acid mixture. By steam distillation camphor was recovered, melting at 174° after crystallization from petroleum ether and sublimation.

Acetic Acid.—The acids obtained from the saponification of the ester were neutralized with caustic soda and treated with $AgNO_3$. Five fractional precipitates were collected, containing 62.98–63.17% Ag without further purification. It is very probable that only acetic acid is present, since silver propionate requires 59.91% Ag, silver acetate 64.64% Ag, and silver formate 70.59% Ag. For further confirmation the silver salts were recrystallized from water and analyzed as follows: 0.1970 gram silver salt gave 0.1266 gram Ag = 64.26% Ag.

"*Green Oil.*"—After removal of the esters, 14.5 grams of oil distilled between 125–151° at 20 mm. and 6.5 grams between 151–205° at 20 mm., $d_{20°}$ 0.9370. The first fraction was colored pale greenish yellow and the second deep green. No solid derivatives could be obtained from either fraction. The following color reactions were obtained for the "green oil:" A drop dissolved in acetic anhydride and treated with a drop of H_2SO_4 produced a deep blue color, changing to green; when glacial acetic acid was used in place of acetic anhydride the solution turned crimson, and on standing two hours became purple; a glacial acetic acid solution of the oil treated with bromine vapors became first purple, then ammoniacal copper blue.

Summary.

The constituents of the leaf oil of Douglas fir with their approximate percentages are as follows:

Constituent.	Percentage.
1-α-Pinene	25
1-β-Pinene	48
i or *l*-Limonene	6
Furfural	..
Ester as bornyl acetate	6.1
Free alcohol as borneol	6.5
"Green oil"	3.0
Losses by polymerization	5.0

FOREST PRODUCTS LABORATORY,
MADISON, WISCONSIN.

[CONTRIBUTION FROM THE HARRIMAN RESEARCH LABORATORY, ROOSEVELT HOSPITAL, NEW YORK.]

STUDIES ON ENZYME ACTION. VIII. A CONTINUATION OF THE STUDY OF THE ACTION OF AMINO ACIDS AND CASTOR BEAN LIPASE ON ESTERS.

BY MARSTON LOVELL HAMLIN.

Received October 4, 1913.

I. Introduction.

For a long time many enzymes have been considered to be protein in character, and this supposition holds well in the case of the castor bean

lipase. Falk has shown[1] conclusively that the differences in the behavior of this enzyme towards different esters—that is, its specificity—are due largely to differences, probably chiefly physical, in the medium in which it acts; the chemical nature of the enzyme itself, however, undoubtedly plays a considerable part in this selective action. If the lipases are proteins, their activity may be due to one or several specific groupings of the building stones of the protein molecule, the amino acid radicals. If this is so, it is not at all improbable that one or more of the amino acids or polypeptides has a like effect by itself, and since most proteins lack this lipolytic property, it is not surprising to find it lacking or slight in most of the simpler groupings of their constituents. In experimental testing of such substances, therefore, negative results would be expected in the greater number of cases.

In two previous papers of this series[2] experiments on the lipolytic effect of certain amino acids on various esters were described and discussed. This work has been further extended; alanine and phenylalanine and castor bean lipase[3] have been tested with the same esters that were used before, and an attempt was made to discover conditions under which the partial catalytic effect of the hydrogen ion could be distinguished from the total effect. Such conditions were found, and it has been possible to show not only that hydrolysis of methyl acetate, for example, in a glycine-sodium chloride-hydrochloric acid solution is not the same as in an isohydric hydrochloric acid solution, but that in some cases a change of conditions that caused an increase in the hydrolysis of methyl acetate caused a decrease in the hydrolysis of ethyl butyrate.

II. Experimental.

The experimental method used in testing the amino acids appearing in Table I was the same as the one previously employed by Hamlin,[4] except that 1 cc. of ester, instead of three, was used in making the tests. Glycine was retested with this change in conditions. Separate portions of amino acid (in most cases between one and two-tenths of a gram) were weighed out to one-tenth of a milligram, dissolved in 25 cc. pure, neutral water, and the ester added from a pipet; if the mixture was heated to dissolve the acid, the solution was cooled before adding the ester. The flasks were put in a thermostat and left for twenty-four hours at a temperature as near 38° as the regulator would permit; variations that occurred had no effect on hydrolysis within the experimental error. At the end of this period the contents of the flasks were titrated with standard alkali about one-tenth normal in the presence of sufficient neutralized 35% formaldehyde solution to give a good end point and satisfactory

[1] THIS JOURNAL, **35**, 601, 616 (1913).

[2] Falk and Nelson, THIS JOURNAL, **34**, 828 (1912); Hamlin, **35**, 624 (1913).

[3] Prepared as described by Falk, THIS JOURNAL, **35**, 603 (1913).

[4] *Loc. cit.*

values according to Sorensen's method.[1] With glycine, 10–15 cc. formaldehyde solution were used, with alanine about 30 cc., and with phenylalanine about 20 cc. Alanine gave a less sharp end point than phenylalanine. Solutions containing, respectively, amino acid only and ester only were treated in a similar way. When the value for the ester blank had been subtracted from the corresponding amino acid-ester value, the difference was reduced to cc. of tenth normal alkali solution required per decigram of amino acid, and from this quantity was subtracted the amino acid blank in the same terms. The figure thus obtained expressed the hydrolysis per decigram of amino acid in terms of tenth normal acid set free.[2]

TABLE I.—ACTION OF GLYCINE, ALANINE, PHENYLALANINE AND CASTOR BEAN LIPASE ON SEVERAL ESTERS.

	Glycine.				Alanine.			
1 cc. ester.	No. of detn.	A. D.[3]	Cc. $N/10$ NaOH per 0.1 g.	Action in mols.[4]	No. of detn.	A. D.[3]	Cc. $N/10$ NaOH per 0.1 g.	Action in mols.[4]
MeOAc....	14	±0.04	—0.12	—0.009	7	±0.03	—0.05	—0.004
EtOAc....	4	±0.01	—0.11	—0.008	4	±0.03	—0.05	—0.004
Gl(OAc)$_3$..	8	±0.02	—0.01	—0.001	10	±0.03	—0.02	—0.002
PhOAc.....	8	±0.05	0.31	0.023	10	±0.03	0.39	0.035
EtOBu....	12	±0.02	0.07	0.005	8	±0.02	0.12	0.011
EtOBz....	8	±0.04	0.08	0.006	10	±0.02	0.14	0.012
PhOBz....	8	±0.02	0.03	0.002	10	±0.03	—0.04	—0.004
Castor oil..	..	....	..	..	..	....	....	....
Olive oil...	..	....	..	..	..	....	....	....

	Phenylalanine.				Castor bean lipase.		
MeOAc...	6	±0.02	—0.01	—0.002	4	±0.02	1.21
EtOAc....	4	±0.07	—0.10	—0.017	2	±0.01	0.69
Gl(OAc)$_3$..	2	±0.03	—0.09	—0.015	2	±0.06	2.58
PhOAc....	2	±0.07	0.17	0.028	2	±0.04	3.16
EtOBu....	6	±0.02	0.09	0.015	4	±0.03	1.51
EtOBz....	2	±0.06	0.05	0.008	4	±0.03	0.19
PhOBz....	2	±0.00	—0.01	—0.002	4	±0.03	0.10
Castor oil..	.	..	..	...	4	±0.05	0.19
Olive oil...	.	..	..	...	2	±0.02	—0.02

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

[2] Phenylalanine from three different lots was used in these experiments; two of these were prepared in this laboratory (m. p. 272° and 265°, uncorrected) and the other was prepared by recrystallizing Kahlbaum's product once from water (m. p. 258°; sample from Kahlbaum's original package melted at 235°). In preparing the phenylalanine here, several attempts were made to use the synthesis described by Johnson and O'Brien, *J. Biol. Chem.*, **12**, 205 (1912), as corrected later by Johnson, *Am. Chem. J.*, **49**, 68 (1913) (NH_4CNS and not KCNS was used). This method proved a failure, yielding no phenylalanine, perhaps because the conditions were not sufficiently definitly described. Eventually Fischer's method, *Ber.*, **37**, 3064, was used, and gave very satisfactory results.

[3] A. D. = average deviation of results/$\sqrt{\text{number of determinations}}$.

[4] The figures in this column give the mols of acid set free per mol of amino acid.

Besides these, five sets of experiments were carried out in which the action on ethylbutyrate and methyl acetate of a glycine-hydrochloric acid solution was compared with that of an isohydric hydrochloric solution. The method of determining the action was the same as that previously described, except that the solutions of the catalysts were measured out volumetrically, and the duration of the tests was shorter.

Three catalytic solutions were used. The first, called solution I in the tables, was made up of ten parts of a solution of 7.505 grams glycine and 5.85 grams sodium chloride per liter (0.1 N), to ten parts tenth normal hydrochloric acid and its hydrogen ion concentration is given by: $[H^+] = 10^{-1.93}$. The strength of a hydrochloric acid solution isohydric with this was calculated from the degree of dissociation of 0.01 N acid, which was taken as $\alpha_{[HCl=0.01\ N]} = 97.3$.[1] Since $[H^+] = 10^{-1.93} = 1/85.5 = 0.0117$, then $[HCl] = 0.0117/0.973 = 0.01202$. A measured amount of the standard hydrochloric acid, which was 0.1203 N, was therefore diluted to exactly ten times its volume. This is solution II. In one set another solution, solution III, was used, which contained 0.01203 N hydrochloric acid and 0.1 N sodium chloride.

TABLE II.

Time, 20 hrs.; temperature, 38°–40°; formaldehyde, 20 cc.

Catalyst.	Ester (1 cc.).	Cc. 0.0997 N NaOH.		Means.	Action.
I. 30 cc.	MeOAc	66.80	69.45	68.13	38.01
I. 30 cc.	EtOBu	35.50	35.56	35.53	5.55
I. 30 cc.	.....	29.92	29.95	29.94	...
II. 30 cc.	MeOAc	36.20	40.20	38.20	34.29
II. 30 cc.	EtOBu	8.78	9.23	9.01	5.24
II. 30 cc.	.....	3.75	3.70	3.73	...
.....	MeOAc	0.19	0.16	0.18	...
.....	EtOBu	0.04	0.04	0.04	...

TABLE III.

Time, 3 hrs.; temperature, ca. 38°.5; formaldehyde, 15 cc.

Catalyst.	Ester (1 cc.).	Cc. 0.0997 N NaOH.		Means.	Action.
I. 20 cc.	MeOAc	25.10	25.17	25.14	5.12
I. 20 cc.	EtOBu	20.50	20.58	20.54	0.52
I. 20 cc.	.....	19.98	19.97	19.98	...
II. 20 cc.	MeOAc	8.07	7.39	7.73	5.25
II. 20 cc.	EtOBu	2.92	2.83	2.88	0.40
II. 20 cc.	.....	2.43	2.45	2.44	...
III. 20 cc.	MeOAc	8.07	7.92	8.00	5.52
III. 20 cc.	EtOBu	2.88	2.90	2.89	0.41
III. 20 cc.	.....	2.48	2.40	2.44	...
.....	MeOAc	0.04	0.04	0.04	...
.....	EtOBu	0.04	0.04	0.04	...

[1] Noyes and Falk, THIS JOURNAL, 34, 496 (1912).

TABLE IV.

Time, MeOAc, 3 hrs.; EtOBu, 5 hrs.; temperature, ca. 40°; formaldehyde, 15 cc.

Catalyst.	Ester (1 cc.).	Cc. 0.0997 *N* NaOH.		Means.	Action.
I. 20 cc.	MeOAc	25.56	25.48	25.52	5.41
I. 20 cc.	EtOBu	21.01	20.92	20.97	0.88
I. 20 cc.	.....	20.00	20.09	20.05	...
II. 20 cc.	MeOAc	8.21	8.17	8.19	5.69
II. 20 cc.	EtOBu	3.35	3.36	3.36	0.86
II. 20 cc.	.....	2.48	2.43	2.46	...
.....	MeOAc	0.04	0.04	0.04	...
.....	EtOBu	0.04	0.04	0.04	...

TABLE V.

Time, 3 hrs.; temperature, ca. 39°; formaldehyde, 15 cc.

Catalyst.	Ester (3 cc.).	Cc. 0.0997 *N* NaOH.		Means.	Action.
I. 20 cc.	MeOAc	33.40	33.46	33.43	13.32
I. 20 cc.	EtOBu	20.80	20.80	20.80	0.71
I. 20 cc.	.....	20.05	20.05	20.05	...
II. 20 cc.	MeOAc	17.30	17.39	17.35	14.85
II. 20 cc.	EtOBu	3.12	3.05	3.09	0.61
II. 20 cc.	.....	2.43	2.45	2.44	...
.....	MeOAc	0.06	0.06	0.06	...
.....	EtOBu	0.04	0.04	0.04	...

TABLE VI.

Time, 3 hrs.; temperature, 40°–41°; formaldehyde, 15 cc.

Catalyst.	Ester (3 cc.).	Cc. 0.0997 *N* NaOH.		Means.	Action.
I. 20 cc.	MeOAc	32.81	32.60	32.71	12.66
I. 20 cc.	EtOBu	20.79	20.73	20.76	0.73
I. 20 cc.	.....	20.00	19.97	19.99	...
II. 20 cc.	MeOAc	15.85	16.20	16.03	13.51
II. 20 cc.	EtOBu	3.05	3.05	3.05	0.55
II. 20 cc.	.....	2.46	2.45	2.46	...
.....	MeOAc	0.04	0.08	0.06	...
.....	EtOBu	0.04	0.04	0.04	...

The data for and results of these five sets of experiments with the three solutions given are summarized in Tables II–VI. In each table the temperature and time of action and also the amount of formaldehyde used in the titration are given at the top. Column one shows the number and amount of catalytic solution, column two the ester tested, columns three and four the amounts of 0.0997 *N* sodium hydroxide used in titrating the solutions in duplicate experiments, and column five the means of these results; in column six is given the action expressed in cc. of 0.0997 *N* alkali, obtained by subtracting the means of the ester and catalyst blanks from the means of the corresponding results.

III. Theoretical Part.

The experimental results described in papers II and VII of this series,[1] and continued in the work described in this paper, exceed expectations in the proportion of active substances encountered. Selective action was observed, and most markedly under the conditions described in this paper for the experiments summarized in Tables II–VI above.

From the results of the experiments described, giving the activities on the seven esters mentioned, the four esters, methyl acetate, ethyl acetate, glyceryl tri-acetate, and phenyl acetate, which are appreciably hydrolyzed by water, may be arranged in the order of the amounts of hydrolysis in the presence of the substances tested, as was done in Table VII of the seventh paper of this series;[2] to these four esters it is interesting to add ethyl butyrate, in view of the results shown in Tables II–VI. This comparison is made in Table VII. There is a marked similarity in

TABLE VII.—ORDER OF HYDROLYSIS OF FIVE ESTERS.

Glycine.	Alanine.	Phenylalanine.
PhOAc	PhOAc	PhOAc
EtOBu	EtOBu	EtOBu
$Gl(OAc)_3$	$Gl(OAc)_3$	MeOAc
EtOAc	EtOAc MeOAc	$Gl(OAc)_3$
MeOAc	.....	EtOAc

all three cases except that phenylalanine causes more hydrolysis of methyl-acetate than does glycine or alanine. This agrees with Falk's and Nelson's results in a previous paper of this series;[3] in their experiments, however, methyl acetate was hydrolyzed in phenylalanine solution even more than ethyl butyrate, a result which could not be substantiated in this work. It was thought that the lesser degree of purity of the phenylalanine used by them (Kahlbaum's) might be responsible for this difference, but no definit conclusion on this point could be arrived at.

The results of the experiments given in Tables II–VI are of special interest in view of the great amount of research on catalysis due to the hydrogen ion and the questions arising as to the role played by the various components of a solution, cations, anions, and un-ionized substance. Divergences from the proportionality of velocity and hydrogen ion concentration—the "salt effect" when observed in the presence of neutral salts—have, in solutions containing lipases, become so great that the role of the hydrogen ion has become entirely secondary.

Although it is beyond the scope of this article to attempt a review of theories of catalysis in solution, it is of interest in this connection, since lipases are practically un-ionized substances, to mention the opinions which the experiments of several recent workers in this field have led to,

[1] THIS JOURNAL, 34, 828 (1912) and 35, 624 (1913).

[2] Hamlin, *loc. cit.*

[3] *Loc. cit.*

Armstrong,[1] as the result of a long series of researches, comes to the conclusion[2] that the hydrolysis (of esters) is an associative process, since a system is first formed, consisting of hydrolyte, catalyst, and water, which is then broken down; or it is the direct interaction of two complexes, one unit of hydrated hydrolyte and one of hydrated catalyst. It is a bimolecular reaction, and its velocity is not proportional to C_H, nor its equilibrium unaffected by the concentration of the catalyst.

Stieglitz, although not committing himself as yet to a definit hypothesis, says[3] "on account of the molecular velocities observed as parallel reactions in other cases, notably by Acree," it is "probable that there *must be some* reactivity of the non-ionized salts," and Rosanoff, as a result of studies on the inversion of sugar, forming part of a study of the general laws of catalysis, has written,[4] "From this point of view the mechanism of sugar hydrolysis appears to depend on the existence of a molecular complex whose concentration determins the velocity of the reaction." Goldschmidt has studied[5] esterification, the reverse action, in alcohol solutions containing weak acids, like tri-chlorobutyric, with small amounts of water and with no water present; he speaks of the partial velocity due to the hydrogen ion; the same with all acids, and that due to acid anion, which varies with the acid. The effect of small amounts of water is best explained in his opinion by assuming a combination of alcohol with the catalyst or its cation.

The importance of the effect of substances other than the hydrogen ion in reactions like these is therefore undoubted, and studies of such effects must sooner or later be brought into close relation to studies of lipolytic ferments. The experimental results given in Tables II–VI present an example of such an effect. They may be condensed for the sake of easy comparison as in Table VIII. Here it is seen that in every

TABLE VIII.—ACTIONS OF TABLES II–VI.

Number of table referred to.	II.		III.			IV.		V.		VI.	
Catalyst.	I.	II.	I.	II.	III.	I.	II.	I.	II.	I.	II.
MeOAc.....	38.01	34.29	5.12	5.25	5.52	5.41	5.69	13.32	14.85	12.66	13.51
EtOBu.....	5.55	5.24	0.52	0.40	0.41	0.88	0.86	0.71	0.61	0.73	0.55

case except II (run 20 hours) the action is greater on methyl acetate in the presence of hydrochloric acid alone (catalyst II) than in the presence of hydrochloric acid and glycine (catalyst I), though in both cases the hydrogen ion concentration is practically the same. Any deduction from this observation would necessarily depend on the

[1] *Roy. Soc. Proc.*, 1904–1912.
[2] *Ibid., Ser. A.*, 87, 612 (1912).
[3] THIS JOURNAL, 34, 1694 (1912).
[4] *Ibid.*, 35, 257 (1913).
[5] *Physik. Chem.*, 81, 66.

strict equality of this concentration in both solutions, but the further fact, shown in this table, that in every case except the first, the action on ethyl butyrate is less in the absence of glycine, makes this strict equivalence unnecessary. It proves that whatever the hydrogen ion concentrations in the two solutions are, the variation that causes an increase of hydrolysis of methyl acetate causes a decrease in that of ethyl butyrate. This further confirms the results given in Table VI of the seventh paper of this series already referred to. Here the ratio of hydrogen ion concentrations in two solutions is $10^{-2.607}/10^{-3.341} = 5.42$, while the ratios of hydrolysis under like conditions of three esters in these solutions are, for methyl acetate, $17.41/2.14 = 8.13$; for ethyl acetate $13.34/1.45 = 9.40$, and for ethyl butyrate, $1.18/0.35 = 3.36$. Clearly, in the presence of glycine the velocity of hydrolysis of the methyl acetate and ethyl butyrate is not proportional to the hydrogen ion concentration.

III. Summary.

1. The selective lipolytic actions of glycine, alanine, phenylalanine and castor bean lipase on methyl, ethyl, glyceryl tri- and phenyl acetates, ethyl butyrate, and ethyl and phenyl benzoates have been compared, and that of castor bean lipase on castor oil and olive oil has been measured.

2. It has been shown that the selective lipolytic action of glycine on methyl acetate and ethyl butyrate is more marked in the presence of certain concentrations of hydrochloric acid, and this selective action has been measured.

3. It has been shown that in the glycine-hydrochloric acid solution used the hydrolysis of methyl acetate and ethyl butyrate is not proportional to the hydrogen ion concentrations of the solutions, and that the disproportionality, which has been measured, is comparatively large.

[CONTRIBUTION FROM THE HARRIMAN RESEARCH LABORATORY, ROOSEVELT HOSPITAL, NEW YORK.]

STUDIES ON ENZYME ACTION. IX. EXTRACTION EXPERIMENTS WITH THE CASTOR BEAN LIPASE.

BY K. GEORGE FALK.

Received October 4, 1913.

The experimental study of the lipolytic activity of the castor bean preparation, the results of which were presented in previous papers,[1] dealt with the mixed soluble and insoluble substances present in the preparation. This method of working was essential in the first part of an extended investigation, for it is evident that any treatment of the lipase material, which, in part, showed the properties or characteristics of a colloid, will produce some change in the material and undoubtedly modify the behavior

[1] THIS JOURNAL, **34**, 375 (1912); **35**, 210, 602, 616 (1913).