reaction is not constant and where the rate of the reaction is taken as proportional to the mole fractions of the reacting substances. The quaternary salts melt at low temperatures without decomposition.

Diethylaminomethyl-methyl ether has been prepared for the first time.

BERKELEY, CALIFORNIA

[CONTRIBUTION FROM THE HARRIMAN RESEARCH LABORATORY, THE ROOSEVELT HOSPITAL]

STUDIES OF ENZYME ACTION. XXXVI ESTER-HYDROLYZING ACTIONS OF THE CASTOR BEAN[1]

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Introduction

The experiments described in this paper form a development of the work on castor beans published some years ago. They also parallel the study of the ester-hydrolyzing or lipase actions of animal tissues and tumors which is in progress. The topics treated here include the use of various substrates, the actions at different hydrogen-ion concentrations, some studies of the kinetics of the actions, and the solubility of the active enzyme materials in water and in sodium chloride solution. The work of others will be taken up in connection with the new experimental work.

Experimental Methods and Results

Enzyme Material.—Several preparations of castor beans[2] were used. The method of obtaining the material hull- and fat-free was described elsewhere.[3]

Substrates.—Soluble esters only were used. This may appear to limit the usefulness and applicability of the results because such esters are found only to small extents in natural sources. If comparable results could be obtained with fats and similar bodies, it would be desirable to use them as well. The enzyme material used, frequently is insoluble or in colloidal solution. The use of an insoluble substrate may introduce added complications in the way of obtaining uniform and reproducible mixtures and therefore comparable estimations of the enzyme actions. It was decided to study only esters soluble under the conditions of the experiments and in this way attempt to eliminate one of the most troublesome of the experimental factors.

[1] Taken in part from the thesis presented by I. Lorberblatt in partial fulfilment of the requirements for the degree of Bachelor of Science at the Brooklyn Polytechnic Institute, June, 1925. Thanks are due to Professor B. P. Caldwell for his advice and suggestions in the various phases of the work and for permission to include the results in this paper.

[2] Supplied by the Baker Castor Oil Company, New York.

[3] Noyes, Lorberblatt and Falk, *J. Biol. Chem.*, **68**, 135 (1926).

The following esters were used.

	Acetate						
Ester	Methyl	Ethyl	*iso*Butyl	Glyceryl tri		Phenyl	Benzyl
B. p., °C.	57	77	108	258 (decomp.); 140 (10 mm.)		196.7	206

	n-Butyrate		Benzoate	
Ester	Methyl	Ethyl	Methyl	Ethyl
B. p., °C.	102	121	199	213

These esters were used in an extended study of the ester-hydrolyzing actions of animal tissues and tumors, the results of which are being presented elsewhere.[4] In addition, the following esters were used in part of the work.

	Acetate			Propionate		*n*-Butyrate	Valerate
Ester	Propyl	*n*-Butyl	*iso*Amyl	Methyl	Propyl	Propyl	Methyl
B. p., °C.	100	124	138.5	79	121.5	142	127

3.4 Milli-equivalents of ester, or some multiple thereof, were used in each experiment.

Method of Testing.—The enzyme actions were determined by titration with 0.1 *N* sodium hydroxide solution with phenolphthalein as indicator, after suitable incubation at 37–38°. Toluene was present throughout. Each result is the mean of duplicate determinations. The necessary blanks were tested in every case and the results as given here corrected for these. The enzyme actions are stated in terms of cc. of 0.1 *N* alkali used in the titrations, corrected for blanks, or in tenths of milli-equivalents of acid produced from the ester by the enzyme material under the conditions of the experiments.

Effect of Hydrogen-ion Concentration.—A number of series of determinations of the ester-hydrolyzing actions of several castor bean preparations, starting at different hydrogen-ion concentrations were carried out.

TABLE I

ESTER-HYDROLYZING ACTIONS OF PRESSED CASTOR BEAN PREPARATION AT DIFFERENT HYDROGEN-ION CONCENTRATIONS

0.1 g. of enzyme material; 3.4 milli-equivalents of esters

Ester	P_H 4.0	P_H 5.0	P_H 6.0	P_H 7.0	P_H 8.0
Phenyl acetate	2.08	2.47	2.24	2.07	2.11
Glyceryl triacetate	4.27	4.63	2.28	2.00	1.82
Methyl *n*-butyrate	0.59	0.69	0.84	0.80	0.75
Benzyl acetate	.22	.22	.27	.38	.40
Ethyl acetate	.19	.26	.42	.45	.39
Methyl acetate	.26	.30	.52	.59	.55
Ethyl *n*-butyrate	.25	.47	.80	.69	.70
Methyl benzoate	.00	.00	.00	.01	.00
Ethyl benzoate	.00	.07	.00	.02	.00
*iso*Butyl acetate	.64	.51	.32	.35	.32

[4] Compare Falk, Noyes and Sugiura, *J. Biol. Chem.*, *J. Gen. Physiol.*, and *J. Cancer Res.*, for papers published since 1921.

The mixtures were brought to the requisite hydrogen-ion concentration initially with hydrochloric acid or sodium hydroxide, the indicators recommended by Clark[5] being used. One such series of results is shown in Table I.

These results indicate clearly that with glyceryl triacetate optimum action is found at about P_H 5.0 or slightly more acid, with phenyl acetate, at about P_H 5.0 although the trend is not so clear; with methyl and ethyl butyrates, however, at P_H 6.0 to 7.0. The differences between glyceryl triacetate and the butyrates are unmistakable. *iso*Butyl acetate appears to be more closely allied to phenyl acetate. The benzoates gave no action, while ethyl, methyl and benzyl acetates may be grouped together. No buffer substances were added in these tests so that for the solutions more alkaline than P_H 5.0, a gradual reversion to this value took place. Buffer mixtures were not used because of the probable influence of added substances with these sensitive enzyme actions. The striking feature of these results, which was borne out repeatedly, is the difference in optimum hydrogen-ion concentration of the material depending upon the specific substrate used.

Haley and Lyman[6] found the value for the optimum action of the castor bean lipase on olive oil to be close to P_H 5.0, while Willstätter and Waldschmidt-Leitz[7] found the value P_H 4.7 for olive oil and castor oil. Compared with the results presented here, it is of interest to note the similar optimum actions with olive and castor oils as compared with glyceryl triacetate and, to a less extent, with phenyl acetate.

A comparison of these values for optimum actions with those of the optimum actions of lipases of animal origin would lead too far here. One point may, however, be mentioned since it is of significance for all enzyme actions although naturally to different extents. In studying the conditions for the effect of hydrogen-ion concentration on lipase actions, two separate factors are involved; first, the enzyme action at the given hydrogen-ion concentration, and second, the stability (or rate of inactivation) of the lipase at that hydrogen-ion concentration. A detailed discussion of these questions and the experimental evidence bearing on them, was presented elsewhere[8] and will not be repeated here.

Kinetics of Reaction.—Two series of results are shown in Fig. 1 (Expts. 58 and 59). These were obtained with tests of 0.2 g. of Pressed Castor Bean Preparation No. 1, 5.1 milli-equivalents of glyceryl triacetate (Curve A) and of methyl *n*-butyrate (Curve B), 15 cc. of water, and 1 cc. of toluene.

[5] Clark, "The Determination of Hydrogen Ions," Williams and Wilkins Co., Baltimore, 2nd edition, 1923.

[6] Haley and Lyman, THIS JOURNAL, **43**, 2664 (1921).

[7] Willstätter and Waldschmidt-Leitz, *Z. physiol. Chem.*, **134**, 161 (1924).

[8] Noyes, Sugiura and Falk, *J. Biol. Chem.*, **55**, 653 (1923).

The tests were started at P_H 5.0. Each result shown in the figures is the average of two separate determinations, corrected for blanks in the customary way. Each measurement represented an independent determination from the beginning. This may have resulted in greater irregularities in the curves but, on the other hand, the cumulative effect of errors which might result if portions were taken from a large volume of mixture is eliminated.

Ester-hydrolyzing actions of the two preparations were read from the curves of the actions for definite time intervals and these values used to

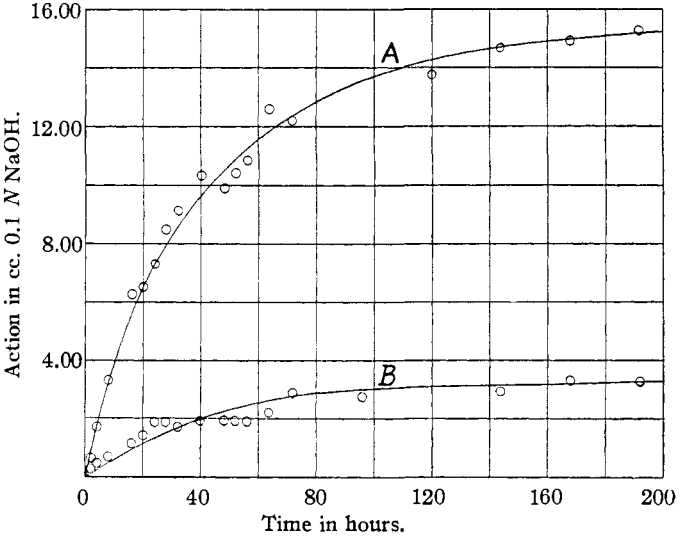

Fig. 1.—Curve *A*—Hydrolyzing actions of castor bean preparation on glyceryl triacetate. Curve *B*—Hydrolyzing actions of castor bean preparation on methyl *n*-butyrate.

calculate the constants for three of the kinetic equations which have been proposed. These calculated constants are given in Table II. K represents the constant of the simple monomolecular reaction velocity equation. K_S represents the constant of Schütz's equation, $K = \frac{x}{\sqrt{T}}$, in which x is the amount of acid formed (or ester hydrolyzed) in time T. "K_N" represents the constant of Northrop's equation,[9] $K_N = \frac{A \log e \frac{A}{A-x} - x}{ET}$, in which A represents the initial substrate concentration, x, the amount hydrolyzed in time T, and E, the enzyme concentration, assumed constant in these experiments.

[9] Northrop, *J. Gen. Physiol.*, **2**, 471 (1919–20).

TABLE II

CONSTANTS OF REACTION VELOCITY EQUATIONS CALCULATED FROM RESULTS TAKEN FROM CURVES IN FIG. 1

Time Hours	$K \times 10^4$ Expt. 58	$K \times 10^4$ Expt. 59	$K_S \times 10^2$ Expt. 58	$K_S \times 10^2$ Expt. 59	$K_N \times 10^4$ Expt. 58	$K_N \times 10^4$ Expt. 59
5	16.0	76	17.0	86.3	56	36
10	14.0	76	22.8	119	..	131
20	13.0	68	28.4	145	20	226
30	11.0	60	30.7	153	7	257
40	10.0	53	31.9	155	8	272
50	9.0	48	31.8	155	9	277
60	8.1	44	31.1	153	13	272
80	6.5	37	29.1	145	40	248
100	5.6	32	27.3	140	10	255
120	4.8	28	25.9	132	7	203
140	4.3	24	25.1	123	7	183
160	3.9	21	24.4	116	5	163
180	3.7	19	24.2	110	6	147

These calculations show essentially the same features that have been shown in the results of others with other enzymes as well as with lipases. At times, constant values have been obtained over extended time ranges, but such results appear to be exceptional. As found here, the values of K decrease constantly, those of K_S increase, remain constant and then decrease, those of K_N show essentially the same changes. The theoretical bases are, however, more satisfactory for K_N than for K_S, although Arrhenius[10] has developed a theoretical significance for the latter. A closer analysis of the results will not be given here. It would cover essentially the ground covered by Northrop in connection with proteolytic actions and that given elsewhere with the kinetics of ester-hydrolyzing actions with enzyme material from animal sources.[11]

Solubility Experiments.—Some years ago[12] a number of experiments were described in which it was shown that water extraction removed certain ester-hydrolyzing material from castor beans and that sodium chloride solutions removed substances showing different ester-hydrolyzing properties. Recently Willstätter and Waldschmidt-Leitz[7] stated that the lipase of castor beans is completely insoluble in water and in sodium chloride solutions, and that the actions found previously were due to proteolytic actions of the castor beans. They also described certain experiments which do not bear upon the question and devote much space to an entirely different technique.

Repetition of the extraction experiments with water and with sodium chloride solution confirmed in every particular the experimental results

[10] Arrhenius, *Medd. Vetenskapsakad. Nobelinst.*, **1908**, 1, No. 9.

[11] Sugiura, Noyes and Falk, *J. Biol. Chem.*, **56**, 903 (1923).

[12] Falk and Sugiura, THIS JOURNAL, **37**, 217 (1915). Falk, *J. Biol. Chem.*, **31**, 97 (1917).

given in the previous papers and contradicted the statements of Willstätter and Waldschmidt-Leitz. In addition to testing the hydrolyzing actions of the different extracts on the esters used before, additional esters were used, and also the protease actions were studied on the proteins present in the extracts themselves, and upon added protein preparations. The formol titration method was used for testing the protease actions. After incubation for suitable lengths of time, the solutions to be tested were titrated with 0.1 *N* sodium hydroxide solution using phenolphthalein as indicator, to a definite pink color, 10 cc. of 40% formaldehyde solution neutral to phenolphthalein was added, and the mixture titrated again to a definite pink color with sodium hydroxide solution. The first titration value (of free acid present) will be designated as the "direct" titration, the second (after formaldehyde addition) will be designated as "formol" titration. It was shown in a previous study of protease actions of various tumor and tissue extracts that the results obtained in this way were practically identical with the results obtained by the Van Slyke amino nitrogen method where the increases in free amino groups on proteolysis were determined.[13] The method as used may be accepted as showing with a reasonable degree of accuracy the extent of protease action as the term is commonly understood. The necessary blanks were tested in every experiment. These included titration, both direct and formol, of the mixtures as soon as prepared, as well as similar titrations of the extracts without the addition of substrates, and of substrates without the presence of extracts containing enzymes, after incubation.

On complete experiment (Expt. 70) will be given. The results obtained were confirmed repeatedly but in place of giving a large mass of individual results, this experiment will serve to show their general nature. One hundred g. of castor bean preparation was made up to 2500 cc. with water, toluene was added and the mixture was allowed to stand at room temperature overnight and occasionally shaken, then centrifuged and the liquid filtered through hardened paper. A clear filtrate (B) was obtained. The residue from the water extraction was made up to 2250 cc. with 10% sodium chloride solution. (Certain losses are unavoidable in the manipulations. In order to obtain approximately the same concentrations for extraction with the different solvents a loss of 10% of the solid was assumed to occur in the manipulations attending the extraction with water. The error introduced by this approximate estimate of loss would be of minor magnitude in the present instance, in the determination of the enzyme actions.) Toluene was added and the mixture allowed to stand overnight with occasional shaking. A clear filtrate (C) was obtained by centrifuging and filtering through hardened paper. The residue (D) was dried by suction

[13] Falk, Noyes and Sugiura, *J. Biol. Chem.*, **53**, 75 (1922). Compare also J. H. Northrop, *J. Gen. Physiol.*, **3**, 715 (1920–21).

and its lipase content determined. The lipase content of the original Castor Bean Preparation A was also determined. The experimental results are shown in Fig. 2.

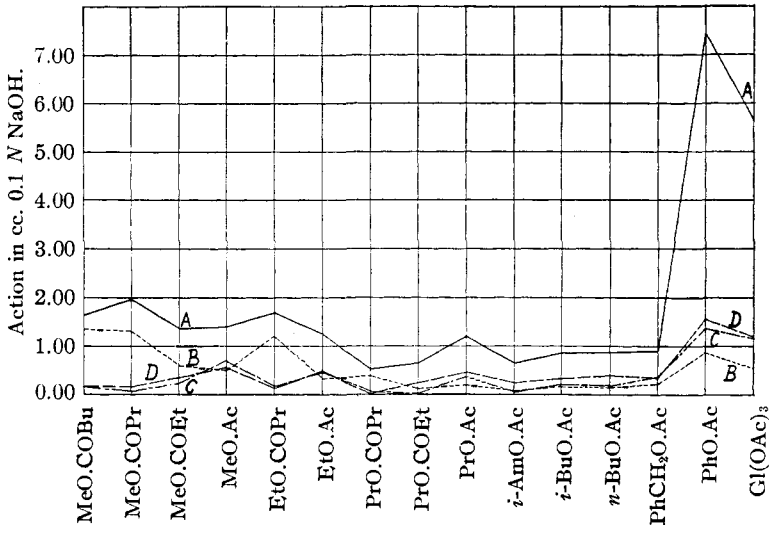

Fig. 2.—Ester-hydrolyzing actions.

A. Portions of 0.3 g. of Castor Bean Preparation in 15 cc. water, equal to 20 mgs. per cc.

B. Water extract, diluted to double its volume with water and 15cc. portion used. Solution equivalent to 20 mg. of Castor Bean Preparation per cc. of solution.

C. Sodium chloride solution extract, diluted to double its volume with water and 15cc. portions used. Solution equivalent to 20 mg. of Castor Bean Preparation per cc. of final solution (5% sodium chloride content).

D. Portions of 0.3 g. of residue in 15 cc. of water, equivalent approximately to 20 mg. of Castor Bean Preparation per cc. of solution.

Three and four-tenths milli-equivalents of the different esters were used. Toluene was added to each flask; duplicates and the customary blanks were run. The tests were started at P_H 7.0, run at 37.5° for 22 hours and the liquids titrated in the usual way. (Protease actions were measured by the formol titration method in each case. These results will be taken up presently.) The results in the figure are given as cubic centimeters of 0.1 *N* sodium hydroxide solution used corrected for blanks or, in other words, as tenths of milli-equivalents of the esters hydrolyzed by the enzymes, under the conditions of the experiments. In the figure, these values are plotted as ordinates. The esters are arranged at equidistant intervals along the abscissa axis.

The percentage actions in terms of the enzyme actions of the original Castor Bean Preparation are shown in Table III.

TABLE III
ESTER-HYDROLYZING ACTIONS OF CASTOR BEAN PREPARATION
PERCENTAGE OF TOTAL ACTIVITIES FOUND IN EXTRACTS

Ester	Water extract	NaCl extract	Residue	Lost
Methyl valerate	82	9	9	0
Methyl *n*-butyrate	67	2	8	23
Methyl propionate	44	16	25	15
Methyl acetate	35	50	38	−23
Ethyl *n*-butyrate	65	5	7	23
Ethyl acetate	26	36	38	0
Propyl *n*-butyrate	76	10	4	10
Propyl propionate	17	2	33	48
Propyl acetate	16	28	38	18
*iso*Amyl acetate	14	11	38	37
*iso*Butyl acetate	17	22	37	24
n-Butyl acetate	16	21	44	19
Benzyl acetate	25	42	38	−5
Phenyl acetate	12	18	21	49
Glyceryl triacetate	10	20	21	49

Several facts stand out clearly and unmistakably in the results shown in Fig. 2 and Table III. (1) Water and sodium chloride solution extract ester-hydrolyzing materials from castor beans. (2) The water extract hydrolyzes the esters to different extents; with the same alcohol group, the actions increase with increase in the molecular weight of the acid with the same acid group, the actions decrease with increase in the molecular weight of the alcohol. (3) The sodium chloride solution extract as tested shows little or no action on the valeric, butyric and propionic esters; on the acetic esters with the simple alcohol groups the actions decrease with increase in the molecular weight of the alcohol. (4) Methyl valerate and methyl and ethyl butyrates show the greatest actions with the water extracts; phenyl and glyceryl acetates, with the sodium chloride solution extracts, the residues, and the original Castor Bean Preparation. (5) The sums of the three separate activities B, C and D, equaled the action of the original material (A) only in a few instances. Experimental errors account for a part of these differences (especially with methyl acetate the difference found is obviously due to an error); differences in method, such as testing in the presence of sodium chloride in C, the use of dialyzed or undialyzed extracts, etc., also will account for part. Including these possibilities, differences remain which cannot be accounted for and which may be ascribed to inactivation or loss of enzyme properties. This is especially true with propyl propionate, *iso*-amyl acetate, phenyl acetate and glyceryl triacetate.

These results raise a number of questions and suggest various experiments in the way of extraction, etc. A number have been performed but will not be described in detail here. The main object has been to show that water and sodium chloride solutions extract ester-hydrolyzing substances

from castor beans, contrary to the statement of Willstätter and Waldschmidt-Leitz. One experiment has been given. Other experiments give results which vary to a certain extent from those shown, due to differences in the nature of the materials, uncontrollable conditions which influence the actions of materials as sensitive to external influences as the ester-hydrolyzing enzymes, etc. At the same time, the general relations obtained have been the same and, if similar conditions of working are followed, the results should be readily reproducible, qualitatively even if not quantitatively.

The effect of the hydrogen-ion concentration on the activities of the different extracts and the residue was studied in several series. Without giving the detailed experiments, it may be stated that the following results were obtained. Working with the dialyzed aqueous and sodium chloride solution extracts, optimum actions on all the esters were found at about P_H 7.0. With the residue, the optimum appeared at about P_H 5.0 with glyceryl triacetate, but at about P_H 7.0 for the rest. This difference in the action of the residue on glyceryl triacetate is striking. These results fall in line with the results on the whole castor bean preparation at different hydrogen-ion concentrations described in the earlier part of this paper.

Some additional facts may be presented to supplement the conclusions arrived at from the experiment given in Fig. 2 and Table III. In Conclusion 5 the question of adding the values of the activities of the two extracts and residue was considered. It was stated that different conditions might influence the results so that the sums of the separate actions would not be equal to the action of the original castor bean preparation. Several experiments showed that testing the sodium chloride solution extract with the sodium chloride present gave smaller actions on several of the esters, notably phenyl acetate, than when the sodium chloride had been removed by dialysis. This difference may amount to 50% of the action of the undialyzed extract. In this way a part of the action of the original castor bean preparation which is apparently lost in the different treatments may be accounted for, but there is still a large percentage of the action on phenyl acetate for example, which does not reappear in the separate fractions.

With reference to some of the properties of the soluble ester-hydrolyzing enzymes of the castor bean it was shown in the earlier work that the water-soluble esterase is inactivated on standing in solution, more rapidly in slightly acid (P_H 4.0) than in neutral (P_H 7.0) solution. Precipitated with acetone or with alcohol, washed and dried, only a small fraction of the original activity is retained. The salt-soluble enzyme material precipitated by dialysis, filtered off, dried by washing with alcohol and ether, loses its activity entirely. When the material is kept in suspension in water, as precipitated by dialysis, or even concentrated by filtration to remove part of the water, the activity appears to be retained indefinitely.

One question remains to be considered, that is, in how far may protease actions be involved in the observations recorded. The fact that blanks were tested with the enzyme material in the absence of esters and the values were subtracted from the results of the enzyme-ester experiments in each case should be sufficient answer.[14] In order to check up the possibility of protease action, the formol titration was carried out with each enzyme and enzyme-ester mixture. In no case was the formol titration greater in the enzyme-ester mixture than in the enzyme material alone. The presence of a protease in castor beans, however, is shown by the fact that with 0.3 g. of the material in 15 cc. of water, the direct titration immediately after mixing was 0.10 cc. of 0.1 *N* sodium hydroxide solution, the formol titration, 0.16 cc.; after 22 hours of incubation at 37.5°, the direct titration was 0.46 cc. and the formol titration, 0.60 cc. With the water and sodium chloride solution extracts such differences were not observed, the proteolysis having evidently gone as far as it could during the period of extraction. These proteolytic actions do not come into question in the lipase tests, since the enzyme material blanks used were those found after incubation.

The castor bean preparation as well as the water and sodium chloride solution extracts showed definite proteolytic actions on added proteins. For the proteins tested, the activities were greatest on casein, next on a peptone preparation, and smallest on gelatin. These protease activities were greater at P_H 7.0 than at P_H 5.0.

It has been shown definitely that the castor bean preparation can be extracted successively by water and sodium chloride solution and different ester-hydrolyzing fractions obtained. One additional experiment (Expt. 73) needs to be described. The castor bean preparation was divided into two parts, one part extracted with water, the other part with 10% sodium chloride solution. These were filtered and the actions of aliquot portions tested on phenyl acetate, glyceryl triacetate, methyl butyrate and ethyl butyrate, separately. Other portions of the two clear filtrates were dialyzed and the resulting liquids (that from the sodium chloride extract containing a large amount of precipitate) tested similarly. In these enzyme tests, each solution as tested was diluted to correspond to 20 mg. of original castor bean preparation per cc. of final solution as tested. The tests were carried out at 37–38° for 22 hours starting at P_H 7.0, with 3.4 milli-equivalents of the esters, and the corrections for blanks introduced as usual. The results are given in terms of cubic centimeters of 0.1 *N* acid produced by the enzyme.

This short table illustrates a number of facts. Sodium chloride solution extracts enzyme material active on all four esters after removal of the salt. The action of the dialyzed salt extract after dialysis is the same as that of

[14] This was also done in the experiments described in the earlier papers, as stated at the time.

the water extract, but the water extracts very much less of the material acting on phenyl acetate and glyceryl triacetate. Other facts appearing from these results have been already stated in different connections and need not be repeated.

TABLE IV

ESTER-HYDROLYZING ACTIONS OF UNDIALYZED AND DIALYZED CASTOR BEAN EXTRACTS

	—10% NaCl extract—		—Water extract—	
Ester	Undialyzed (Tested at 5% NaCl concn.)	Dialyzed	Undialyzed	Dialyzed
Phenyl acetate	2.01	3.07	0.94	0.62
Glyceryl triacetate	1.58	1.46	0.46	0.28
Methyl *n*-butyrate	0.39	1.30	1.49	1.31
Ethyl *n*-butyrate	.36	1.26	1.31	1.11

The original statement made in the earlier papers that water extracts enzyme material from castor beans which acts to a greater extent on simple alkyl butyrates than on glyceryl triacetate (and phenyl acetate) and that sodium chloride solution extracts enzyme material acting to a greater extent on the latter than on the former must be modified in the sense that while the first part of the statement with reference to the water extract holds, the second part should be amended in the sense that sodium chloride solution is able to extract both enzyme materials from castor beans but that the actions on butyrate are obscured by the sodium chloride which, under the conditions used, appears to produce a reversible inactivation, and the action on these esters is evident only after the removal of the salt. Obviously if water is used first to extract the enzyme material active towards butyrates, then the sodium chloride solution acting on the residue from the aqueous extract can only extract the enzyme material active on the acetates.

The results given clearly refute the view of Willstätter and Waldschmidt-Leitz in regard to the solubility of the ester-hydrolyzing materials of the castor bean. Most of the work published by Willstätter and Waldschmidt-Leitz involved the use of olive oil as substrate, and little water in the enzyme tests. Conditions for emulsification were favorable. There is no direct comparison between these results and the solubility results described here. It has been definitely shown that protease activities are not involved here. If the matter is one of nomenclature and it is desired to denote the actions on olive oil as lipase actions and those on the simple fatty esters as esterase actions, there can be no dispute, both actions being fundamentally enzymic in character.

It is inadvisable, to say the least, to be dogmatic in enzyme studies. All that should be said in connection with these experiments is that soluble enzyme materials were obtained in this Laboratory by the methods described previously and in this paper. The failure of Willstätter and

Waldschmidt-Leitz to confirm these experiments, may have been due to the use of different material or to a different method of working.

Recently Platt and Dawson[15] showed that a pancreatic extract having definite hydrolytic actions on ethyl butyrate and on olive oil, had its actions modified by the addition of albumin which increased the first action and decreased the second, and by the addition of edestin (a globulin) which increased both, the second to a much greater extent than the first. They compared these actions with the ester-hydrolyzing actions of the water extract (containing albumin) and sodium chloride solution extract (containing globulin) of the castor bean which showed actions analogous to the pancreatic extract actions after the addition of albumin and a globulin. They consider the same enzyme present in the two sets of preparations and that the properties of the enzyme were largely determined by the accompanying protein.

An attempt was made to test these conclusions by adding small amounts of albumin and edestin to the water and sodium chloride solution extracts of the castor bean preparation. No differences in the ester-hydrolyzing actions on the different esters were observed in the presence of the added proteins, using 20 mg. of the protein to 15 cc. of the extracts corresponding to 0.3 g. of original castor bean preparation. It is possible that the protein material present, derived from the castor beans themselves, obscured the possible actions of the added proteins. It is also possible that the conclusions of Platt and Dawson obtained from their experiments with pancreas do not apply to castor beans where the association of enzyme property, whether connected with a definite chemical group or not, and protein may be so close as to form part of the same molecule. In any event, the results of Platt and Dawson are most suggestive and may aid in throwing additional light on the chemical nature of the active enzyme and its behavior under different conditions.

Summary

An experimental study of the ester-hydrolyzing actions of oil- and husk-free castor beans is presented.

Optimum conditions for the action of the whole preparation on glyceryl triacetate were found at about P_H 5.0, on a number of other simple soluble esters at about P_H 7.0.

The kinetics of the enzymic actions on glyceryl triacetate and on methyl butyrate gave results similar to those with other ester-hydrolyzing enzymes and with proteolytic enzymes. The values of the constants of some of the velocity equations which have been proposed were shown to vary as the reactions proceed. One of the complicating factors in such a study was indicated and reference made to a more complete discussion elsewhere.

[15] Platt and Dawson, *Biochem. J.*, **19**, 869 (1925).

The solubility of ester-hydrolyzing material of the castor bean in water and in sodium chloride solution was confirmed. The action of the extracts and of the residue were studied under various conditions. The statement of Willstätter and Waldschmidt-Leitz with reference to the insolubility of the ester-hydrolyzing material of the castor bean was shown to be incorrect.

NEW YORK, N. Y.

[CONTRIBUTION FROM THE BIOCHEMICAL DEPARTMENT, STATE SCHOOL OF HYGIENE OF WARSAW]

IMPROVEMENTS IN ELEMENTARY MICRO-ANALYSIS. DETERMINATION OF SULFUR AND HALOGENS

BY CASIMIR FUNK AND STANISLAS KON

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In continuation of our study of micro methods[1] we have arrived at certain useful modifications of the sulfur and halogen analysis of the classical and epoch-making methods of Pregl.

We have found first that the so-called "Perlenrohr's" supplied to us were of porous porcelain and therefore retained considerable amounts of material used for cleaning the combustion tube as well as of the material to be determined. To improve this condition we have adapted a different model of combustion tube (Fig. 1) which consists of a Jena glass tube with a capillary tube attached at right angles. The end of the tube is filled with glass beads made of Pyrex glass and carries a constriction to hold the beads in place. The capillary tube reaches to the bottom of a wide Pyrex test-tube in which the precipitation and from which later the filtration on a small Pregl's asbestos filter is effected. Pregl in his book[2] claims that the precipitate of barium sulfate, on account of its high specific gravity, cannot be transferred by vacuum filtration. By diminishing the bore of the filtering tube to a capillary size (about 1 mm.) the filtration is accomplished without difficulty. To avoid the occlusion of barium chloride which leads Pregl to adopt a rather complicated procedure, we have diminished the concentration of barium chloride solution from 10 to 1% and therefore diminished the possible error ten times. The procedure as modified by us renders the sulfur analysis simpler and speedier and, as a matter of fact much better, than the macro-analysis. We have convinced ourselves that the tube used by us can be used with equal success for the halogen determination. For determination of halogens in substances free from sulfur (and probably phosphorus) we have adapted the excellent procedure

[1] Casimir Funk, "Mikroanalyse nach der Mikro-Dennstedt Methode." Bergmann, Munich, 1925. Funk and Kon, *J. Chem. Soc.*, **127**, 1754 (1925).

[2] Pregl, "Die quant. organische Mikroanalyse," 2nd ed., Springer, Berlin, 1923.