhibiting actions on the hydrolysis of ethyl butyrate by a castor bean lipase preparation under comparable conditions, the amount of inhibition increasing with the concentration. Solutions of glucose and glycerol showed no inhibiting action except perhaps in the most concentrated solution.

The view is suggested that simple esters exert an inhibiting action on lipase similar to that exerted by simple alcohols, and that higher esters (such as the glycerol esters) exert less inhibiting action similar to that exerted by glycerol.

The lipolytic activity of the castor bean preparation was tested with solutions of methyl acetate, ethyl acetate, ethyl butyrate, and glyceryl triacetate (triacetin) of considerable ranges of concentration, and the results were correlated and explained by the aid of the theory outlined.

Possible applications of the theory to the action of other hydrolyzing agents on esters compared with the action of lipase, to lipases of animal origin, and to the effect on the determination of the activity of lipase under various conditions of added substances, were mentioned.

This theory, together with the specific actions of various groupings in the (presumably) protein molecule of lipase on the hydrolysis of esters as demonstrated in the second paper,[1] will probably explain most, if not all, of the selective actions of the lipases.

Finally, in agreement with A. E. Taylor, the use of triacetin as substrate for testing lipolytic activity is recommended.

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STUDIES ON ENZYME ACTION. VII. A FURTHER STUDY OF THE HYDROLYTIC ACTION OF AMINO ACIDS ON ESTERS.

BY MARSTON LOVELL HAMLIN.

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1. Introduction.

In a previous paper of this series[2] Falk and Nelson described the lipolytic action of certain amino acids on several esters. The work was undertaken with the hope of throwing light on the character of lipases

[1] *Loc. cit.* Dr. M. L. Hamlin is developing further in this laboratory the lipolytic actions of aminoacids. Cf. the following article.

[2] THIS JOURNAL, 34, 828, (1912).

and, indeed, of finding out whether lipolytic activity "is limited to a single substance or group of substances." They found that certain amino acids and polypeptides aided the hydrolysis of methyl acetate, ethyl butyrate and olive oil, and that this action was to some extent selective in character. The work then begun has been extended along broader lines, and in this paper are reported the results of studies of the lipolytic action of glycine, on methyl and ethyl acetates and ethyl butyrate in various solvents, and of glycine, glutamic acid and aspartic acid on methyl, ethyl, glyceryl tri-, and phenyl acetates, ethyl butyrate, and ethyl and phenyl benzoates in water.

2. Experimental Part.

The following sets of experiments were carried out:

The lipolytic action of:	*was tested on:*
1. Different amounts of glycine.	Different amounts of methyl acetate and ethyl butyrate.
2. Glycine, glutamic acid, aspartic acid.	Methyl, ethyl, glyceryl tri-, phenyl acetates, ethyl butyrate, and ethyl and phenyl benzoates.
3. Glycine in 0.2 *M*, 0.5 *M*, 2 *M*, NaCl and $MgSO_4$, and 0.2 *M* and 0.5 *M* Na_2SO_4 solutions.	Methyl acetate and ethyl butyrate.
4. Glycine + $^1/_2$, 1 and 2 equivalents acetic acid.	Methyl acetate and ethyl butyrate.
5. Glycine + HCl, two different solutions of known hydrogen ion concentration.	Methyl and ethyl acetates and ethyl butyrate.

Experimental Method.—When glycine was tested in water or in salt solutions, separate portions were weighed out to one-tenth of a milligram for each measurement, and dissolved in 25 cc. water or salt solution in a 150 cc. Erlenmeyer flask, the ester was added from a pipet, the flask stoppered and let stand in a thermostat at 38°–40° for twenty-four hours. Since, for each result, three measurements were necessary, ester and glycine blanks were also made up, containing like amounts of ester and solvent or like amounts of solvent and weighed amounts of glycine. At the end of the given time, formaldehyde, neutralized toward phenolphthalein was added to each flask[1] and after ten to fifteen minutes' standing, the contents were titrated with a standard solution of approximately 0.1 normal sodium hydroxide. From the result thus obtained for glycine, ester and solvent, was subtracted the value found for ester and solvent, and the remainder was multiplied by ten times the normality of the alkali and divided by ten times the weight in grams of the amino acid; from this was subtracted the amount in cubic centimeters of 0.1 normal alkali required by 0.1 gram glycine (found from the glycine blanks), and the result was the amount of 0.1 normal alkali required to neutralize the acid set free by the action of one decigram of glycine. The figures in all the

[1] S. P. L. Sörensen, *Biochem. Z.*, 7, 45.

following tables showing the action of the amino acids, are the means of the indicated number of determinations in which each separate result is given a weight proportional to the amount of amino acid used.

Owing to the fact that glutamic and aspartic acids dissolve in water comparatively slowly at 38°, standard solutions of these substances, containing approximately four grams per liter, were used when their lipolytic action was tested. Standard solutions of glycine, too, were used in the experiments with acetic and hydrochloric acids. These solutions were standardized by titrating carefully measured volumes of the solutions, and weighed samples of the respective acids dissolved in water, in the manner already described. For each test 25 cc. were used and amino acid solution blanks were made up with each set to check the standard.

In the course of preliminary work, it was found that often 5 cc. formaldehyde were not sufficient for the amounts of glycine present, and consistently low results were obtained. In all the work summarized below, 14–15 cc. of neutralized 40% formaldehyde were used in titrating glycine solutions, and about 30 cc. in titrating the other amino acids. Eight titrations of weighed amounts of glycine gave a weighted mean:

0.1 gram glycine ≡ 13.17 cc. 0.1 *N* NaOH (98.9% theoretical).

A. D. = 0.023.[1]

Twelve titrations of glutamic acid gave:

0.1 gram glutamic acid ≡ 13.08 cc. 0.1 *N* NaOH (96.2% theoretical).

A. D. = 0.016.

Two titrations of aspartic acid gave:

0.1 gram aspartic acid ≡ 14.30 cc. 0.1 *N* NaOH (95.2% theoretical).

A. D. = 0.039.

Experimental Results.—The five sets of experiments outlined above are summarized in the following tables: The object of the first set, in which the action of amounts of glycine between 0.08+ and 0.2+ gram was tested on different amounts of methyl acetate and ethyl butyrate, was to find the most convenient amount of ester to use in testing, and to determin whether the action was closely proportional to the amount of glycine used. The results are given in Table I; in the second, third and fourth columns, respectively, are given the number of determinations,

TABLE I.—ACTION OF GLYCINE ON METHYL ACETATE AND ETHYL BUTYRATE.

Amount of ester. Cc.	Methyl acetate.			Ethyl butyrate.		
	No. of det.	A. D.	Cc. *N*/10 NaOH per dg.	No. of det.	A. D.	Cc. *N*/10 NaOH per dg.
0.5	2	±0.014	0.00	1	.....	0.00
1	5	±0.027	0.12	4	±0.027	0.14
2	..	.....	....	2	±0.049	0.17
3	7	±0.037	—0.30	7	±0.018	0.26
5	5	±0.058	—0.64	5	±0.065	0.45

[1] Here and below A. D. is average deviation of results/$\sqrt{\text{number of determinations}}$.

TABLE II.—ACTION OF GLYCINE, GLUTAMIC ACID AND ASPARTIC ACID ON SEVERAL ESTERS.

		Glycine.				Glutamic acid.				Aspartic acid.			
Ester.	Ester blank.	No. of detn.	A. D.	Cc. $N/10$ NaOH per dg.	Action in mols.	No. of detn.	A. D.	Cc. $N/10$ NaOH per dg.	Action in mols.	No. of detn.	A. D.	Cc. $N/10$ NaOH per dg.	Action in mols.
MeOAc	with bl.	7	±0.037	—0.30	—0.023	4	±0.17	3.66	0.54	4	±0.34	7.44	0.99
	no bl.			0.59	0.044			4.55	0.67			8.33	1.10
EtOAc	with bl.	2	±0.14	—0.75	—0.056	4	±0.066	2.64	0.39	4	±0.30	5.89	0.78
	no bl.			1.10	0.083			4.49	0.66			7.74	1.03
$Gl(OAc)_3$	with bl.	2	±0.031	—0.64	—0.048	2	±0.039	1.86	0.27	2	±0.014	4.46	0.59
	no bl.			0.95	0.071			3.45	0.51			6.05	0.81
PhOAc	with bl.	2	±0.025	0.83	0.062	2	±0.028	1.11	0.16	2	±0.044	1.17	0.16
	no bl.			1.39	0.100			1.67	0.25			1.73	0.23
EtOBu	with bl.	7	±0.018	0.26	0.020	4	±0.025	0.64	0.094	4	±0.027	0.79	0.11
	no bl.			0.26	0.020			0.64	0.094			0.79	0.11
EtOBz	with bl.	2	±0.039	0.25	0.019	2	±0.014	0.46	0.068	2	±0.17	0.47	0.063
	no bl.			0.25	0.019			0.46	0.068			0.47	0.063
PhOBz	with bl.	2	±0.007	0.06	0.0045	2	±0.028	0.16	0.024	2	±0.053	0.29	0.039
	no bl.			0.06	0.0045			0.16	0.024			0.29	0.039

TABLE III.—ESTER-WATER BLANKS.

MeOAc.		EtOAc.		$Gl(OAc)_3$.		PhOAc.		EtOBu.		EtOBz.		PhOBz.	
0.89	±0.044 (19)	1.85	±0.002 (13)	1.59	±0.032 (2)	0.56	±0.032 (2)	0.00	....	0.00	....	0.00	....

A. D., and amount of acid set free from methyl acetate by 0.1 glycine in terms of cubic centimeters of 0.1 normal alkali, and the fifth, sixth, and seventh columns the same data for ethyl butyrate. Three cubic centimeters seemed the most convenient amount and was used in making the tests given in Table I.

The action of glycine, glutamic acid, and aspartic acid on three cubic centimeters of each of the seven esters mentioned is given in like terms in Table II. Column one indicates the ester tested; column two shows in which of the two results for each ester the ester-water blank was subtracted (the reasons for giving two results are discussed below); columns three, four, seven, eight, eleven, and twelve give the number of determinations and A. D. for the amino acids which head them; columns five, nine and thirteen give the amounts of acid set free from the esters per decigram of amino acid in terms of cubic centimeters of 0.1 *N* alkali; finally, columns six, ten and fourteen give the mols of acid set free per mol of amino acid. Table III shows the magnitude of the ester water blanks in cubic centimeters of 0.1 normal alkali; A. D. for each ester is indicated by a plus or minus sign and the number of determinations is given in parenthesis.

Table IV gives the hydrolytic action of glycine on 3 cc. methyl acetate and ethyl butyrate in 25 cc. of the indicated salt solution.

TABLE IV.—HYDROLYTIC ACTION OF GLYCINE IN THE PRESENCE OF SALTS.

		MeOAc.			EtOBu.		
Salt.	Concentration.	No. of detn.	A. D.	Cc. *N*/10 NaOH per dg.	No. of detn.	A. D.	Cc. *N*/10 NaOH per dg.
NaCl	0.2 *M*	2	±0.011	—0.45	1	...	0.36
	0.5 *M*	1	...	—1.82	2	±0.021	0.30
Na_2SO_4	2 *M*	0	...	..	2	±0.011	0.25
	0.2 *M*	2	±0.011	0.07	2	±0.092	0.25
	0.5 *M*	2	±0.032	0.06	2	±0.042	0.19
$MgSO_4$	0.2 *M*	2	±0.014	—0.02	2	±0.035	0.21
	0.5 *M*	2	±0.025	—0.35	2	±0.065	0.42
	2 *M*	2	±0.00	—0.09	2	±0.007	0.26

In Table V is shown the hydrolytic action of glycine and acetic acid (upper part of table) and of acetic acid alone (lower part). The solutions were made up as follows: A glycine solution, containing about 4 grams per liter, and an acetic acid solution were standardized against alkali; the glycine solution contained 0.0978 gram glycine in 25 cc. and the acetic acid was 0.1084 *N*. Of the acetic acid solution 11.9 cc. were equivalent to the same amount of alkali as 25 cc. glycine; hence, in the experiments results of which are given under the heading "With glycine," 5.9 cc., 11.9, and 23.8 cc. acetic acid were added to 25 cc. glycine solution; and the action tested on 3 cc. ester. This is indicated in the first column by "$^1/_2$ equivalent," "1 equivalent," and "2 equivalents." In the experi-

ments results of which are headed "Without glycine," equivalent amounts of 0.1066 normal acetic acid (6.0 cc., 12.0 cc., and 24.0 cc.) were added to 25 cc. water and the action tested on 3 cc. of the indicated esters.

TABLE V.—ACTION OF GLYCINE AND ACETIC ACID TOGETHER AND SEPARATELY ON METHYL ACETATE AND ETHYL BUTYRATE.

	With glycine.					
	MeOAc.			EtOBu.		
Acetic acid.	No. of detn.	A. D.	Cc. $N/10$ NaOH per dg.	No. of detn.	A. D.	Cc. $N/10$ NaOH per dg.
None	7	±0.037	—0.30	7	±0.018	0.26
$^1/_2$ equivalent	2	±0.032	0.96	2	±0.046	0.21
1 equivalent	2	±0.046	1.77	2	±0.11	0.42
2 equivalents	2	±0.018	3.34	2	±0.00	0.59

	Without glycine.					
	MeOAc.			EtOBu.		
Acetic acid.	No. of detn.	A. D.	Cc. $N/10$ NaOH per dg.	No. of detn.	A. D.	Cc. $N/10$ NaOH per dg.
None	19	±0.044	0.89	>10	±0.00	0.00
$^1/_2$ equivalent	2	±0.088	4.98	2	±0.06	0.38
1 equivalent	2	±0.039	6.83	2	±0.05	0.57
2 equivalent	2	±0.035	8.81	2	±0.035	0.78

Two sets were tested with glycine solutions of known hydrogen ion concentration. For this work a solution of glycine was made up containing in one liter 7.505 grams glycine and 5.85 grams sodium chloride.[1] In Table VI are given the results obtained when this solution was used. The results under "Solution I" show the action of 21.0 cc. of glycine solution and 9.0 cc. 0.1 normal hydrochloric acid on 3 cc. ester; for this solution $C_{H^+} = 10^{-2.607}$. Those under "Solution II" show the action of 27.0 cc. of glycine solution and 3.0 cc. 0.1 normal hydrochloric acid on 3 cc. ester; for this solution $C_{H^+} = 10^{-3.341}$.

TABLE VI.—THE ACTION OF GLYCINE AND HYDROCHLORIC ACID ON THREE ESTERS.

	Solution I.			Solution II.		
Ester.	No. of detn.	A. D.	Cc. $N/10$ NaOH per dg.	No. of detn.	A. D.	Cc. $N/10$ NaOH per dg.
MeOAc	2	±0.32	17.41	2	±0.035	2.14
EtOAc	2	±0.011	13.34	2	±0.032	1.45
EtOBu	2	±0.035	1.18	2	±0.025	0.35

3. Theoretical Part.

The Relative Values of the Results.—The first point to be considered in discussing the above results is the relative weight to be assigned to each. The most obvious source of error is an uncertain end point. The fact that negative results were obtained (see Tables I and II) with methyl and ethyl acetates, precisely those esters which are most easily hydro-

[1] S. P. L. Sörensen, *Comptes-Rendus du Laboratoire de Carlsberg*, 8ème volume, *1ère* livraison, p. 41 (1909).

lyzable with excess of alkali, and hence give more or less quickly vanishing end points, suggests a reason for questioning their validity. These values are, however, not abnormal. In a study of the ionization of water, in which he followed the course of its hydrolytic action on methyl acetate for several weeks, Wijs[1] found that the rate of hydrolysis fell to a minimum and then rose. This he shows to be a necessary consequence of the fact that the hydroxyl ion is about 1400 times as rapid a catalyzer as the hydrogen ion; when the concentration of the former is repressed by the acid formed during the hydrolysis, the rate must decrease until the concentration of hydrogen ions shall have risen sufficiently to offset the loss of the comparatively more active hydroxyl ions. From this it follows that hydrolysis in a water solution must be greater than in a very dilute acid solution. Glycine is an extremely weak acid (for a 0.1 normal solution,[2] $C_{H+} = 10^{-6.106}$) and hence the negative values for the first three esters, which mean that hydrolysis in water solution is greater than in glycine solution, are quite in accord with the theory. In the same way the negative results obtained by Falk and Nelson,[3] using methyl acetate with leucine, leucylglycine, glycylleucine and diglycylglycine may be accounted for.

Because of these conditions the negative values obtained when the ester blank is subtracted do not indicate the lipolytic power of glycine; the conditions in the ester-water and glycine-ester-water solutions are not comparable. On the other hand, since there is such a marked hydrolysis of four of the esters by water alone, the ester blank cannot be disregarded. Hence in Table II are given, on the second line, opposit each ester, results that are the sum of those on the first line and the corresponding ester blank in Table III. In other words, in the results on the second lines, opposit "no-bl.," the ester-water blank is disregarded.

In general, the accuracy of each result may be judged by the corresponding A. D. and the number of determinations. The A. D. is in each case equal to 1.18 times the probable error.

Specificity and Effect of Added Substances.—In the two immediately preceding papers of this series[4] Falk has pointed out the two factors probably most important in determining specificity of lipase action—the effect of enzyme on substrate and of substrate on enzyme—and has shown the effect of esters and other neutral substances on castor bean lipase. In studying specificity from the other point of view—that of the enzyme—the internal structure of the molecule may be expected to play a leading role. Since the lipase molecule is probably protein in character,

[1] *Z. f. Physik. Chem.*, 11, 492; 12, 514 (1893).

[2] Sörensen, *loc. cit.*

[3] *Loc. cit.*

[4] Compare Falk, This issue, pp. 602, 616.

any indication of selective action by amino acids in the hydrolysis of esters will be of interest. Such indications are at hand in these experimental results.

In Table II the esters are arranged in the order of decreasing hydrolysis by glutamic and aspartic acids, which is also the order of decreasing molecular weight of the corresponding alcohols, but this order is seen to be quite different from the order referring to glycine either with or without the ester blank, and this order again is different from that of the ester blanks, if those esters giving zero blanks be excluded. Table VII summarizes these differences in hydrolysis of the four esters giving positive blanks.

TABLE VII.—ORDER OF HYDROLYSIS OF FOUR ESTERS.

Water.	Glycine (with blanks).	Glycine (without blanks).	Glutamic and aspartic acids.
EtOAc	PhOAc	PhOAc	MeOAc
$Gl(OAc)_3$	$[Gl(OAc)_3]$	EtOAc	EtOAc
MeOAc	MeOAc	$Gl(OAc)_3$	$Gl(OAc)_3$
PhOAc	$Gl(OAc)_3$	MeOAc	PhOAc
$[Gl(OAc)_3]$	EtOAc	$[Gl(OAc)_3]$	$[Gl(OAc)_3]$

Glyceryl tri-acetate alone may be considered from two points of view—that of mols of acid formed (given by results in the table) or of mols of ester hydrolyzed (the results in the table divided by three). Rated according to the latter standard, this ester takes the position indicated by brackets. Disregarding this change in order to simplify consideration of the case, phenyl acetate is seen to be at the foot of the column for water blanks, where the hydrolysis is catalyzed by hydrogen and hydroxyl ions, and at the foot of the column for hydrolysis by glutamic and aspartic acids where the catalysis must be affected by the comparatively large hydrogen ion concentration, while it stands first under glycine (whether the ester blank is considered or not), showing the greatest hydrolysis among those four esters. This indicates some sort of selective action; a difference in hydrolytic action between glycine and glutamic acid, if due only to a difference in the hydrogen ion concentration of the solutions or some other non-selective factor, would cause proportionate differences with the esters, and hence result in no difference in their order when arranged according to hydrolysis.

From Table IV it can be seen that in the case of ethyl butyrate the effect of neutral salts was neither great enough nor consistent enough to be of importance in this work. With methyl acetate the ester-salt-solution blanks were large, particularly with sodium chloride, so that the negative results obtained were larger than usual.

Table V is of particular interest. Here the interesting result is shown that methyl acetate is much less hydrolyzed by a solution of acetic acid and glycine than by a solution of a like amount of acetic acid alone, al-

though glycine is a stronger acid than base;[1] with ethyl butyrate the difference in the two solutions is the same in sense but not so great.

Summary.

Glycine, glutamic acid and aspartic acid exert a varying lipolytic action on methyl, ethyl, glyceryl tri- and phenylacetates, ethyl butyrate, and ethyl and phenyl benzoates. If these be arranged in the order of decreasing amounts of hydrolysis, the order will be different in the three cases where the action is caused by water, by glycine, and by glutamic or aspartic acids. This indicates selective action.

The effect of sodium chloride, sodium sulfate, and magnesium sulfate in solutions from 0.2 to 2 normal is not marked or consistent enough to be important for this work.

The hydrolytic action of solutions of glycine and acetic acid on methyl acetate and ethyl butyrate is less than that of corresponding solutions of acetic acid alone; this difference is proportionately much less with ethyl butyrate.

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STUDIES ON THE CHEMISTRY OF EMBRYONIC GROWTH. I. CERTAIN CHANGES IN THE NITROGEN RATIOS OF DEVELOPING TROUT EGGS.[2]

BY ROSS AIKEN GORTNER.

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Introduction.

The Problem.—The development of an apparently lifeless body into a living, active organism is the one great wonder of the world, and any insight into the chemical processes by means of which this development takes place, must necessarily be of considerable interest. The egg is a chemical laboratory where we find fats, lecithins, cholesterol, carbohydrates, various proteins, a mixture of enzymes, inorganic salts and other compounds. During the development of this egg into an independent animal, all of these compounds are called upon to play their part in the formation of the new structure, but, *do they enter the growing tissue in the same form in which they are laid down in the egg or are synthetic changes also taking place so that some of the material that is present in the egg is used, not in its original form, but in a modified condition?* It seemed to me that synthetic action must take place, for otherwise we must think of the

[1] Lundén, *Sammlung Chem. und Chem.-tech. Vortr.*, 14, 82 (1909).

[2] Read before the Division of Biological Chemistry at the Spring Meeting of the American Chemical Society, Minneapolis, Minn., March, 1913.