EVIDENCE FOR THE COENZYMATIC NATURE OF A SYNTHETIC COFACTOR INVOLVED IN THE BIOTIN RELATED DEAMINASE SYSTEMS[1, 2]

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We have reported previously (Christman and Williams, 1952) concerning the synthesis of a specific activator for aspartic acid deaminase by the sulfuric acid degradation of carbohydrates. The immediate conclusion arising from this observation was that biotin and adenylic acid could not be constituents of the "coenzyme" although the mode of action of the enzyme system and its activators remained to be elucidated.

Evidence was presented in this same report which showed that this carbohydrate degradation product was identical in physico-chemical behavior to the substance present in yeast and liver extracts which has been designated by Lichstein (1950) to be the coenzyme of aspartic acid, serine, and threonine deaminases, and succinic acid and oxalacetic acid decarboxylases.

It is the purpose of this communication to resolve some of the speculation concerning these systems, and to present evidence that this synthetic material behaves as the coenzyme of the deaminases of aspartic acid, serine, and threonine. Evidence also will be presented to the effect that biotin and adenylic acid also are involved intimately in these same systems, albeit unrelated to the chemical structure of the coenzyme.

METHODS

The organism used to prepare the partially resolved aspartic acid, serine, and threonine deaminases was *Bacterium cadaveris*. The method of enzyme resolution by the use of acid phosphate buffer has been described previously (Lichstein and Umbreit, 1947; Lichstein and Christman, 1948). Although this technique has encountered some controversy (Axelrod *et al.*, 1948; Lichstein, 1949), apparently it produces results consistent with other methods of enzyme resolution (Lichstein, 1950). We have found that refrigeration of washed cells at −18 C for 24 to 72 hours prior to phosphate aging always leads to consistent resolution. Prolonged refrigeration leads to complete inactivation of the system. A second method of aspartic acid deaminase resolution has been described by Boyd and Lichstein (1951) and was employed in certain of the experiments.

Dried cell preparations of *B. cadaveris* were made by vacuum drying washed cell paste over silica gel or by vacuum sublimation of the moisture in a modified lyophile apparatus.

Biotin assays were performed by the method of Snell *et al.* (1940) using *Saccharomyces fragilis* as the assay organism.

The glucose cofactor solutions were prepared according to the method previously described (Christman and Williams, 1952). The furfural cofactor solutions were prepared in the following manner. Five per cent solutions of furfural in 2 N sulfuric acid were autoclaved at 121 C for two hours. Charred and resinous material was removed by filtration and the filtrate neutralized with calcium carbonate. The slurry so obtained was filtered and the filtrate decolorized with carbon. Following the carbon treatment, excess calcium ion was removed as the oxalate salt and excess sulfate ion as the barium salt. The solution was decolorized a second time with activated carbon and was filtered. The filtrate, now a straw-colored liquid, was evaporated to dryness and the solid material ground to a fine powder. This preparation then was extracted with hot 75 per cent alcohol, and the crystalline material obtained on evaporation of the solution was used in the experiments.

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[2] Reported in part at the South Central Branch Meeting, Society of American Bacteriologists, May 17, 1952.

EXPERIMENTAL RESULTS

The following experiments have been performed: (1) examination of a number of carbohydrates and carbohydrate related materials for enzyme stimulating properties; (2) investigation of the glucose cofactor in partially resolved serine and threonine deaminase systems; (3) study of the effect of cofactor addition on the biotin content of partially resolved cells of *B. cadaveris*; (4) investigation of the time dependency of the stimulatory effect of the furfural cofactor; (5) examination of concentration effects with the furfural cofactor; (6) examination of the effect of yeast extract and the cofactor on cells grown with glucose; (7) study of dried cell systems; and (8) further examination of highly resolved systems which require the cofactor, biotin, and adenylic acid for maximum stimulation.

Examination of carbohydrates and carbohydrate related materials for enzyme stimulating properties. In order to determine whether this synthetic cofactor was one of the known chemical products resulting from acid degradation of glucose, a number of compounds were examined to determine whether they possessed any stimulating activity *per se* and whether they would yield the cofactor on acid hydrolysis. The following compounds were examined: formic, acetic, propionic, levulinic, and reductic acids; glyceraldehyde, erythrose, arabinose, glucose, mannose, galactose, fructose; and mannitol, erythritol, furfural, reductone (as the PABA complex[3]), and ascorbic acid.

None of the ordinary products of acid degraded glucose which were tested had any stimulating activity in partially resolved aspartic acid deaminase. An examination of some of the above materials for their ability to yield the stimulating factor by sulfuric acid degradation gave the results shown in table 1. From these data one can conclude that the prime known reaction of sulfuric acid on glucose to yield levulinic acid is not the reaction which is involved. Secondly, since furfural gives rise to the cofactor in as good yield as does glucose, one can conclude that the product probably has less than six carbon atoms and that the stereoisomerism of the polyhydrated molecule, glucose, has little or no bearing on the synthesis. Thirdly, it would seem that an active aldehyde is essential in the production of the material.

[3] Courtesy of Dr. W. W. Umbreit, Merck Institute for Therapeutic Research, Rahway, New Jersey.

Investigation of the cofactor in partially resolved serine and threonine deaminase systems. Since the natural material found in yeast and liver extracts is capable of stimulating partially resolved serine and threonine deaminases as well as aspartic acid deaminase, experiments were performed to determine if the synthetic material prepared from glucose degradation has similar biological activity (table 2). It can be seen from these data that the glucose degradation product has exceptional ability to stimulate all three amino acid deaminases. Thus, from a biochemical as well as physiochemical viewpoint, this synthetic substance acts in a manner identical to the material present in yeast extract. By inference (Lichstein, 1950) it would appear that this material should be involved also in the succinic and oxalacetic acid decarboxylases. These experiments have not yet been undertaken.

TABLE 1

Substances which yield the aspartase cofactor on sulfuric acid degradation

YIELD*			
Excellent	Good	Poor	None
Glucose	Galactose	Fructose	Erythritol
Mannose	Arabinose	Reductic† acid	Mannitol
Furfural			Levulinic acid

* Values rated according to ability to stimulate partially resolved aspartic acid deaminase of *Bacterium cadaveris*.

† May have been due to contaminating glucose.

Study of the effect of cofactor addition on the biotin content of partially resolved cells of B. cadaveris. Since various investigators have established that biotin and adenylic acid are primarily involved in the deaminase reactions (Lichstein and Christman, 1948; Wright *et al.*, 1949), and since the foregoing data seemingly contradict or at least complicate these observations, an examination of the biotin content of the cells was made on partially resolved resting cell systems, activated by the synthetic degradation product of glucose, in order to show whether or not the addition of cofactor led to biotin synthesis. The cells were grown for 18 hours on two liters of one per

cent yeast extract, one per cent tryptone, and 0.5 per cent KH_2PO_4 medium; harvested; washed once; equally distributed among four flasks; and treated as follows: The cells in the first flask were maintained at room temperature in distilled water during the aging period, followed by the addition of buffer and water to a volume of 70 ml. The second group of cells was aged in one molar phosphate buffer, pH 4, for 30 minutes and incubated with buffer and water. The contents of the third flask were aged in the same manner as the second and incubated with aspartic acid; the cells in the fourth flask were aged and incubated with aspartic acid and the glucose degradation cofactor. Following a two hour incubation, cellular activity was destroyed by the addition of trichloroacetic acid. The supernate was assayed for ammonia (100 per cent yield—4,900 μg) while the cells were hydrolyzed in 2 N sulfuric acid for two hours at 121 C and then assayed for biotin. The results are presented in table 3. One can conclude immediately that increasing the biotin concentration in the cells is not the function of the cofactor. These data can be interpreted further in two ways: either cellular biotin is not involved in aspartic acid deaminase during cofactor stimulation or there is sufficient biotin present to stimulate the cells with respect to biotin and both biotin and the synthetic factor are required for maximum deamination.

TABLE 2

Activity of cofactor on partially resolved amino acid deaminases

ADDITIONS	AMINO ACID SUBSTRATES		
	Aspartic acid	Serine	Threonine
	*Micrograms ammonia per ml**		
Experiment 1:			
No additions	11.3	7.2	12.1
Biotin (1 μg) and adenylic acid (0.1 mg)	24.4	7.9	13.0
Yeast extract (1 mg)	21.4	9.0	16.6
Synthetic cofactor (10 μg)	45.5	39.3	32.1
Experiment 2:			
No additions	11.6	7.3	5.3
Biotin (1 μg) and adenylic acid (0.1 mg)	35.0†	8.7	7.2
Yeast extract (1 mg)	29.2	10.2	6.4
Synthetic cofactor (10 μg)	35.0†	35.0†	14.5

* Cells grown on AC medium 16 hr, at 37 C, harvested, washed, and aged 30 min, at 25 C in M phosphate buffer, pH 4. Incubation at pH 5, 2 hr, 37 C. Controls without added amino acid substracted to give quoted values.

† Approximate value.

TABLE 3

*Biotin content of cells on aging and stimulation by synthetic cofactor**

CELL TREATMENT	SUBSTRATE	NH_3 PRODUCT PER FLASK IN μG	TOTAL BIOTIN CONTENT OF CELLS† IN μG
None	None	140	0.17
Aged	None	130	0.24
Aged	Aspartic acid	370	0.28
Aged	Aspartic acid and cofactor	2,560	0.20

* For details, see text.

† Biotin content of the supernates approximately 0.01 μg each.

Investigation of rate curves. It seemed that these hypotheses could be attacked by examining the rates of deamination of partially resolved aspartic acid deaminase when stimulated by a mixture of biotin and adenylic acid[4] or by the cofactor. These rate curves are presented in figure 1. In the experiment presented, representative of a number of similar experiments, the resolution of the enzyme was of very high order since even after a two hour incubation there was no increased

[4] Kindly supplied by Ernst Bischoff Company, Ivoryton, Connecticut.

capacity of the cells to deaminate aspartic acid. Similarly, biotin and adenylic acid, which ordinarily produce some reactivation immediately (Lichstein and Christman, 1948), show a lag of sixty minutes before any indication of activation of the deaminase appears; at two hours there is only a twofold activation of the system. With the synthetic cofactor, however, there is an im-

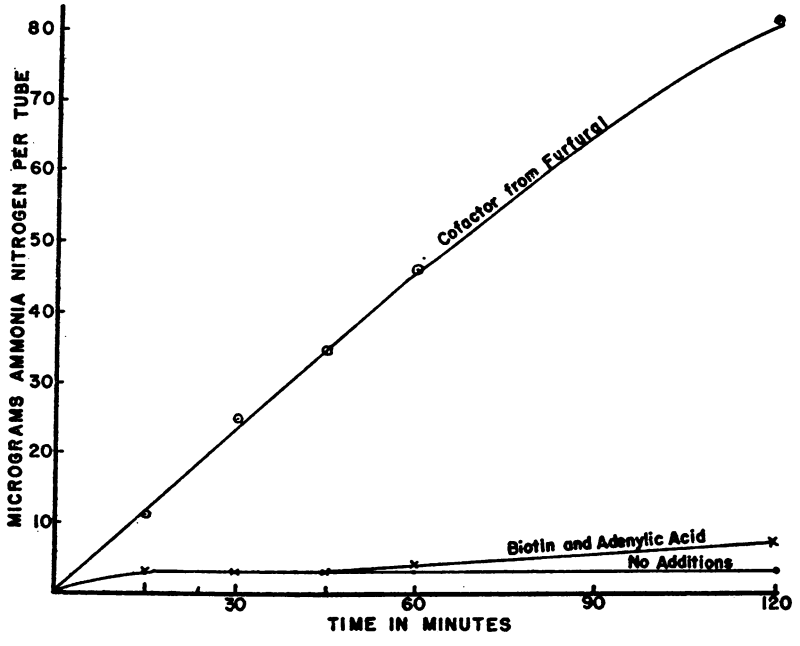

Figure 1. Effect of the synthetic cofactor from furfural, and biotin with adenylic acid on aspartic acid deaminase of *Bacterium cadaveris*. Reaction run at pH 5, 0.5 M phosphate, 37 C. Concentrations of additions: biotin, 1 μg; adenylic acid, 0.1 mg; cofactor, 10 μg.

mediate activation which progresses linearly with time and proceeds to near maximum ammonia production in two hours.

Examination of concentration effects. As a part of the elucidation of the nature of cofactor stimulation, the extent of stimulation obtainable with graded amounts of synthetic cofactor was studied. Figure 2 represents a typical experiment from these studies. The three curves are homogeneous in nature, the stimulation effect being linear with respect to concentration. It is evident immediately that the aqueous alcohol extract is more concentrated than the crude furfural hydrolyzate and that the activity of both of these fractions is considerably greater than corresponding concentrations of yeast extract. In order to produce magnitudes of stimulation by yeast extract corresponding to levels attained by the synthetic product, it becomes necessary to use 10 to 15 times as much yeast extract as crude hydrolyzate. Values obtained at these concentrations of yeast extract must be viewed with caution since at levels of 1,000 μg or more the endogenous reactions producing ammonia are of such magnitude that the deamination determinations have a correction factor larger than the amount of ammonia produced.

Examination of the effect of synthetic cofactor and yeast extract on cells grown with glucose. Enzyme resolution by the method of Boyd and Lichstein (1951) was employed to determine whether results obtained by the "glucose-aging" techniques were consistent with those obtained by phosphate aging. Data from several experiments are presented in table 4. It would appear that "glucose-aging" produces a system that is extremely well resolved with respect to the cofactor. Both synthetic cofactor and yeast extract are capable of stimulation, the greater concentration of the cofactor in the synthetic preparation probably accounting for the differences observed between the natural and synthetic material.

Particularly worth noting, however, is the high order of stimulation obtained with cofactor in the presence of biotin and adenylic acid, suggesting that the glucose-aged cells were resolved with respect to both stimulants.

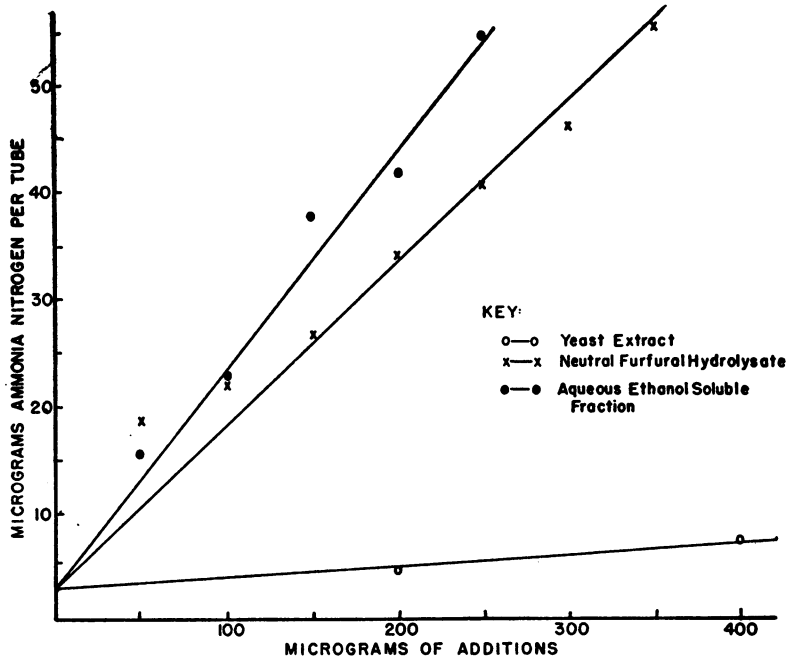

Figure 2. A comparison of the effects of yeast extract, a crude furfural hydrolyzate, and a purified furfural hydrolyzate fraction on aspartic acid deaminase of *Bacterium cadaveris*. Reaction run at pH 5, 0.5 M phosphate, 37 C, two hours.

TABLE 4

Cofactor stimulation of glucose grown cells

ADDITIONS	MICROGRAMS NH_3 PRODUCED*		
	Exp 1	Exp 2	Exp 3
None	0.1	0.7	8.9
Biotin (1 μg) and adenylic acid (0.1 mg)	0.4	1.7	8.6
Yeast extract (1 mg)	6.3	5.6	48.7
Synthetic cofactor (10 μg)	9.6	7.8	53.8
Synthetic cofactor (10 μg) and biotin (1 μg) and adenylic acid (0.1 mg)	18.8	12.5	59.9

* Cells grown on AC medium plus 0.5 per cent glucose, 16 hr, 30 C, harvested, washed, and incubated with and without substrate and additions at pH 5, 90 min, 37 C; controls without added substrate substracted to give quoted values.

Study of dried cell preparations. Dried cells were prepared by rapid drying (3 hr) in a vacuum sublimation apparatus and by slow drying (16 hr) in a vacuum desiccator containing silica gel. Both harvesting and drying of cells were carried out at 4 C. The data are presented in table 5. The large magnitude of stimulation obtainable with the synthetic cofactor is apparent immediately. As was noted with the glucose-aged cells, here likewise the combined effect of cofactor plus biotin and adenylic acid produced the largest response. This effect was noticeable particularly with the lyophilized cells.

Rate curves with highly resolved systems which require the cofactor together with biotin and adenylic acid for maximum stimulation. Both of the preceding experiments indicated that highly resolved systems required not only the cofactor but also biotin and adenylic acid for maximum stimulation. To examine further this possibility, experiments were performed wherein the combination of biotin and adenylic acid along with the synthetic cofactor was compared with yeast extract, synthetic cofactor, and biotin plus adenylic acid. Highly resolved cells were obtained by storing washed cell paste for a week at −18 C. The time curves obtained are shown in figure 3. These

TABLE 5

*Cofactor stimulation of dried cellular preparations**

ADDITIONS	MICROGRAMS NH_3 PER ML†	
	Lyophilized cells	Cells dried over silica gel
None	6.1	7.9
Biotin (1 μg) and adenylic acid (0.1 mg)	12.2	5.0
Yeast extract (1 mg)	6.9	9.1
Synthetic cofactor (10 μg)	38.4	18.8
Synthetic cofactor (10 μg) and biotin (1 μg) and adenylic acid (0.1 mg)	46.4	20.9

* For details of preparation of dried cells, see text.

† Experiments performed using 4 mg dried preparation per tube, pH 5, 1 hr, 37 C; controls without added aspartic acid substracted to give values quoted.

rapid response to the combination of cofactor plus biotin and adenylic acid.

DISCUSSION

In regard to the coenzymatic nature of the synthetic cofactor, we have demonstrated the following characteristics of this material: (a) it is apparently identical chemically to the yeast factor known to be involved in the biotin linked deaminase systems; (b) it rapidly stimulates all three of the biotin linked deaminases; (c) apparently it is not involved in cellular synthesis of biotin; (d) it is capable of immediate activation of partially resolved cell systems, which activation proceeds linearly with time; (e) it stimulates a cellular response which is linear with respect to concentration of the cofactor; (f) it is highly stimulatory to cells resolved by "glucose-aging"; and (g) it is highly stimulatory to vacuum dried and lyophilized cells aged by washing the cell paste. From these observations we can conclude that

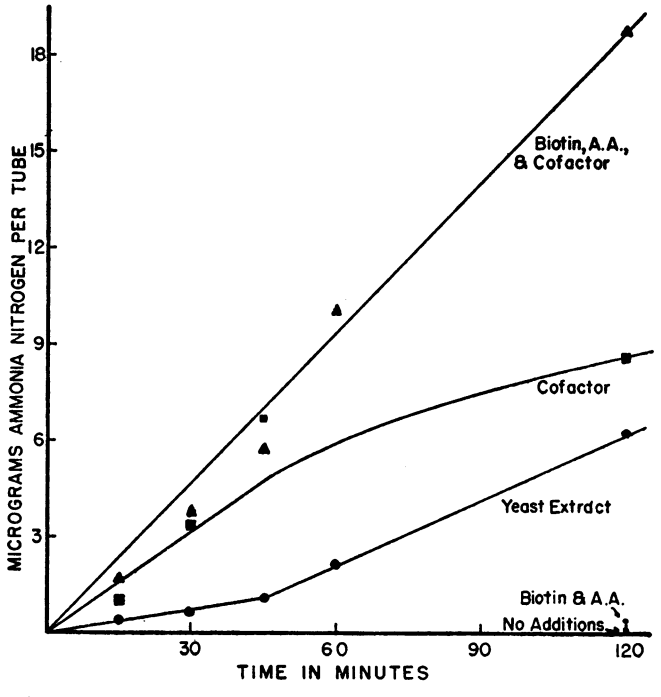

Figure 3. A comparison of the rates of reactivation of partially resolved bacterial aspartic deaminase by biotin with adenylic acid, yeast extract, synthetic cofactor, and synthetic cofactor with biotin and adenylic acid. Reaction run at pH 5, 0.5 M phosphate, 37 C. Concentrations of additions: biotin, 1 μg; adenylic acid, 0.1 mg; cofactor, 10 μg; yeast extract, 1 mg.

findings bear out the indications noted in the preceding experiments and clearly demonstrate that highly resolved cell systems show the most this cofactor is probably the coenzyme of the biotin linked deaminases.

During the past five years much evidence has

been accumulated concerning these deaminases, a thorough summary of which is now available (Lichstein, 1951). In the light of the data presented here, certain aspects of these investigations merit emphasis: (a) biotin and adenylic acid are concerned somehow with these systems; (b) a factor is present in yeast and liver extracts which apparently is the coenzyme of these systems; and (c) a factor, prepared by sulfuric acid degradation of carbohydrates and carbohydrate related substances, is involved in these systems and appears to be identical to the yeast factor. It is equally apparent that neither biotin nor adenylic acid is a chemical part of this synthetic material. An attempt to harmonize these points leads one to several alternatives. One can assume that biotin and adenylic acid are (a) indirectly involved, e.g., via the synthesis of the coenzyme or the apoenzyme, or (b) directly involved in these deaminase systems. If hypothesis "a" represents the correct interpretation, one might reason as a consequence that aging depletes the cells of the coenzyme and that the addition of biotin permits the cells to metabolize stored glucose to produce more coenzyme since the coenzyme can arise by chemical reaction from glucose. This viewpoint appears to be eliminated on the grounds that the biotin-adenylic acid additions show an increased effect on systems containing an excess of coenzyme and bring about this effect without a time lag.

If hypothesis "b" is taken as an alternative, then one must explain how biotin and adenylic acid can function directly in the deamination reaction and yet not be chemical parts of the far more active cofactor. To do this, one must conclude that biotin and adenylic acid are involved in some manner different from that of chemical transfer agents, considered the normal mode of action of vitamins.

Close examination of the evidence seems to favor this second interpretation, this evidence being: (a) although the cofactor invariably stimulates cell activity without lag, biotin and adenylic acid often do likewise even in the suboptimal pH 5 system where synthesis of intermediates is at a minimum; (b) biotin and adenylic acid frequently stimulate dried cell preparations; (c) an additive effect can be observed in well resolved systems which require both the cofactor and the biotin-adenylic acid combination for maximum stimulation; and (d) frequently systems can be isolated (figure 3) wherein biotin and adenylic acid alone have no effect in deamination and still cause a significant increase over cofactor alone when added along with the cofactor. It is conceivable that the deaminase system requires not only a coenzyme but also a vitamin stimulator that is unrelated to the coenzyme.

SUMMARY

Evidence is presented favoring the coenzymatic nature of a synthetic cofactor involved in the aspartic acid, serine, and threonine deaminase systems found in resting cell and dried cellular preparations resolved by a variety of techniques. The cofactor can be prepared from a variety of carbohydrates and their derivatives by sulfuric acid degradation; none of these materials has cofactor activity before degradation.

Cofactor stimulation of resting cellular suspensions is not accompanied by any increase in biotin content.

The existence of a joint requirement for the cofactor and the biotin-adenylic acid combination in highly resolved cellular preparations is demonstrated.

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