viously in this laboratory by the method of Sherman and Schlesinger. We have confirmed and extended many of their observations regarding the adsorption and elution of the enzyme, and, by careful determination of optimal conditions have obtained data which indicate that pancreatic amylase like malt amylase[8] has a definite isoelectric point or zone at essentially the same pH at which it exerts its optimum enzymic activity.

These new experiments, including the direct repetition of the work of Willstätter and his co-workers, confirm the conclusion, reached in our previous investigations in which the problem of the chemical nature of pancreatic amylase has been approached from several different angles, that the enzyme either is of protein nature or contains protein as an essential constituent.

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Further proof of the "bios" character of crystalline bios 223.*

RALPH W. KERR, WALTER H. EDDY and R. R. WILLIAMS.

[*From the Laboratory of Physiological Chemistry, Teachers College, Columbia University, New York City.*]

In our original report[1] on the isolation and properties of Bios 223 we reported failure to obtain either the ninhydrin or mustard oil reaction for a primary amine, but did obtain a reaction with benzene-sulfon-chloride. In pursuit of the chemical structure of the crystals we have since obtained a reaction with HNO_2 and a Van Slyke nitrogen determination that checks our previous value obtained by the Dumas method. These results have not only given us new leads in the study of structure, but also a means of determining definitely whether the yeast growth stimulatory power resides in the crystals of Bios 223, or is due to contamination of the crystals by the active factor. The experimental data by which we have demonstrated that the crystals are responsible for the activity follow and constitute the subject of this report.

* To avoid confusion we have elected to identify our Crystalline Bios by using the melting point 223° C. in the designation.

[1] Eddy, Kerr, and Williams, R. R., *J. Am. Chem. Soc.*, 1924, xlvi, 2846.

EXPERIMENTAL.

First Step: Preparation of an addition compound of Bios 223 and benzene-sulfon-chloride.

Two preparations of the sulfonamide have been made, identical in properties, one from a sample of Bios 223, isolated in September, 1925, and another from a sample isolated January, 1926. As shown by the sample exhibited, the compound is in the form of fine, white, needle shaped crystals melting sharply at 138.5° C. They were prepared as follows:

0.399 grams (0.003 moles) of a sample of Bios 223 were dissolved in the minimum amount of water. To this solution was added 0.48 grams of NaOH (4 x 0.003 moles) in 12 per cent solution. (N. B. Stronger solution of NaOH inhibited the formation of the compound.) To this mixture was then added, a little at a time, a total of 0.797 grams of benzene-sulphon-chloride. As this substance does not mix with water the combination was agitated from time to time over a period of 48 hours. At the end of this period the mixture was warmed to expel all traces of free b-s-c. The solution was then acidified with HCl and a precipitate of crude sulfonamide formed. Purification was accomplished by the following steps: The product being soluble in ether and relatively insoluble in acidified water its removal from the HCl solution was accomplished by shaking with ether, the sulfonamide passing into solution in the ether layer. This layer was removed and evaporated. The residue was next treated to eliminate any di-sulfonamides that might have been formed due to excess of b-s-c used. 8 cc. of sodium alcoholate solution (0.8 grams Na in 20 cc. of C_2H_5OH) was added directly to the residue and warmed for twenty minutes. Water was then added and the solution boiled to remove the alcohol. This left the product in solution as the sodium salt. Acidifying the water solution of the salt precipitated the compound again and by ether extraction it was freed from the water and the sodium chloride. The product was then purified further by crystallization from ether in which Bios 223 is insoluble, and two successive recrystallizations from hot water in which it is freely soluble. The yield was .220 grams of fine white needles melting sharply at 138.5° C. Successive recrystallizations failed to change the melting point. No known benzene-sulfonamide was found answering to this description. The compound is now under analytical investigation and data on the structure will be reported later.

Second Step: Tests for yeast growth stimulating power.

The product was then tested for activity. Clark's sugar-salt medium was used, the sugar being purified by recrystallization from 80 per cent alcohol. In each test tube was placed 8 cc. of medium, 1 cc. of water carrying the stimulatory material in definite dilution, and 1 cc. of a yeast suspension containing a definite and constant number of yeast cells (250,000) as determined by the hemocytometer. The tubes were incubated at 31° C. for 48 hours, the contents then transferred to a Hopkins vaccine tube and centrifuged at definite speed. The readings are given in fractions of cubic centimeters and in numbers of cells. *Neither the product obtained from the September,* 1925, *Bios 223 nor the January sample showed activity.* The replacement of a single hydrogen of the NH_2 group in Bios 223 by benzene-sulfonchloride is sufficient to inactivate it. (See Table 1.) (N. B. To

TABLE I.
Sample activity tests on the benzene-sulfonamide of Bios 223.

Dosage	Results with September product		Results with January product.	
	Vols.	Cells.*	Vols.	Cells.
Controls	0.003 cc.	42	0.002 cc.	28
0.2 mg. sulfonamide per cc.	——	—	0.002	28
0.1 mg. sulfonamide per cc.	0.004	56	——	—
0.02 mg. sulfonamide per cc.	——	—	0.003	42
0.01 mg. sulfonamide per cc.	0.004	56	——	—
0.001 mg. sulfonamide per cc.	0.003	42		

*A seeding of 250,000 cells per 10 cc. of combined culture medium, yeast suspension and test substance is called a count of 1. 42 cells then means that at the end of 48 hours' incubation the tubes contained 42 × 250,000 cells.

insure the presence of the benzene-sulfonamide in solution, the test fraction was first dissolved in hot water and on cooling did not precipitate out in the dilution used.)

Third Step: Restoration of activity by hydrolytic removal of the attached B-S-C Group.

Having now a crystalline compound of Bios 223 with no activity we proceeded to attempt its hydrolysis with sulfuric acid. Boiling the compound in 10 per cent sulfuric acid under a reflux condenser for six hours failed to split the compound. The hydrolysis was finally accomplished as follows:

100 mg. of the recrystallized, inactive sulfonamide was placed

in a tube with 8 cc. of water and 2 cc. of concentrated sulfuric acid and the tube sealed. The tube with its contents was then heated for one hour at 150° C. On cooling no precipitate formed and since the sulfonamide is difficultly soluble in cold acid solution, hydrolysis was assumed to have occurred. The hydrolysate was neutralized with baryta, filtered and evaporated to dryness. The residue contained considerable barium-benzene sulfonate. (Before testing this residue for activity the hydrolysate was slightly acidified with sulfuric acid and filtered. This split the barium from the sulfonate and removed all soluble barium salts.)

The hydrolysate residue was a mixture of benzene-sulfonic acid and bios but using this mixture definite evidence of activity was obtained. (See Table II.)

TABLE II.

Sample activity tests with the Bios 223, the sulfonamide made from it, and the hydrolysate residue left after splitting the benzene sulfonic acid from the inactive sulfonamide.

Dosage	Results A.		Results B.	
	Vols.	Cells.	Vols.	Cells.
Controls	0.003	42	0.003	42
0.1 mg. Bios 223 per cc.	0.016	227	0.017	245
0.01 mg. Bios 223 per cc.	0.004	56	0.005	71
0.2 mg. sulfonamide per cc.	——	—	0.002	28
0.02 mg. sulfonamide per cc.	——	—	0.003	42
0.2 mg. hydrolysate per cc.	0.009	128	0.011	156
0.02 mg. hydrolysate per cc.	0.004	56	0.004	56

Incubation 48 hours at 31 C.

CONCLUSIONS.

The isolation of a crystalline substance from an active substrate always leaves existent the doubt as to whether the activity is due to the crystals themselves or to adhering traces of the potent factor. The complete inactivation of Bios 223 crystals by forming the crystalline benzene sulfonamide and the recovery of activity by hydrolysing this inactive crystalline product seems to remove all reasonable doubt and to demonstrate that the yeast stimulating power of Bios 223 resides in the crystals themselves. The nature of the addition compound also suggested that the NH_2 group is concerned in the exercise of this function.

We also take pleasure in acknowledging the suggestions and advice of Professor John M. Nelson of Columbia University in developing these results.