Summary.

The actions of neutral salts of the uni-uni-, uni-bi-, and bi-bivalent types were tested on the activity of a castor bean lipase preparation toward ethyl butyrate under comparable conditions.

In every case the change in activity, whether increase or decrease, was found to be a continuous function of the concentration of the salt added.

Decreased activities, as compared with aqueous solutions, were shown by all the uni-univalent salts, by the chlorides and nitrates of barium and calcium (except for the most dilute solutions) and magnesium, by sodium oxalate, and by dilute solutions of sodium sulfate.

Increased activities were shown by dilute solutions of the chlorides of barium and calcium, by more concentrated solutions of sodium sulfate, by magnesium sulfate, and by the chloride and sulfate of manganese. Potassium sulfate solutions gave the same results as purely aqueous solutions.

The observed regularities for the actions of the individual substances of each type are discussed in the body of the article following the tables in which the results are presented.

If an explanation of the retarding actions of the various salts be looked for, it may perhaps be found in the coagulation of the enzyme (either alone or together with other substances) by the addition of the salts, the ions of which produce their individual specific effects in each case. The unionized molecules may also take part in these reactions. The accelerations cannot be explained in as simple a manner except, perhaps, for the cases where increased formation of active lipase (as by manganous salts)[1] may be assumed.

HARRIMAN RESEARCH LABORATORY,
ROOSEVELT HOSPITAL, NEW YORK.

STUDIES ON ENZYME ACTION. VI. THE SPECIFICITY OF LIPASE ACTION.

BY K. GEORGE FALK.

Received March 1, 1913.

In the preceding paper, the action of neutral salts on the activity of a castor bean lipase preparation was described in detail. In this paper, the action of some organic substances on the activity of the lipase preparation will first be described and then the possibility of explaining the selective action of lipases on different esters as a logical deduction from these results will be discussed.

The following substances were studied: methyl alcohol, ethyl alcohol, acetone, glycerol, and glucose. On account of the presence of a urease

[1] Cf. Falk and Hamlin, Third Paper, *loc. cit.*

in the preparation[1] it was impossible to study the lipolytic action with urea solutions.

The results which were obtained for solutions of methyl alcohol, ethyl alcohol, and acetone, for concentrations from 2 molar down are presented in Table I. The method of experimenting was the same as with the inorganic salts and was described in detail in the preceding paper. The results are given in terms of the number of cc. of 0.1 normal acid solution formed from 1 cc. of ethyl butyrate by 0.2 grams of husk- and oil-free castor bean preparation in contact with 25 cc. of the solution of the organic substance. The significance of the columns is the same as before; "Molar conc." referring to the concentration of the organic substance added, "No. of determ." and "Aver. dev. of mean" giving a measure of the agreement of the various results with each other for each concentration, "Action found" giving the average values of the lipolytic actions corrected for the lipase and ester blanks, and "From curve" the final results from the smoothed curves obtained by plotting the actions found and the logarithms of the concentrations. The duration of the tests was 48 hours and the temperature 38° to 40°. The lipase blank corrections were as follows:

Molar conc. =	2.0	1.0	0.5	0.2	0.1	0.05	0.02
CH_3OH	0.38	0.45	0.48	0.50	0.51	0.52	..
C_2H_5OH, $(CH_3)_2CO$	0.35	0.37	0.42	0.47	0.49	0.51	0.52

The ester blank corrections were taken to be zero throughout, although for some of the higher concentrations a value of 0.04 cc. was indicated. The value for the action at zero concentration of added substance was again taken to be 2.50 cc.

The retarding action of the two alcohols and acetone on the lipolytic activity of the preparation is very marked and similar to that observed with solutions containing uni-univalent salts. The inhibiting effect increases continuously with the amount of substance added until a value of practically zero is reached.[2] With regard to the actions shown by the individual substances, the table shows that for the same molar concentration, methyl alcohol exerts a greater inhibitory effect than ethyl alcohol, and ethyl alcohol a greater than acetone down to 0.01 molar concentration at which the retardation is practically the same for all three.

On account of the similarity of the retarding actions of these substances with those observed for the simple uni-univalent salts, it seems reasonable to suggest a similar explanation for the phenomenon, namely, a coagula-

[1] Cf. Fourth Paper of this Series, THIS JOURNAL, **35**, 292 (1913).

[2] In the First Paper, THIS JOURNAL, **34**, 735 (1912), it was shown that the lipase preparation in moist acetone solution showed distinct lipolytic activity. The conditions of that experiment were entirely different from the conditions of the experiments described in the present paper, but the action may perhaps be compared with the lipolytic activity shown by some of the more concentrated fluoride solutions given in the preceding paper.

TABLE I.—ACTION OF CASTOR BEAN LIPASE ON ETHYL BUTYRATE IN AQUEOUS SOLUTIONS OF METHYL ALCOHOL, ETHYL ALCOHOL AND ACETONE.

Molar conc.	Subst. used.	No. of determ.	Aver. dev. of mean.	Action found.	From curve.	Subst. used.	No. of determ.	Aver. dev. of mean.	Action found.	From curve.	Subst. used.	No. of determ.	Aver. dev. of Mean.	Action found.	From curve.
2.0	CH_3OH	4	±0.01	0.24	0.21	C_2H_5OH	3	±0.02	0.16	0.16	C_3H_6O	4	±0.03	0.03	0.04
1.0	"	4	2	0.32	0.34	"	4	2	0.34	0.34	"	4	2	0.41	0.42
0.5	"	4	2	0.53	0.56	"	4	2	0.68	0.68	"	4	2	0.82	0.92
0.2	"	4	4	1.06	1.05	"	4	5	1.37	1.37	"	4	5	1.55	1.53
0.1	"	4	8	1.49	1.44	"	6	6	1.68	1.67	"	4	10	1.87	1.77
0.05	"	4	5	1.74	1.72	"	6	10	2.05	1.88	"	4	5	1.85	1.92
0.02	"	3	5	1.90	2.00	"	4	5	2.02	2.06	"	3	5	2.03	2.07
0.01	"	4	3	2.17	2.16	"	4	5	2.12	2.17	"	4	4	2.18	2.16
0.005	"	4	5	2.33	2.28	"	6	4	2.14	2.27	"	4	6	2.18	2.23
0.002	"	2	5	2.15	2.39	"	.	..	..	..	"	.	..	..	..
0.001	"	2	6	2.33	2.45	"	2	3	2.20	..	..	.	..	..	..

tion of substances, in the course of which the active lipase material is partially or wholly removed from the sphere of action. The mechanism or reactions involved in this precipitation or coagulation may be different for the inorganic and the organic substances added. This question will be taken up again when more experimental data are at hand.

A number of experiments were carried out with solutions of glucose and of glycerol, up to a concentration of 2.0 molar with the former and of 25% with the latter. No distinct change in the lipolytic activity of the preparation could be observed with either of these substances in solution. Armstrong and Ormerod[1] showed that glycerol up to a concentration of 25% exerted little, if any, influence, but in more concentrated solution retarded the action of castor bean lipase.

The differences observed in the actions of methyl alcohol and ethyl alcohol and of glycerol on lipolytic activity suggested the study of the possible inhibiting effect of esters, substances similar to the alcohols in many of their physical and chemical properties.

The lipolytic activity of the castor bean preparation was therefore determined for a series of concentrations of methyl acetate (MeAc), ethyl acetate (EtAc), ethyl butyrate (EtBu), and triacetin. The results are presented in Table II for experiments of 48 hours duration. at 38–40° in which the ester given in the column, "Substances used" were tested at the concentrations stated in the preceding column. In all other respects the experiments were carried out in the same way as those given in Table I. The lipase blank was taken to be 0.52 and the ester blanks (in water) were found to be as follows:

Molar conc. =	1.0	0.5	0.2	0.1	0.05	0.02	0.01	0.005
MeAc.....	0.85	0.24	0.06	0.03	0.01	0	..	...
EtAc.....	3.30	0.87	0.26	0.12	0.06	0.02	0	...
EtBu.....	0	..	..	..	..	..	..	...
Triacetin.....	..	..	..	0.35	0.15	0.05	0.02	0

The values given in the columns headed "Action found" are corrected for both lipase and ester blanks. In plotting these results, concentrations (in place of the logarithms of the concentrations as before) were used, and the results in the columns headed "From curve" taken from the curves except for the two most concentrated solutions of methyl acetate and three most concentrated solutions of ethyl acetate. For these, the ester-water corrections are very large and for the most concentrated solution of ethyl acetate gives a negative value for the action found. Evidently here the hydrolytic action of the water on the ester exceeds that of the lipase solution on the ester for the conditions of the experiment. This anomaly may readily be explained as due in the latter case to the neutralization, in part at any rate, by the protein material of the prepara-

[1] *Proc. Roy. Soc. London*, **78**, (*B*) 380 (1906).

tion, of the acid formed by the hydrolysis. For the five solutions, in which the ester corrections are at all considerable, the values given in the columns "From curve" are uncorrected for the ester blanks. The values for these actions, given in parentheses in the table, are undoubtedly too large. For triacetin, the increase in the action above the concentration of 0.02 molar was too great to permit the drawing of a satisfactory curve to represent the results.

TABLE II.—ACTION OF CASTOR BEAN LIPASE ON AQUEOUS SOLUTIONS OF METHYL ACETATE, ETHYL ACETATE, ETHYL BUTYRATE AND TRIACETIN.

Molar conc.	Subst. used.	No. of determ.	Aver. dev. of mean.	Action found.	From curve.	Molar conc.	Subst. used.	No. of determ.	Aver. dev. of mean.	Action found.	From curve.
1.0	*MeAc*	2	±0.05	0.75	(1.60)	...	*EtAc*	4	±0.08	—1.43	(1.87)
0.5	"	6	7	1.41	(1.65)	...	"	6	7	0.69	(1.56)
0.2	"	6	9	1.28	1.28	...	"	6	8	1.02	(1.28)
0.1	"	6	5	1.04	1.02	...		8	5	0.81	0.89
0.05	"	6	5	0.65	0.75	...	"	8	5	0.80	0.71
0.02	"	6	8	0.49	0.44	...	"	8	5	0.43	0.48
0.01	"	5	5	0.25	0.25	...	"	6	7	0.40	0.34
1.24	*EtBu*	2	±0.04	2.41	...	0.1	*Triacetin*	4	±0.67	20.10	...
0.62	"	4	6	2.30	...	0.05	"	7	23	6.96	...
0.31	"	4	2	2.41	2.50	0.02	"	4	20	2.86	2.86
0.16	"	4	6	2.29	2.26	0.01	"	4	4	1.91	1.96
0.08	"	4	6	1.90	1.90	0.005	"	4	7	1.26	1.28
0.04	"	6	6	1.52	1.53	0.002	"	4	6	0.80	0.70
0.02	"	6	3	1.16	1.20	...	"	.	.	..	...
0.01	"	6	4	0.97	0.93	...	"	.	.	..	...

The consideration of the results of Table II shows a very small increase in the activity of the lipase preparation for a very large increase in the concentration of ester added for methyl acetate, ethyl acetate and ethyl butyrate. With triacetin on the other hand, there is a large increase in the activity observed as the concentration of the ester is increased. In order to be compared with equivalent concentrations of the other esters, the concentrations of the triacetin must be tripled. An approximate comparison when this is done reveals the fact that for the most dilute solution, the actions found with ethyl butyrate and tiracetin are very nearly the same, and both considerably greater than for the acetic esters, and that as the concentrations are increased, the activity shown by the triacetin solutions increases with far greater rapidity than those by the equivalent ethyl butyrate solutions. The latter again show somewhat greater activities than do the acetic ester solutions. The values for the solutions of methyl acetate and ethyl acetate do not differ much from each other for equivalent concentrations.

These results may be compared with the results obtained with the alcohols. Methyl alcohol and ethyl alcohol show marked retarding action on the lipolytic activity, this retardation increasing rapidly with increasing concentration of the alcohol. Glycerol shows no retarding action except in very concentrated solutions. Methyl and ethyl esters show a small increase in lipolytic action with increasing concentration of ester, while the glycerol ester shows a great increase in action with increasing concentration of ester.

Now, when these results are taken into consideration it seems justifiable to extend the explanation advanced for the action of the simple alcohols to the action exerted by the simple esters; *i. e.*, the ester causes a precipitation or coagulation of substances in the course of which the active lipase material is partially or wholly removed from the sphere of action.[1] Methyl acetate and ethyl acetate show least increase in activity with increasing concentration of ester and therefore the greatest inhibiting action. Glyceryl acetate (triacetin) shows the greatest increase in activity with increasing concentration of ester and therefore the smallest inhibiting action. These results are exactly similar to those obtained with methyl and ethyl alcohols and glycerol. That the actions are not controlled entirely by the alcohol radicals is apparent from the fact that with triacetin, even the dilute solutions do not show a proportionality between the amount of ester and the action.

With methyl acetate, increasing the concentration of ester in the solution increases the action, although only very slowly. Between 0.01 and 0.2 molar, the concentration was increased twenty-fold but the action only five-fold. With the most dilute solution of ester 2.50 cc. 0.1 normal alkali solution would be required to neutralize the acid if the ester were hydrolyzed completely. The inhibiting action of the ester was therefore very great especially as the solutions became more concentrated. This is brought out clearly by the maximum action at 0.5 molar, the inhibiting action above this concentration overbalancing the increase in action due to the added ester, so that the actual values obtained decreased. With ethyl acetate, the inhibiting action was just as marked as with methyl acetate except that no maximum action was observable. It must be noted, however, that the ester blanks are not included for the final results given in the columns "From curve" for the most concentrated solutions. These would lower the values to some extent, especially since, in the most concentrated solutions, the ester blanks for ethyl acetate gave greater values

[1] It is of secondary importance whether this explanation is correct. The important feature of this point of view is the similar results, whether due to precipitation, coagulation or some other phenomenon, obtained with neutral salts, alcohols, and esters, which makes possible the understanding of some of the specific actions of lipases. The alcohol formed in the hydrolyses of the esters is too small in quantity to cause an appreciable inhibiting effect.

than those for methyl acetate. With ethyl butyrate, more action was shown for the most dilute solution than with the esters just considered, and the increase, with increasing concentration, was either equal to or greater than the increase for them. A maximum action was obtained at about 0.3 molar, above which no increase was observed. This may, however, have been due to the limited solubility of the ethyl butyrate. The dilute solutions showed that the inhibiting action of the ethyl butyrate was less than that of methyl acetate and ethyl acetate. With triacetin, a comparatively small inhibiting action was shown in the dilute solutions, since the actions were not proportional to the concentrations of the ester. For the more concentrated solutions, the rate of increase was more than proportional to the increase in the concentration of ester. This may have been due to the increased acidity of the medium which in itself accelerates the action of the castor bean lipase.[1]

In view of the similar retardations exerted by uni-univalent salts described in the preceding paper, for castor bean lipase and by Terroine[2] for an animal lipase and in view also of the commonly accepted inhibiting action of alcohol on all lipases, it may be assumed as very probable that the same relations with regard to the inhibiting actions of certain esters on their own hydrolysis hold for lipases of animal as well as for those of vegetable origin.

The fats as glycerides of fatty acids of high molecular weight doubtless exert practically no inhibiting action. The rate of their saponification will then depend upon the possibility of dissolving the fat or of bringing it in direct contact with the enzyme. To how great an extent accelerators such as bile salts act in this way does not appear to be quite certain as yet.

In addition to the changes which may be produced in the lipase by the specific inhibiting action of the ester, another factor must be considered. The lipolytically active substance is probably protein in character, and the hydrolyses observed may be due to or influenced greatly by certain groupings present in the molecule. In the second paper[3] of this series, the lipolytic actions of a number of simple amino acids and peptides toward certain esters were tested, and some interesting differences in action found. For instance, glycine exerted a greater hydrolytic action on ethyl butyrate than on methyl acetate; phenyl alanine, a greater on methyl acetate than on ethyl butyrate.

These two explanations, first, the specific action of the esters on the lipase material, and second, the action of lipase material containing various groupings on esters, both based upon experimental investigation,

[1] Connstein, Hoyer and Wartenberg, *Ber.*, **35**, 3988 (1902).

[2] *Biochem. Z.*, **23**, 429 (1910).

[3] THIS JOURNAL, **34**, 828 (1912).

should be sufficient to account for all of the specific actions observed with lipases.

In the preceding paper the results obtained for the action of neutral salts on the lipolytic activity of the castor bean preparation on ethyl butyrate were presented. The inhibiting action of the ethyl butyrate itself would be exerted in every experiment and whatever actions were observed should be corrected for this if the actual effects to be attributed to the salts alone are desired. Since the same amount of ethyl butyrate was present in each solution, the actions are comparable among themselves. If the work were to be repeated with an ester such as triacetin, it may be predicted that the retardations observed would be smaller, and the accelerations greater than with ethyl butyrate. This is also true for the experiments described in this paper with methyl alcohol, ethyl alcohol, and acetone.

This work also has bearing on another point of practical interest; that is, the choice of an ester for testing lipolytic activity. On the basis of the foregoing results, triacetin is the most suitable one of those used, since it is fairly soluble and shows less inhibiting action than any of the simpler esters. The use of triacetin as the best routine test for a fat-splitting ferment was clearly pointed out by A. E. Taylor.[1] The following results show the amount of hydrolysis in terms of cc. of 0.1 normal sodium hydroxide solution produced with the weights of lipase preparation given in the first column in 25 cc. water on 0.22 cc. triacetin (0.04 molar solution) for the times of action 5 hours and 20 hours given in the second and third columns, at 38–40°. The necessary corrections for lipase and ester blanks have been introduced.

TABLE III.—ACTION OF CASTOR BEAN LIPASE ON TRIACETIN.

Lipase preparation. Gram.	5 hours.	20 hours.
0.1	0.26	0.63
0.2	0.56	1.20
0.4	1.12	3.04
0.6	1.70	4.40

For the time interval of 5 hours, the amount of action was proportional to the amount of lipase preparation. For 20 hours, and 0.4 gram and 0.6 gram material the action was greater than would be expected from a strict proportionality. This may, perhaps, have been due to the accelerating action of the acid formed. The amounts of action were not proportional to the two time intervals, less action being found for 20 hours. This was doubtless caused by the inactivation of the lipase by the water solution and aided in part perhaps by the small inhibiting action of the triacetin.

A possible objection to the use of triacetin is of a theoretical nature.

[1] *J. Biol. Chem.*, **2**, 90 (1906).

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.

HARRIMAN RESEARCH LABORATORY,
ROOSEVELT HOSPITAL, NEW YORK.

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

BY MARSTON LOVELL HAMLIN.

Received March 18, 1913.

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).