hydroxide solution and to this solution contained in a glass-stoppered bottle was added an excess over the calculated amount of dimethyl sulfate. The solution was shaken well. More alkali and dimethyl sulfate were added alternately from time to time and the contents of the bottle were shaken well after each addition. A colorless oil separated and later solidified. The ester is easily recrystallized from hot methyl alcohol, in which it is very soluble. It separates in colorless crystals melting at 80–81.5°. This indicates that it is identical with the second ester mentioned by Meyer and Turnau. Some of the desiccator-dried ester was analyzed.

Analyses. Subs., 0.1795, 0.1778: CO_2, 0.4678, 0.4636; H_2O, 0.0844, 0.0827. Calc. for $C_{16}H_{14}O_4$: C, 71.09; H, 5.22. Found: C, 71.09, 71.13; H, 5.26, 5.25.

Summary

1. The sodium, silver, barium, calcium and zinc salts of *p*-hydroxybenzoyl-*o*-benzoic acid have been prepared.

2. The ethyl and methyl esters of *p*-hydroxybenzoyl-*o*-benzoic acid have been prepared and the action of ammonia on them has been observed.

3. The dibromo derivative of *p*-hydroxybenzoyl-*o*-benzoic acid has been prepared by direct bromination of the acid.

4. The phenylhydrazone of *p*-hydroxybenzoyl-*o*-benzoic acid has been prepared.

5. Hydroxy-phenylphthalide and its acetate have been prepared.

6. *p*-Methoxybenzoyl-*o*-benzoic acid and its sodium and potassium salts and methyl ester have been prepared.

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THE INFLUENCE OF VARIOUS ANTISEPTICS ON THE ACTIVITY OF LIPASE[1]

BY LEROY S. PALMER

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One of the prerequisites of a suitable technique for measuring lipase activity is an antiseptic which does not interfere with the fat hydrolysis but which will effectively prevent the growth of bacteria and the activity of other acid producing enzymes. Very few data on this subject are found in the literature.

Kastle and Loevenhart[2] made the most comprehensive study. Their experiments were with the esterase of the pancreas and liver, using ethyl butyrate as substrate. The period of incubation in their experiments was only 15 minutes. The antiseptics examined were hydrocyanic acid, potassium cyanide, mercuric chloride, silver nitrate,

[1] Published with the approval of the Director as Paper No. 280, Journal Series, Minnesota Agricultural Experiment Station. The studies here presented were begun at the Missouri Agricultural Experiment Station and carried to completion at the Minnesota Agricultural Experiment Station. Reported at the Philadelphia Meeting of the American Chemical Society, September, 1919.

[2] Kastle and Loevenhart, *Am. Chem. J.*, **24**, 491 (1900).

sodium fluoride, phenol, thymol, toluene, chloroform, iodoform, bromoform, salicylic acid and formaldehyde. Of these salicylic acid and sodium fluoride, only, inhibited the esterases completely. Several had a marked retarding effect, *e. g.*, silver nitrate, mercuric chloride and potassium cyanide. Some were paralyzers, *e. g.*, hydrocyanic acid, thymol, toluene, phenol, and iodoform. Toluene, which is very widely used at present in lipase work, acted irregularly, at times retarding the hydrolysis slightly and in other trials not at all. Iodoform also acted irregularly, retarding the pancreatic esterase 20%, the liver esterase being unaffected. Neither chloroform nor formaldehyde had any marked effect on either esterase, although it appeared as though the liver esterase was accelerated slightly by the formaldehyde and the pancreatic esterase retarded slightly (about 15%).

Falk[3] studied the effect of alcohols and acetone on the activity of castor bean esterase, using ethyl butyrate as substrate, and found that methyl and ethyl alcohols and also acetone retarded the activity. Acetone proved to have the most marked effect, 2 *M* solutions practically inhibiting the action.

Graves and Kober[4] have examined the effect of a 0.2% solution of tricresol on the action of the lipase in castor bean extract and pancreatin, using ethyl butyrate and triacetin as substrates. The lipase in the castor bean extract was retarded when either substrate was used. The enzyme in pancreatin which hydrolyzes tri-acetin was also retarded. The pancreatic esterase, however, was accelerated by the tricresol.

Vandevelde[5] proposed a 3% solution of iodoform in acetone as a suitable antiseptic for lipase work. He added this to cow's milk in such amounts that the resulting solution contained 0.3% of iodoform in experiments in which he sought to determine whether protease and lipase are natural constituents of cow's milk. He failed, however, to determine the effect of this antiseptic on proteolytic and lipolytic enzymes of known origin.

So far as the writer has been able to discover even fewer studies have ever been made on the effects of antiseptics in experiments in which the substrate consisted of emulsions of neutral fats. Connstein, Hoyer and Wartenberg[6] mixed 10 cc. of 1% chloral hydrate solution with 5 g. of crushed castor beans and found that this antiseptic permitted 75–92% of the theoretically possible fatty acids to be liberated from castor oil in the seeds and also from various other oils and fats in 24 hours. The chloral hydrate is stated to have kept the mixture sterile. Under the same conditions, however, the castor beans had practically no hydrolyzing effect on tri-acetin or tributyrin. These investigators found, also, that practically no fat hydrolysis occurred when 0.5 g. of castor bean seeds was ground with 5 g. of 0.5% formaldehyde solution.

Kita and Osumi[7] have examined the activity of castor bean lipase when acting on soya bean oil, and found that the enzyme retained its activity in dilute acetone but not in ethyl alcohol.

Scope of the Work

In the experiments reported in this paper the effect of the following substances was studied on the hydrolysis of emulsions of butter fat, namely, formaldehyde, chloroform, acetone, acetone solution of iodoform, iodoform, iodine, bromine, mercuric chloride and chloral hydrate.

The results with mercuric chloride and chloral hydrate will not be re-

[3] Falk, THIS JOURNAL, **35,** 616 (1913).

[4] Graves and Kober, *ibid.*, **36,** 751 (1914).

[5] Vandevelde, *Mem. Acad. Royal Belgique*, Sciences Series II, **2,** 1 (1907–10).

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

[7] Kita and Osumi, *J. Tokyo Chem. Soc.*, **39,** 13 (1918).

ported in detail. The former inhibited lipase activity completely in 0.1, 0.2, and 0.3% concentrations, and chloral hydrate not only retarded the lipase but also failed as a germicide when using cow's milk as substrate.

In most cases the substrate used was an artificial acacia "milk" prepared according to the directions given below. The enzyme used was usually a suspension of commercial steapsin in water.

A few experiments were carried out with formaldehyde and chloroform using a glycerin extract of pig's pancreas as the source of the enzyme and cow's milk as substrate. The results were unsatisfactory because the enzyme preparation contained proteases which acted on the proteins of the milk. These experiments showed that it does not appear possible to prevent proteolytic activity effectively by antiseptics without, at the same time, interfering with lipase activity, if the substrate contains both protein and fat. Lipase studies, therefore, must be carried out as far as possible on an emulsion free from protein, such as the artificial acacia milk used in the experiments reported in this paper.

Methods Employed

In the experiments using artificial milk as substrate the technique was not identical in all cases. The variations, however, were in certain details which were intended to simplify the procedure or yield the maximum titratable acidity. These various modifications eventually led to the the adoption of the following method.

Preparation of Artificial Acacia Milk.—Fifteen-g. portions of powdered U.S.P. acacia were ground in a mortar with 20 to 25 cc. of distilled water until the gum was thoroughly hydrated. Twenty g. of fat (melted butter fat was used in all cases) was then added, 2 or 3 cc. at a time with thorough grinding after each addition. Fifteen cc. of warm water was now gradually added with continued trituration. The emulsion was then gradually diluted with distilled water with continued grinding until a volume of 500 cc. was reached. The resulting "milk" contained 4.0% fat, and had a Sörensen value of approximately 5.0.

Measuring the Lipase Activity.—Seventy-five-cc. portions of artificial milk were measured into 200cc. sterile Erlenmeyer flasks. The antiseptic was next added to each flask, except the check, to which no antiseptic was added. One cc. (usually) of the lipase preparation[8] was now added to Flask 1, and after thorough shaking 25 cc. was withdrawn with a pipet and added to 100 cc. of 3:1 acetone-ether, and the mixture titrated at once to a faint permanent pink with 0.1 *N* alcoholic potassium hydroxide solution, using 5 drops of 1% alcoholic phenolphthalein as indicator.

[8] For the commercial steapsin this was usually a 6% suspension in distilled water. In some of the later experiments the amount of steapsin was increased because the preparation (then several months old) was not so active.

One cc. of the steapsin was now added to Flask 2 and the acidity of 25 cc. of the mixture determined as before. This was repeated for each successive flask in turn, including a flask to which 1 cc. of lipase was added to 75 cc. of water only. In this case the acetone-ether mixture was omitted for determining the acidity, water only being used as solvent. After the initial acidity of all the experimental flasks and of the lipase preparation had been determined in this manner, the remainder of the lipase preparation was heated rapidly to the boiling point and quickly cooled. One cc. of this lipase was now added to 75 cc. of milk, also containing the antiseptic, as a control, and the acidity of a 25cc. aliquot determined in the same manner as for the experimental flasks. All the flasks were now placed in the incubator at 38° and the titrations repeated on 25cc. aliquots after 24- and, in some cases, also after 48-hour intervals, the flasks being rotated occasionally. The flasks were stoppered either with cotton plugs or corks covered with cotton throughout the experiment.

Advantages of Method.—This method of measuring lipase activity has the following advantages: (1) it provides a uniform standard emulsion of fat; (2) the substrate is free from extraneous substances which are likely to yield titratable acids through the action of other enzymes, particularly proteases; (3) the substrate has a uniform hydrogen-ion concentration which is independent of the kind of fat or oil employed; (4) the method provides for the determination of all the free fatty acids produced by the action of the lipase through the addition of a suitable fat solvent to the substrate and the employment of alcoholic in place of the usual aqueous alkali for the neutralization of the fatty acids.

Although butter fat was used as the fat in the substrate in the experiments reported in this paper, other fats or oils could also have been employed without affecting the hydrogen-ion concentration of the substrate. This conclusion is supported by the data shown in Table I, the hydrogen-

TABLE I

HYDROGEN-ION CONCENTRATION OF 4.0% ACACIA "MILK" MADE WITH VARIOUS FATS AND OILS

Kind of fat	P_H	Kind of fat	P_H
Butter fat	4.965	Lard	4.978
Olive oil[a]	4.932	Acacia alone	4.945
Corn oil (refined)	4.957		

[a] Caution must be observed in the use of olive oil since recent observations [Rector, *J. Ind. Eng. Chem.*, **12**, 156 (1920)] indicate that olive oil itself may contain lipase.

ion concentration being determined by the potentiometer method. In the author's experience it does not appear advisable to adjust the reaction of the acacia milk to a hydrogen-ion concentration nearer neutrality. In a brief study of this point it was found that the addition of 2 cc. of saturated lime water to 100 cc. of milk lowered the hydrogen-ion con-

centration from P_H 4.96 to P_H 6.69, the addition of 2.4 cc. of lime water changing it to P_H 6.90. However, when the gum was ground up with the same amounts of lime water, preparatory to the dispersion of the oil, this adjustment was not secured, presumably because of a reaction which occurs between the concentrated colloidal gum and the lime water, which does not occur when the calcium hydroxide solution is added to the diluted gum suspension. Probably this reaction would occur in time even with the dilute solution.

The advantages which follow the employment of alcoholic instead of the usual aqueous alkali for titrating fatty acid mixtures containing acids of both high and low molecular weight are clearly down in Table II. The

TABLE II

COMPARISON OF AQUEOUS AND ALCOHOLIC POTASSIUM HYDROXIDE FOR TITRATING FATTY ACIDS IN LIPASE ESTIMATION

Expt.	Titrating solution 0.1 *N* KOH	Initial titration[a] Cc.	After 24 hours' incubation Cc.	Expt.	Titration solution 0.1 *N* KOH	Initial titration[a] Cc.	After 24 hours' incubation Cc.
1	aq.	0.70	10.35	6	alc.	3.30	13.10
2	alc.	0.70	13.95	7	aq.	3.10	9.05
3	aq.	0.70	9.65	8	alc.	3.10	13.25
4	alc.	0.70	13.05	9	aq.	3.10	9.05
5	aq.	3.30	8.50	10	alc.	3.10	13.40

[a] 0.1 *N* alkali required for 25 cc. of acacia "milk."

higher initial titration values of Expts. 5 to 10 in contrast with the results secured in Experiments 1 to 4 are due to the fact that 100 cc. of 3:1 acetone-ether mixture was added to the initial as well as to the 24-hour aliquots in the tests showing the higher initial acidity, the fat solvent being added to only the 24-hour aliquots in the tests showing the low initial acidity. These results emphasize the importance of using a suitable fat solvent in the estimation of acidity due to action of lipase.

Calculation of Results.—In calculating the lipase activity the acidity of the lipase preparation alone was first subtracted from all the other results. The initial acidity of the control (boiled lipase + milk) was next subtracted from the initial acidity of each experimental flask (less the acidity of the lipase preparation). A similar subtraction was made of the 24- or 48-hour control flask acidities from the 24- or 48-hour experimental acidities. The net initial acidity of each flask was then subtracted from the net 24- and 48-hour acidities, respectively, and the result calculated in terms of cubic centimeters of 0.1 *N* acid developed per 100 cc. of milk. A comparison of the acidity which developed in the presence of the preservative with that which developed in the check flask permits a calculation of the percentage retardation or acceleration due to the antiseptic.

Experimental

Formaldehyde.—Several experiments were run at different times in which formaldehyde was examined for its effect on lipase activity. The data are directly comparable only in so far as the relative effects of the formaldehyde are concerned because the actual acidity developed in various experiments varied to an extent which makes a direct comparison impossible. A typical experiment is given in Table III and the net effects

TABLE III

EFFECT OF VARIOUS CONCENTRATIONS OF FORMALDEHYDE ON THE ACTIVITY OF ANIMAL LIPASE

Description of test	Amount of antiseptic	Initial acidity[a] 25cc. portions "Blank"[b] = 0.40 cc. "Milk"	Final acidity[a] 25cc. portions "Blank"[b] = 0.50 cc. "Milk"	Net increase in acidity due to lipase (per 100 cc. milk)	Effect on lipase
	Parts/1000	Cc.	Cc.	Cc.	%
Milk + lipase	None	1.20	9.85	34.2	....
Milk + HCHO + lipase	1.0	1.30	10.90	38.0	−11.3
Milk + HCHO + lipase	0.5	1.30	10.65	37.0	+ 8.2
Milk + HCHO + lipase	0.25	1.30	10.10	34.4	+ 0.6

[a] Cc. 0.1 *N* acid.

[b] Acacia solution without fat + HCHO + lipase.

of the formaldehyde in the various experiments are summarized in Table IV. The data given in Table III are, however, omitted from Table IV.

The data show that only in one case, namely, Expt. 6, Table IV, was

TABLE IV

EFFECT OF FORMALDEHYDE ON ACTIVITY OF ANIMAL LIPASE

Expt.	Concentration of formaldehyde	Net acidity developed[a] Without antiseptic	With antiseptic	Effect of antiseptic
		Cc.	Cc.	%
1	1 :1,000	43.00	45.60	+ 7.9
2	1 :2,000	43.00	44.73	+ 4.0
3	1 :1,000	29.20	32.80	+ 9.2
4	1 :2,000	29.20	30.80	+ 5.5
5	1 :1,000	49.40	45.90	− 9.3
6	1 :1,000	49.40	31.20	−36.8
7	1 :1,500	34.80	40.40	+16.1
8	1 :1,500	39.20	40.45	+ 3.2
9	1 :1,500	39.20	41.20	+ 5.1

[a] Cc. of 0.1 *N* acid developed in 100 cc. of milk in 24 hours.

there any indication of a retarding effect of the formaldehyde when using moderate concentrations of the antiseptic. Because of this result and also because the author[9] has shown that formaldehyde 1:1500 is an effective germicide for milk preservation from a bacteriological standpoint,

[9] Palmer, *Missouri Agr. Exp. Sta. Res. Bull.*, **34**, 1-29 (1919).

a concentration of formaldehyde between 1:1,000 and 1:1,200 is recommended as best suited for lipase determinations.

The results of these experiments are of considerable interest in connection with the theories advanced by Falk[10] of an active enol-lactim structure in esterases and lipases, such as may exist in substances of a protein nature. The fact that formaldehyde in relatively high concentration has no detrimental effect on the activity of animal lipase when acting on fat emulsions seems to throw grave doubt, at least, on the possible protein nature of this enzyme. Besides the ready reaction of formaldehyde with the free amino groups of proteins to form methylene compounds, a serious denaturing effect is also produced, which not only prevents the coagulation of albumins and globulins by heat, but produces other marked changes in their physical and chemical properties. Just what these chemical changes are is not known, but it seems very probable that they modify, in some way, the peptid linkages or at least would interfere greatly with tautomeric changes in the keto-lactam and enol-lactim structures.

Chloroform.—Chloroform has several disadvantages as an antiseptic for lipase work in spite of its effective germicidal powers. It has been used, however, in lipase determinations by several workers, *e. g.*, by Rogers[11] and co-workers and by Thatcher and Dahlberg.[12] Harding and Van Slyke,[13] a number of years ago, showed that chloroform, to be an effective germicide for milk, must be present in a concentration of at least 1.5%, in order to dissolve the fat and saturate the serum with the vapors. These results show, therefore, that chloroform has a distinct disadvantage for lipase work in that the lipase does not act on a fat emulsion but in reality on a chloroform solution of fat. In view of the fact, however, that chloroform has been used as an antiseptic in lipase determinations, it was deemed advisable to determine the effects which it produces on lipase activity under controlled conditions.

The experiments on this point were run at various times, as in the case of the formaldehyde experiments. The results are summarized in Table V. They show a marked retarding effect of the reagent on the lipase activity. Whether this effect is due to the mechanical disadvantages pointed out above or to an actual chemical effect on the lipase was not investigated.

Acetone.—The influence of several concentrations of acetone on lipolytic activity was tested in the same manner as in the experiments with formaldehyde and chloroform, already reported. The acetone used

[10] Falk, *J. Biol. Chem.*, **31**, 97 (1917).

[11] Rogers, Berg and Davis, *U. S. Dept. of Agr., Bureau of Animal Industry Circular*, **189**, 307–326 (1912).

[12] Thatcher and Dahlberg, *J. Agr. Res.*, 11, 437 (1917).

[13] Harding and Van Slyke, *N. Y. Agr. Exp. Sta. Tech. Bull.*, **6**, pp. 41–82 (1907).

TABLE V

EFFECT OF VARIOUS CONCENTRATIONS OF CHLOROFORM ON THE ACTIVITY OF ANIMAL LIPASE

Expt.	Concentration of chloroform	Net acidity developed[a] Without antiseptic	With antiseptic	Effect of antiseptic
	%	Cc.	Cc.	%
1	1.5	43.00	33.60	−21.8
2	2.5	43.00	25.00	−41.9
3	2.0	29.20	12.60	−56.9

[a] Cc. of 0.1 *N* acid developed in 100 cc. of milk in 24 hours.

was in some cases a commercial product of reagent grade, and in others a product redistilled from technical acetone. The results are summarized in Table VI. They show that acetone alone, in concentrations of 6 to

TABLE VI

EFFECT OF VARIOUS CONCENTRATIONS OF ACETONE ON THE ACTIVITY OF ANIMAL LIPASE

Expt.	Concentration of acetone	Net acidity developed[a] Without antiseptic	With antiseptic	Effect of antiseptic
	%	Cc.	Cc.	%
1	6.0	49.40	43.60	−11.7
2	6.0	51.60	44.58	−13.3
3	12.0	51.60	39.20	−24.0

[a] Cc. of 0.1 *N* acid developed in 100 cc. of milk in 24 hours.

12%, has a noticeable retarding effect on lipase activity when using fat emulsions as substrate. As in the case of chloroform this result is at least theoretically attributable to the solvent action of acetone on fat, and to the effect this would have on the stability of fat emulsions.

Acetone Solution of Iodoform.—It was Vandevelde's[5] proposal, especially, which prompted an examination of an acetone solution of iodoform as a suitable antiseptic for lipase determinations. A 3% solution of iodoform in purified acetone was used, as in Vandevelde's experiments. This was added in different amounts to the artificial milk, gradually, with shaking, because the iodoform precipitates in concentrations of water-acetone, such as are used in these tests. The results are summarized in Table VII. They show that Vandevelde's antiseptic for lipase work may,

TABLE VII

EFFECT OF VARIOUS CONCENTRATIONS OF ACETONE SOLUTION OF IODOFORM ON LIPASE ACTIVITY

Expt.	Concentration Of iodoform	Of acetone	Net acidity developed[a] Without antiseptic	With antiseptic	Effect of antiseptic
	%	%	Cc.	Cc.	%
1	0.02	1.4	43.00	20.80	− 51.6
2	0.30	10.2	29.20	2.80	− 90.5
3	0.15	5.1	29.20	0.00	−100.0

4	0.03	1.0	29.20	3.20	−89.1
5	0.30	10.0	34.80	25.00	−28.1
6	0.30	10.0	34.80	25.60	−26.5
7	0.30	10.0	34.80	20.20	−42.0
8	0.30	10.0	34.80	26.00	−25.3

[a] Cc. of 0.1 *N* acid developed in 100 cc. of milk in 24 hours.

at times, inhibit entirely the activity of the lipase and, in any case, has a marked retarding action on the enzyme.

Iodoform Alone.—In view of the fact that acetone itself has a marked retarding action on lipase, experiments were carried out to determine how much of the effects secured was due to the acetone and also with the view of throwing some light on the marked irregularity in the results secured in the different tests. For this purpose a saturated solution of iodoform in acetone was prepared. This contained approximately 25% iodoform and reduced the amount of acetone to be added to the milk to a minimum. Formaldehyde, 1:1500, was also added to each flask except the check flask, to which no antiseptic was added. The results of this experiment are given in detail in Table VIII. The data show a direct correlation

TABLE VIII

EFFECT OF IODOFORM ON LIPASE ACTIVITY

Flask	Concentration of CHI_3	Acidity[a] Initial	Acidity[a] After 24 hours	Net acidity developed per 100 cc. of milk	Retardation due to CHI_3
	%	Cc.	Cc.	Cc.	%
1	0.00	1.40	12.60	44.80	00.0
2	0.03	1.35	11.10	38.00	15.1
3	0.06	1.45	10.55	36.40	18.7
4	0.15	1.55	9.85	33.20	25.9
5	0.30	1.55	8.95	29.60	33.9
6	0.30	1.50	8.85	29.40	34.3
7	0.45	1.60	7.60	24.00	46.5
8	0.60	1.60	6.70	20.40	54.5

[a] Cc. of 0.1 *N* acid in 25 cc. of milk.

between the amount of retardation and the concentration of iodoform added. The data do not, however, offer an explanation for the fact that the results of the experiments with an acetone solution of iodoform were very irregular.

Iodine.—A study of the possible causes of the irregularities of the preceding two experiments led to the conclusion that these results, especially those reported in Table VII, may have been due to the fact that the acetone solution of iodoform used for Expts. 2, 3 and 4, Table VII, was a solution which undoubtedly contained free iodine. This was found to be the case, the solution being shown by analysis to contain 0.912% of free iodine. The percentage of free iodine added to the flasks in Expts. 2, 3, and 4, Table VII, was therefore 0.09, 0.045 and 0.009%, respectively.

In order to determine the effect of these concentrations of iodine on lipase activity two sets of experiments were run, in one of which 0.05, 0.25 and 0.5 cc. of a 17% acetone solution of iodine was used as antiseptic, and in the other 2 cc. of a 5% solution of iodine in potassium iodide. Seventy-five cc. of acacia "milk" was used in each experiment and formaldehyde, 1:1500, also was added to each flask, including the check flasks to which no iodine was added. The results of the experiments are summarized in Table IX. The data show that iodine in the concentra-

TABLE IX

EFFECT OF VARIOUS CONCENTRATIONS OF IODINE ON LIPASE ACTIVITY

Expt.	Concentration of iodine	Net acidity developed[a] Without iodine	With iodine	Retardation due to iodine
	%	Cc.	Cc.	%
1	0.090[b]	40.85	1.60	96.10
2	0.045[b]	40.85	1.20	97.10
3	0.045[b]	40.85	0.40	99.00
4	0.009[b]	40.85	3.20	92.20
5	0.100[c]	39.72	0.00	100.00
6	0.100[c]	39.72	0.00	100.00
7	0.100[c]	39.72	0.00	100.00
8	0.100[c]	39.72	0.00	100.00

[a] Cc. of 0.1 *N* acid developed in 24 hours.

[b] Acetone solution of iodine.

[c] KI solution of iodine.

tions employed effectively prevents lipase activity. The data also explain the results shown in Table V, summarizing experiments in which an acetone solution of iodoform, containing free iodine, was employed.

Several interesting questions arise in connection with the results with iodine. One is whether the iodine merely inhibits the activity or actually destroys the lipase. Another is whether the effect of iodine throws any light on the probable constitution of lipase.

In connection with the probable action of iodine itself a brief experiment was run in which artificial milk was treated with 0.1% iodine in the form of an iodine solution in potassium iodide. Lipase was then added and the initial acidity determined on a 25cc. aliquot. The remainder of the mixture was incubated for 24 hours and the acidity determined again on a 25cc. aliquot. Sufficient sodium thiosulfate solution of known strength was now added to the remainder of the milk to react with all the free iodine present and the flask placed in the incubator for another period of 24 hours. The acidity after this incubation was compared with the initial acidity and the acidity after 24 hours in the presence of iodine. Identical results were secured in each of the three titrations indicating that the lipase had been actually destroyed by the iodine. The destroying action of

iodine on other enzymes has also been noted. According to Zunz,[14] iodine destroys pepsin. Its action on trypsin is not stated.

Regarding the possible bearing of these results on the constitution of lipase, the data indicate that lipase has an unsaturated structure, which the author believes to be aliphatic rather than cyclic.

This suggestion is supported by the results, reported above, on the failure of formaldehyde to retard lipase, even in fairly high concentrations.

Bromine.—The results secured with iodine suggested an examination of the effect of bromine on lipase activity. The results of these experiments are summarized in Table X. The bromine was added in the form

TABLE X

EFFECT OF BROMINE ON LIPASE ACTIVITY

Expt.	Concentration of bromine	Net acidity developed[a] Without antiseptic	With antiseptic	Retardation due to antiseptic
	%	Cc.	Cc.	%
1	0.250	40.75	2.80	93.2
2	0.250	40.75	2.60	93.6
3	0.090	40.75	34.60	15.1
4	0.090	40.75	34.80	14.6
5	0.008	53.04	50.92	4.0

[a] Cc. of 0.1 *N* acid developed in 100 cc. of milk in 24 hours.

of a saturated aqueous solution. The data show that higher concentrations of bromine than of iodine are required to destroy lipase. The data, however, lend support to the suggestion of an unsaturated aliphatic structure for the lipase molecule.

Summary

Data are presented on the influence of various concentrations of several antiseptics on the hydrolysis of emulsions of milk fat in gum acacia solutions, using commercial steapsin as the source of lipase.

Formaldehyde in concentrations up to 1 part in 250 had no detrimental effect on the activity of the lipase, 1% solutions being required to produce a retardation of the enzyme.

Chloroform in concentrations from 1.5 to 2.5% retarded the lipase activity from 20 to 60%.

Acetone in concentrations of 6 and 12%, retarded the lipolysis 12 to 25%.

A freshly prepared 3% solution of iodoform in acetone added to give a concentration of 0.3% iodoform retarded the lipase action 25 to 40%. When using a similar solution of iodoform, which had stood for some time, practically complete inhibition was obtained with this and even smaller concentrations of iodoform.

[14] Zunz, E. Abderhalden's, "*Biochem. Handlexikon*," J. Springer, Berlin, 1911, vol. 5, p. 584.

In the experimental examination of these results it was found that iodoform alone retarded lipolysis in direct proportion to the concentration of the iodoform present. This varied from a 15% retardation with 0.03% concentration of iodoform to a 55% retardation with 0.5% concentration of the antiseptic.

It was found, also, that old solutions of iodoform in acetone contain free iodine which has a marked effect on lipase activity. Iodine, in concentrations of 0.045% or more, inhibited lipolysis entirely.

Results similar to those with iodine were obtained using bromine water. Higher concentrations of bromine were found necessary to inhibit the lipase, however, concentrations of 0.25% retarding the enzyme activity only 93 to 94%.

The results with the formaldehyde and the halogens are discussed with reference to their bearing on the possible constitution of lipase.

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HALOGENATION. XXI. SOME DERIVATIVES OF CARBAMIC ESTERS. CHLORINE AS A SIMULTANEOUS OXIDIZING AND CONDENSING AGENT

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It has been shown[1] that when chlorine is passed through an aqueous solution of various alkyl carbamic esters, mono- and dichloro substitution products are formed, the chlorine replacing the hydrogen of the amido group to form substituted nitrogen chlorides. It has been found that when chlorine is passed into carbamic esters in alcoholic solutions, the alcohol is oxidized to the corresponding aldehyde which immediately condenses with the carbamic esters to form dicarbamic esters. Thus, when chlorine is passed into a methyl alcoholic solution of urethane, the methyl alcohol is first oxidized to formaldehyde which then condenses with two molecules of urethane to form methylene diurethane.

$$CH_3OH \longrightarrow CH_2O$$
$$CH_2O + 2NH_2COOC_2H_5 = CH_2(NH_2COOC_2H_5)_2 + H_2O$$

In a similar manner, chlorination of methyl carbamic ester in methyl alcoholic solution, gives rise to methylene dicarbamic methyl ester, $CH_2(NHCOOCH_3)_2$.

These compounds were prepared previously by Conrad and Hock[2] by the condensation of formaldehyde with ethyl carbamic and with methyl carbamic esters, respectively.

[1] Datta and Gupta, THIS JOURNAL, **36**, 386 (1914); Ref. 6.

[2] Conrad and Hock, *Ber.*, **36**, 2206 (1903).