5-hexyl-1-phenyl-barbituric acid were tested with mice. The former was inert and the latter toxic without preliminary hypnotic effect.

Summary

A number of derivatives of *n*-hexylmalonic acid are described. Chief among these in point of interest is ethylhexylbarbituric acid, which is a powerful hypnotic and may prove to be of therapeutic value.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY, No. 450]

THE ISO-ELECTRIC POINT OF MALT AMYLASE[1]

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The experiments reported in this paper originated in attempts to standardize further the methods which have been developed here for obtaining purified and highly active malt amylase preparations. Work in this Laboratory has repeatedly pointed either to the protein nature of this amylase or to protein as an essential constituent of it. Since proteins are least soluble at their iso-electric points, it was thought, provided the enzyme is protein or intimately associated with protein, that it might be separated from inactive proteins found in crude extracts by precipitating at the iso-electric point of the enzyme. We have, therefore, studied the iso-electric point by electrophoresis experiments with solutions of malt amylase adjusted to a wide range of hydrogen-ion concentrations by means of buffer solutions.

Extensive preliminary experiments with commercially concentrated malt extracts adjusted to different hydrogen-ion concentrations showed that while there was some evidence of electrophoresis of the active substance, consistent results could not be obtained with the crude material. This was probably due to the presence in the extracts of relatively large amounts of inert proteins of varying iso-electric points which may have masked the electrophoresis of the amylase.

Entirely consistent evidence of electrophoresis was, however, obtained from experiments upon solutions of purified malt amylase which had been made in this Laboratory by our modification of the Osborne method as described in previous papers.[2,3]

Experimental Part

The Apparatus and Technique finally adopted as satisfactory may be described briefly as follows. The U-tube apparatus of Michaelis[4] was

[1] The expenses of this investigation were shared by the Carnegie Institution of Washington and the Department of Chemistry of Columbia University.

[2] Osborne, THIS JOURNAL, **17**, 587 (1895); **18**, 536 (1896).

[3] Sherman and Schlesinger, *ibid.*, **35**, 1617 (1913); **37**, 643, 1305 (1915).

[4] Michaelis, *Biochem. Z.*, **16**, 81 (1909).

modified to give less possibility of diffusion and of contamination of the solutions to be tested as shown in Fig. 1.

The connecting flasks (D) were kept as a leveling device but the electrodes were placed in cells[5] (C and A) which dipped into the connecting flasks and which have stopcocks in the connecting tubes or salt bridges. These stopcocks (4 and 5) were greased in such a way that the current would still be carried by a film of the liquid when they were closed. Diffusion of the solutions surrounding the electrodes into the rest of the apparatus was thus decreased and further guarded against by the presence of the connecting flasks (D), which acted as traps as well as leveling devices. Changes in the level of the liquid in the U-tube, which may be caused by the evolution of gases at the electrodes and which, even when slight, give rise to noticeable errors were also thus minimized.

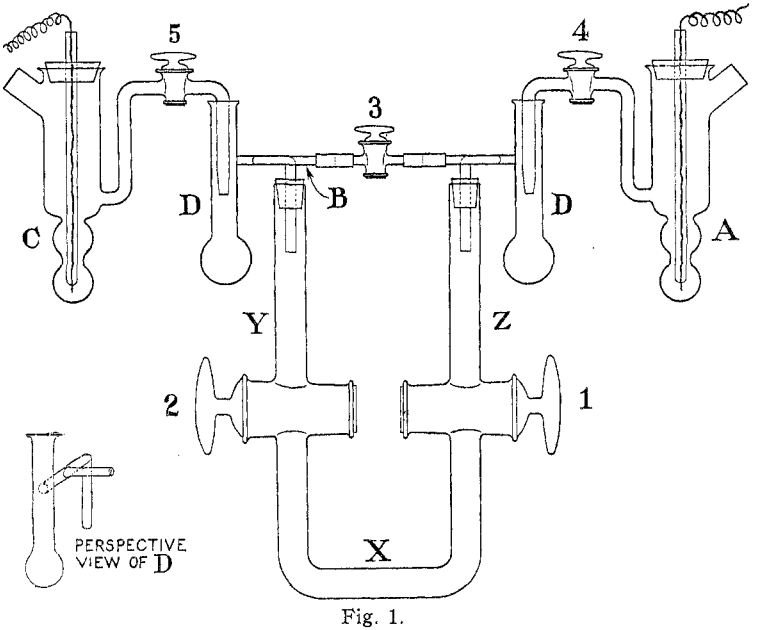

Fig. 1.

Buffer solutions of the desired hydrogen-ion concentrations were prepared by mixing *M* monosodium phosphate with 0.5 *M* disodium phosphate according to electrometric titrations. The enzyme preparation was dissolved in a small portion of buffer solution containing 2% of pure sucrose to increase its density. The bend (X) and stopcocks (1 and 2) of the U-tube were filled with this weighted solution, the stopcocks (1 and 2) were then closed with the careful exclusion of all bubbles and the arms (Y and Z) thoroughly washed. The U-tube was then securely fastened in an ice-bath, the arms (Y and Z) of the tube were filled with buffer solution containing no sucrose, the connecting flasks and leveling bridge (B) inserted and the whole was filled with the same buffer solution, care being taken to exclude all bubbles and to adjust the level of the solution in the connecting flasks. The electrode vessels (C and A) with their salt bridges were also filled

[5] The glass vessel known as the Fales calomel cell was used for these cells. [Fales and Vosburgh, THIS JOURNAL, **40**, 1291 (1918)].

with the same buffer solution and inserted in the connecting flasks (D). Solid copper sulfate and an electrode made by enclosing a copper wire in glass were placed in the electrode vessel (C) on the cathode side, and solid monosodium phosphate[6] and a platinum electrode in the electrode vessel (A) on the anode side. In adjusting the level of the whole apparatus care was taken always to have the solution pass from the connecting flasks into the electrode vessels rather than in the opposite direction in order to avoid contamination of the solutions with the electrode reagents. A 220-volt direct current was then turned on while the stopcocks of the calomel-cell salt bridges and of the leveling bridge were open and those of the U tube closed. The stopcocks 4 and 5 were then closed in such a manner as to have the current conducted by a film of the buffer solution around them. The stopcock (3) of the leveling bridge was next closed as tightly as possible and those in the U-tube (1 and 2) were opened simultaneously, thus allowing the current to pass through the whole system. An ammeter was inserted to give indications of the amount of current obtained during the setting up of the apparatus and during the experimental period. Experience showed that 24 to 36 hours was a satisfactory length of time for the electrophoresis. At the expiration of this time Stopcocks 1 and 2 were simultaneously closed, the current discontinued and the solutions in the arms (Y and Z) and in the bend (X) were tested for enzymic activity. Since inconsistencies due to changes in level and to the tipping of the apparatus have been eliminated, and since the enzyme was originally confined to the bend of the U-tube and the possibility of its diffusion into the arms of the U-tube is equal on both sides, any differences in enzymic activity found in the solutions in the arms of the U-tube can properly be attributed to a migration of the active material caused by the electric current, provided these solutions are tested for amylolytic power under strictly comparable conditions.

The relative concentrations of the amylase in the solutions from the "anode" and "cathode" arms were determined by a modification of the gravimetric method usually employed in this Laboratory.[7,8]

One per cent. starch dispersions activated and adjusted to the proper Sörensen value P_H as described below were prepared and placed in 100cc. portions in cylinders in the 40° thermostat to reach the desired temperature. Equal aliquot portions of the solutions to be tested for activity were pipetted into 200cc. Erlenmeyer flasks. The starch dispersions were then added at intervals of 15 seconds and the flasks placed in the 40° bath. At the end of 30 minutes, enzymic action was stopped by pouring 50 cc. of Fehling's solution into the digestion mixtures at intervals of 15 seconds and in the same order in which the starch was poured onto the enzyme. The amount of reducing sugar formed was determined by immersing the flasks in a boiling water-bath for 15 minutes. The cuprous oxide was filtered into weighed Gooch crucibles, washed with hot water, alcohol and ether, dried at 100° and weighed. Blank determinations with portions of the same starch pastes and buffer solutions were carried out in the same manner and suitable corrections made in the final weights of cuprous oxide which thus represent the relative concentrations of amylase present in the solutions.

[6] The use of sodium chloride at the anode is objectionable when working with a sensitive enzyme, due to the liberation of chlorine.

[7] Sherman, Kendall and Clark, THIS JOURNAL, **32**, 1073 (1910).

[8] Sherman and Walker, *ibid.*, **43**, 2461 (1921).

The solutions obtained from the arms of the U-tube were measured and made up to the same volume, care being taken to dilute them as little as possible. An aliquot portion of each was then taken to determine hydrogen-ion concentration and the remainder was used either in whole or in part to determine its enzymic activity. The hydrogen-ion concentration of the solution in the bend of the U-tube was accurately determined at the end of each experiment. This value was usually the same as that of the original solution although there was occasionally a slight change, as may be seen in Table I.

Since the concentrations of hydrogen ion of the buffer solutions from the different electrophoresis experiments varied widely from one another and often from the optimum which has been established for digestion experiments with this enzyme,[9] careful adjustments had to be made to insure the optimum hydrogen-ion concentration of the digestion mixtures. This was accomplished most satisfactorily by adjusting the P_H value of the starch pastes rather than that of the enzyme solutions. Portions of each original buffer solution corresponding to the amounts to be tested for activity were added to portions of activated starch pastes and the hydrogen-ion concentrations adjusted to the optimum by electrometric titrations. The starch pastes could thus be prepared for each experiment in such a way as to eliminate the effect of changes of hydrogen-ion concentration on the activity of the enzyme and it was possible to measure the activity of the solutions from the "anode" and "cathode" arms of the U-tube under strictly comparable conditions.

In the determination of the enzymic activities aliquot portions of the solutions from the "anode" and "cathode" arms and from the bend of the U-tube were tested side by side with a portion of the original enzyme solution with which the bend of the U-tube had been filled. The activities of the original enzyme solution and of that taken from the bend of the U-tube after electrophoresis were usually about the same. This served as a check on the results of the experiment. Evidence could be obtained in this way of the presence of any deleterious substance or influence in the U-tube and any lack of activity due to such a cause would not be attributed to a failure of the electrophoresis.

It was not possible to regulate all the conditions from time to time so that quantitative comparisons of the results of different experiments could be made. This is always true in working with highly purified amylases but is more noticeable here where the additional difficulties involved in keeping the conditions during the electrophoresis constant, were numerous. For example, the electrical resistance of the system and the temperature were not always the same, the voltage fluctuated somewhat (from a power-house line), the occasional operation of a motor in an adjoining room caused

[9] Sherman, Thomas and Baldwin, THIS JOURNAL, **41**, 231 (1919).

more or less vibration resulting in a slight diffusion into the arms of the U-tube which was more noticeable in some experiments than in others; this of course does not affect the validity of the migration results.

For these and similar reasons a comparison of the results of different experiments given in Table I shows a consistent tendency rather than a fixed quantitative change in the actual weights of cuprous oxide obtained with changes in the concentrations of hydrogen ion.

Results of Typical Experiments with two different purified preparations of malt amylase, Nos. 149 and 153, are given in Table I.

TABLE I

TYPICAL RESULTS OBTAINED IN ELECTROPHORESIS EXPERIMENTS WITH TWO PURIFIED PREPARATIONS OF MALT AMYLASE

The voltage was 110 in the first two experiments and 220 in all of the others

Enzyme preparation	P_H of enzyme solution in bend of U-tube: Before electrophoresis	P_H of enzyme solution in bend of U-tube: After electrophoresis	Relative concn. of enzyme found in "cathode" and "anode" arm solutions after electrophoresis: Cathode	Relative concn. of enzyme found in "cathode" and "anode" arm solutions after electrophoresis: Anode	Time Hours
149	3.74	3.73	13	3	25
149	3.74	3.76	32	8	24
149	3.76	3.77	48	3	42
149	4.02	4.07	40	3	36
153	4.26	4.33	157	32	39
149	4.27	4.26	63	4	36
149	4.32	4.35	17	16	15
153	4.40	4.39	38	23	36
153	4.46	4.47	3	24	24
149	4.50	4.50	65	129	37
153	4.57	4.57	3	105	37
149	4.59	4.59	4	57	36
149	4.80	4.82	3	107	34
149	4.99	5.04	4	39	36
149	6.07	6.03	3	225	26

Discussion and Conclusions

It is evident from these data that a migration of the active material takes place under the influence of an electric current and that the direction of this migration is definitely dependent on the hydrogen-ion concentration of the enzyme solutions.

The two enzyme preparations with which we have worked have given similar results, and in each case the change in the direction of migration occurs between P_H 4.3 and 4.5. On the acid side of this range the activity is greater in the "cathode" solution and on the alkaline side it is greater in the "anode" solution.

These results point to the existence of an iso-electric zone for the active material between P_H 4.3 and 4.5. This shows that the enzyme is itself, or is associated with, an ampholyte and strengthens the theory which

has often been advanced from this Laboratory that the amylase either is protein in nature or is intimately associated with protein. In view of our many experiments, only a few of which are here described, it does not seem probable that such definite and consistent changes in migration would be obtained with changes in hydrogen-ion concentration, in two distinct enzyme preparations if the active substance were merely being carried by inert proteins present in the preparations.

Previous work[9] has shown that the highest activity of this amylase when tested under our usual conditions in digestion experiments of 30 minutes at 40° is found at about P_H 4.4 to 4.5. That the iso-electric zone and the zone of optimum activity should thus coincide is of theoretical interest, showing that this enzyme exerts its optimum effect upon starch when it is in its least ionized and least hydrated condition. This may be accounted for by the possibility of its attraction for the starch particles being greatest when its combination with water is least. In other words, it is best adsorbed by starch when it is in its iso-electric (least hydrated) condition. This is in accord with the work of Abderhalden and Fodor[10] who found that the maximum adsorption of amino acids and of proteins from aqueous solution by charcoal takes place when the hydrogen-ion concentration of the solution is at the iso-electric point of the amino acid or protein. In this connection it is interesting to note that they found the adsorption to be increased from solutions, not at the iso-electric point of the solute, upon the addition of neutral salts. Since the maximum activity of malt amylase is shown at its iso-electric point, that is, the condition of minimum hydration and of maximum degree of adsorption by an adsorbent, its amylolytic activity should also be increased by the addition of neutral salts. This has been found to be true by Sherman and Thomas.[11]

Summary

Our previous work upon pancreatic and malt amylases having pointed either to the protein nature of these enzymes or to protein as an essential constituent of them, experiments were undertaken to determine the iso-electric point of malt amylase by electrophoresis.

Whereas our many similar attempts with malt extracts had proved inconclusive, good results were finally obtained in working with purified enzyme preparations in solutions adjusted to a wide range of hydrogen-ion concentrations by means of buffer solutions. The apparatus and procedure as finally developed are described.

The results show a definite migration of the enzyme under the influence of the electric current and that the direction of this migration is dependent upon the hydrogen-ion concentration of the solution.

[10] Abderhalden and Fodor, *Kolloid. Z.*, **27**, 49 (1920).

[11] Sherman and Thomas, THIS JOURNAL, **37**, 623 (1915).

The two enzyme preparations studied behaved alike and the data of many experiments with both preparations showed that in each case the change in the direction of migration occurs between P_H 4.3 and 4.5. On the acid side of this range the enzyme migrates toward the cathode, and on the alkaline side it migrates toward the anode.

Thus the iso-electric point or zone of malt amylase is definitely established at P_H 4.3 to 4.5 which coincides with that of its optimum enzymic action upon starch.

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THE PHYTOSTEROLS OF WHEAT ENDOSPERM

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Introduction

It has been shown in a previous paper from this Laboratory that corn endosperm[1] contains two different phytosterols. One is identical with sitosterol and the other was found to be a saturated sterol corresponding to dihydrositosterol, $C_{27}H_{47}OH$. We have extended the investigation to include other plant material and in this paper we report upon the results obtained in an examination of the phytosterols occurring in the wheat endosperm.

We have separated the unsaponifiable matter from the fat extracted from wheat endosperm into three parts as follows: (1) an unsaturated sterol identical with sitosterol; (2) a saturated sterol corresponding to dihydrositosterol, $C_{27}H_{47}OH$; (3) a non-crystallizable oil that we have not examined.

The purest dihydrositosterol obtained from wheat endosperm melted[2] between 144° and 145°, and $[\alpha]_D^{20}$ in chloroform was +25.82°. These values are slightly higher than those given by the saturated sterol isolated from corn endosperm but the analyses agree closely with the calculated composition of dihydrositosterol. Whether these sterols are chemically identical or if they are isomers cannot be determined from the present data. The two preparations possess the same crystal form and solubility and give the same reactions and in general are so nearly alike that we are inclined to believe that they are identical.

Experimental Part

The material for this investigation was obtained from a near-by flour mill and consisted of the crushed wheat endosperm after the bran and germ

[1] Anderson, THIS JOURNAL, **46**, 1450 (1924).

[2] Melting points given in this paper are uncorrected.