m. p. 87–87.3°. The compound is soluble in dilute hydrochloric acid, alcohol, ether and benzene; insoluble in dilute sodium hydroxide and petroleum ether.

Anal. Calcd. for $C_{12}H_{11}O_3NS$: N, 5.62; S, 12.87. Found: N, 5.58, 5.60; S, 12.62, 12.67.

o-Benzoylaminophenyl Benzenesulfonate. A. From o-Benzoylaminophenol.—Five grams of *o*-benzoylaminophenol was treated with 5 cc. of 20% sodium hydroxide solution and 4.4 g. of benzenesulfonyl chloride by the Schotten–Baumann reaction. The product was crystallized from hexane; yield, 6.3 g. or 76% of the theoretical; m. p. 90.5–91°. About 200 cc. of hot hexane was required to dissolve each gram of the compound.

B. From o-Aminophenyl Benzenesulfonate.—Ten grams of *o*-aminophenyl benzenesulfonate was refluxed in 150 cc. of ether for two hours with 5.7 g. of benzoyl chloride. The precipitate was filtered off and washed with ether. The ether solution was washed with dilute hydrochloric acid, 5% sodium hydroxide, and water and dried over anhydrous calcium chloride. The ether was evaporated off and the product so obtained crystallized repeatedly from hexane; yield, 11 g. or 78% of the theoretical, m. p. 90.5–91°. It is soluble in ether, alcohol and toluene. It is insoluble in water, dilute acid, dilute alkali and petroleum ether. When mixed with the preparation from A the melting point remained unchanged.

Anal. Calcd. for $C_{12}H_{15}O_4NS$: N, 3.96; S, 9.08. Found: N, 3.79, 3.86; S, 8.70, 8.71.

The authors are indebted to Dr. H. G. Shaw and Mr. G. A. Barber for some of the analytical data.

Summary

1. Satisfactory methods have been developed for preparing *o*-benzenesulfonylaminophenol and *o*-benzenesulfonylaminophenyl benzenesulfonate.

2. A new benzenesulfonyl derivative and a new mixed benzoyl benzenesulfonyl derivative of *o*-aminophenol have been prepared and studied.

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[CONTRIBUTION NO. 294 FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF PITTSBURGH]

The Purification, Specificity and Inhibition of Liver Esterase

BY ZELMA BAKER AND C. G. KING

The primary purpose of the present investigation has been to devise a rapid and uncomplicated method of purifying liver esterase, and then to study the influence of purification upon competitive inhibition and substrate specificity.

The product obtained by Willstätter and Memmen[1] from a procedure involving adsorption, elution and electrodialysis still contained protein material. Using a similar complicated procedure, Kraut and Rubenbauer[2] reported that an esterase preparation had been obtained which was free from protein or protein-like material (apparently based upon a negative Millon reaction in dilute solution). Their product was estimated to be thirty-five times more active than the original extract, and was characterized by a rapid loss in activity after being separated from proteins in solution. It is difficult to evaluate their findings in relation to the degree of purification accomplished per unit of total solids, or to the nitrogen content of the active solution used.

By comparing the activity of enzyme preparations at different stages of purification on a number of possible ester-type substrates, it was possible to differentiate clearly between the ethyl butyrate hydrolyzing enzyme and several other recognized esterases occurring in animal tissues. The primary functional role (*e. g.*, natural substrates) of liver esterase is still largely a matter of conjecture.

The problem of interpreting competitive and non-competitive inhibition phenomena is facilitated by the use of highly purified enzymes, because the surface activity and specific group reactions of concomitant proteins frequently influence the behavior of the enzyme being studied. Our investigations strongly indicate that the liver esterase is itself a typical albumin.

Experimental

Procedure.—The general technique used in preparing an esterase extract of liver powder and in measuring enzyme activity and inhibition has been described previously.[3] After extraction of the enzyme by 0.025 *N* ammonia and precipitation of non-esterase proteins by acetic acid, the solution was 0.4 saturated with sodium sulfate at 37°. The inactive precipitate was removed by filtration and a second precipitation made (*p*H 6.5) by fully saturating with sodium sulfate at 37°. After filtering, the flocculent residue was dissolved in distilled water and dialyzed in a collodion bag against distilled water for three days at 10°, and again filtered. Total organic solids were determined in 20-cc. samples by evaporating to dryness at a tempera-

(1) Willstätter and Memmen, *Z. physiol. Chem.*, **138**, 216 (1924).
(2) Kraut and Rubenbauer, *ibid.*, **178**, 103 (1928).
(3) Glick and King, *J. Biol. Chem.*, **94**, 497 (1931).

ture high enough to remove water from the hydrated salts, and igniting. Nitrogen values were obtained by the method of Koch and McMeekin.[4] Phytin hydrolysis was measured by the method of Adler,[5] except that the phosphate was determined volumetrically.[6] Sodium beta-glycerophosphate[7] and calcium hexose diphosphate were used in 1% solutions, phosphate being determined in the same manner as above. The hydrolysis of lecithin was followed by the method of Akamatsu,[8] except that titrations were carried out in alcoholic solution to obtain sharper end-points. Tannase activity was based upon the hydrolysis of methyl gallate, as described by Freudenberg.[9] Phenyl potassium sulfate was used as a substrate for sulfatase activity as suggested by Neuberg.[10]

Results and Discussion

A number of salts such as $MgSO_4$, $CaCl_2$, K_2SO_4, Li_2SO_4 and $(NH_4)_2SO_4$ were found to yield active precipitates, but all of these were less satisfactory than Na_2SO_4. The lower alcohols and acetone were also found to yield active precipitates which could be centrifuged readily, but there was always a loss in total activity which made their use less satisfactory than Na_2SO_4. The strong dehydrating power of Li_2SO_4 resulted in almost complete destruction of the enzyme during precipitation and centrifuging. In all of these precipitation studies, the most active fractions corresponded to an albumin, in marked contrast to the behavior of pancreas lipase as a typical globulin.[11]

Table I shows the average effect of a series of runs, correlating enzyme activity with total organic solids and non-ammonia nitrogen at different stages in the procedure. The nitrogen content of all active preparations was typical for a protein, *i. e.*, approximately 18%, and the final product was readily dispersed in distilled water.

TABLE I

ESTERASE ACTIVITY PER UNIT TOTAL SOLIDS AND PER UNIT NITROGEN, USING ETHYL BUTYRATE AS SUBSTRATE, AND DIRECT TITRATION OF ACID AT *p*H 7.5 TO 8

Treatment of enzyme	0.01 *N* NaOH per mg. of total solids, during 25 min., cc.	0.01 *N* NaOH per mg. of nitrogen during 25 min.
NH_4OH extract of liver powder	2.1	23
Soln. after HAc pptn.	3.7	42
Soln. of ppt. by satd. Na_2SO_4	9.6	58
Na_2SO_4 ppt. after dialysis	24.0	132

The most active liver esterase preparations showed no activity when tested against phytin, methyl gallate, or phenyl potassium sulfate as substrates, and only a very faint activity upon lecithin. It is therefore clear that the ethyl butyrase activity is entirely distinct from that of phytase, tannase, sulfatase or lecithinase.

The use of sodium β-glycero phosphate and calcium hexose diphosphate as substrates gave very different results, however. The crude extract showed fairly high phosphatase activity, and this increased at the stage of sulfate precipitation, followed by a decrease during dialysis, as summarized in Table II for two typical runs. The results may be taken as an indication that both the esterase and phosphatase are albumins, the latter being more sensitive to denaturation. During dialysis there was always a slight precipitation of protein material which did not involve a significant loss in esterase activity. We have made no attempts to concentrate or study the phosphatases as separate entities.

TABLE II

PHOSPHATASE ACTIVITY OF ESTERASE PREPARATIONS

Mg. of phosphorus hydrolyzed per mg. of organic solids in enzyme soln.

Source of enzyme	Glycerophosphate as substrate, 24 hrs., 37°	Hexose diphosphate as substrate, 48 hrs., 37°
Powder extract after HOAc pptn.	0.11	0.11
	.06	.16
Soln. of satd. Na_2SO_4 ppt.	.38	.23
	.35	.22
Dialyzed soln.	.17	.15
	.13	.13

The effect (*i. e.*, the percentage inhibition) of typical organic competitive inhibitors was not markedly influenced by the degree of purification accomplished. With amyl alcohol, for example, over a concentration range of 10 to 70 $\times 10^{-6}$ mole in 8 cc., the crude extract was inhibited only about 5% less than the highly purified product. Octyl alcohol and hexylresorcinol gave similar results. If the inhibiting effect of such compounds is due in major degree to a reversible combination with the active centers, then it would appear that the amount distributed over the surfaces of concomitant proteins was relatively small compared to the total amount in solution. Otherwise there would be a marked increase in inhibition (with a given concentration of inhibitor) when

(4) Koch and McMeekin, THIS JOURNAL, **46**, 2066 (1924).
(5) Adler, *Biochem. Z.*, **75**, 319 (1916).
(6) Hillebrand and Lundell, "Applied Inorganic Analysis," John Wiley & Sons, New York, 1929, p. 567.
(7) Kay and Lee, *J. Biol. Chem.*, **91**, 135 (1931).
(8) Akamatsu, *Biochem. Z.*, **142**, 184 (1923).
(9) Freudenberg, Blümmel and Frank, *Z. physiol. Chem.*, **164**, 262 (1927).
(10) Neuberg and Kurono, *Biochem. Z.*, **140**, 295 (1923).
(11) Glick and King, THIS JOURNAL, **55**, 2445 (1933).

the enzyme had been purified to twelve times the activity of the original extract. The findings of Willstätter[12] and Rona[13] and associates, that purification did not markedly change the optical specificity toward substrates, are of interest in relation to this interpretation. Rona and Itelsohn-Schlechter followed a complicated procedure, like that of Kraut and Rubenbauer,[2] for purifying the enzyme about ten times beyond that of the ammonia extract. Bamann and Laeverenz[14] found no change in optical specificity when alkaloid inhibitors were used with an enzyme preparation which had been purified to ten times the activity of the dialyzed solution after acetic acid precipitation.

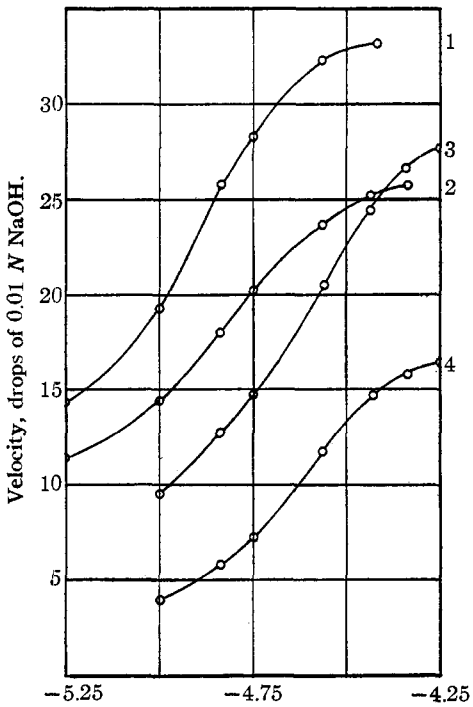

Fig. 1.—Activity–pS curves: 1, without inhibitor; 2, with 9.5×10^{-6} mole of NaF; 3, with 3.65×10^{-6} mole of 2-octyl hydrogen phthalate; 4, with 14.6×10^{-6} mole of 2-octyl hydrogen phthalate.

A number of organic compounds which showed typical competitive inhibition at low concentrations were found, from a study of their influence on the Km value (dissociation constant) for the enzyme–substrate complex,[15] to exert a non-competitive effect at higher concentrations. Figure 1 shows the effect of increasing 2-octyl hydrogen phthalate concentration, in relation to the curve for substrate alone and compared with a typical non-competitive inhibitor, sodium fluoride. As pointed out by Sobotka and Glick,[16] these differences tend to be quantitative rather than qualitative and provide a basis for explaining the phenomena of accelerated enzyme activity by added substances and of inhibition by excess substrate. All of the inhibitors studied (*n*-amyl alcohol, *n*-amyl chloride, methyl hexyl carbinol, 2-octyl hydrogen phthalate, methyl hexyl ketone, hexylresorcinol and phenol) exhibited the same phenomenon in varying degree, *i. e.*, a transition from competitive to non-competitive type inhibition, with the point of 0.5 maximum velocity being displaced only toward the right at low concentrations and downward at higher concentrations. Phenol resembled sodium fluoride more closely than any of the other compounds studied.

The tendency for compounds of different types to combine with different groups on the enzyme is reflected in their exhibiting greater than an additive inhibitory effect when both are present in solution with the enzyme. Typical examples of many such measurements are shown in Table III. The data were taken from carefully constructed curves over a wide range of concentration,

TABLE III

INHIBITION OF LIVER ESTERASE BY MIXED COMPETITIVE INHIBITORS

Present in equiv. amt.	Inhibn. by each alone, %	Inhibn. by 2 × the concn. of first alone, %	Observed inhibn. by mixture, %	Diff.
n-Amyl alc. and	17	31	31.6	+0.6
2-octyl alcohol	27	43	43.4	+ .4
	53	68	70.0	+2.0
2-Octyl alc. and	27	42	49.5	+7.5
methyl hexyl ke-	34	51	59.5	+8.5
tone	39	56	63.0	+7.0
2-Octyl alc. and	30	44	51.2	+7.2
2-octyl hydrogen	42	58	64.5	+6.5
phthalate	51	65	71.6	+6.6

and the solutions with mixed inhibitors were made up directly from the same standard solutions used in determining the individual inhibition curves. It will be noted that inhibitors of similar polarity and similar reactive groups showed simple additive effects, while those of different polarity and different active groups showed a combined inhibitory power distinctly greater

(12) Willstätter, Bamann and Waldschmidt-Graser, *Z. physiol. Chem.*, **173**, 155 (1927).

(13) Rona and Itelsohn-Schlechter, *Biochem. Z.*, **197**, 482 (1928).

(14) Bamann and Laeverenz, *Z. physiol. Chem.*, **193**, 201 (1930).

(15) Michaelis and Menten, *Biochem. Z.*, **49**, 33 (1913).

(16) Sobotka and Glick, *J. Biol. Chem.*, **105**, 199 (1934).

than an additive effect. In other words, when the inhibitors tend to combine with different groups on the enzyme, each exerts a greater inhibition in the presence of the other than would be true if they were competing for the same group and therefore complying with the decreasing slope of the inhibition curve. The fact that divergence from a simple additive effect may begin at relatively low concentrations and then either remain constant or vary in degree with rising concentration, provides strong evidence for there being two or more reactive groups in the so-called active center (which is directly involved in competitive inhibitors).

Summary

A relatively simple procedure has been described for purifying liver esterase to twelve times the activity of a liver powder extract. The product obtained in repeated experiments had the characteristics of a typical albumin.

By checking the activity of the enzyme at different stages of purification on a number of ester-type substrates, it was shown clearly that the ethyl butyrase was entirely distinct from a lecithinase, sulfatase, tannase or phytase. Beta-glycero phosphatase and hexose diphosphatase activity followed ethyl butyrase activity up to the dialysis stage in the purification procedure, but then decreased, indicating their association with the albumin fraction, but also indicating their separate identity from the butyrase.

Inhibition studies with amyl alcohol, amyl chloride, 2-octanol, 2-octanone, phenol, hexylresorcinol, 2-octyl hydrogen phthalate, and sodium fluoride, showed that there was a characteristic tendency for the competitive inhibition type curve to undergo a gradual transition to a non-competitive inhibition type curve at higher concentrations.

With two inhibitors of similar chemical type present in equivalent amount, the inhibition exerted was a simple additive effect, but when the two inhibitors were of different chemical types, the inhibition exerted was greater than an additive effect, indicating their attachment to different groups on the enzyme, and hence offsetting the declining slope of the inhibition curve, with higher concentrations.

PITTSBURGH, PA. RECEIVED NOVEMBER 26, 1934

[CONTRIBUTION FROM THE COBB CHEMICAL LABORATORY, UNIVERSITY OF VIRGINIA, NO. 149]

Reduction Studies in the Morphine Series. V. Dihydro- and Tetrahydro-pseudocodeine Methyl Ethers[1]

BY LYNDON SMALL AND ROBERT E. LUTZ

In the previous papers of this series[2] we have shown that reduction of pseudocodeine types may result in at least seven different products, namely, the dihydro isomers -A, -B, and -C, the tetrahydro derivative, dihydrodesoxycodeines-B and -C, and tetrahydrodesoxycodeine. The relative amounts of these compounds formed depend upon the reduction conditions, and to some extent upon the pseudocodeine analog selected. The direct reductive elimination of the alcoholic hydroxyl group by which the desoxycodeine derivatives are formed is a new type of reaction in the morphine series, and reduction experiments have been extended to the methyl ether series to determine whether a methoxyl group can be similarly removed. Pseudocodeine methyl ether was selected for this purpose because of its relative accessibility and because its reduction should lead to new derivatives for study of the interesting physiological effects associated with the covering of the alcoholic hydroxyl group in the morphine and codeine series.[3]

In respect to catalytic hydrogenation, pseudocodeine methyl ether (I) behaves like pseudocodeine, yielding principally either dihydropseudocodeine-A methyl ether (II) or tetrahydropseudocodeine methyl ether (III) by the different methods of hydrogenation previously described. As in the case of pseudocodeine, no tetrahydro desoxycodeine was found; in contrast to allo

(1) The work reported in this paper is part of a unification of effort by a number of agencies having responsibility for the solution of the problem of drug addiction. The organizations taking part are: The Rockefeller Foundation, the National Research Council, the U. S. Public Health Service, the U. S. Bureau of Narcotics, the University of Virginia, and the University of Michigan.

(2) THIS JOURNAL, (a) **54**, 4715 (1932); (b) **56**, 1741, (c) **56**, 1928, (d) **56**, 2466 (1934).

(3) N. B. Eddy and co-workers, University of Michigan, unpublished results.