STUDIES ON THE METABOLISM OF THREONINE AND RELATED SUBSTRATES IN *NEUROSPORA CRASSA*

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This work I dedicate to my mother.

ABSTRACT

We have investigated the following enzyme systems in extracts of a wild type strain of Neurospora:

1) Threonine deaminase. It catalyzes the reaction giving rise to alpha-ketobutyric acid and ammonia from threonine. It was concluded that alpha-aminobutyric acid, glutamic acid or a deaminated alpha-ketobutyric acid precursor in equilibrium with alpha-ketobutyric could not be intermediates in this reaction. Pyridoxal phosphate activates the system, and a number of methods were tested in order to improve the resolution of enzyme and coenzyme in the preparations.

2) Serine deaminase. Yields pyruvic acid and ammonia from serine, and is also activated by pyridoxal phosphate. The responses of serine and threonine deaminases to pyridoxal phosphate are at variance, suggesting that two different enzymes are involved.

The effect of p^H and temperature on serine and threonine deaminase was investigated.

3) Glutamic-alphaketobutyric transaminase, which is activated by pyridoxal phosphate.

4) A system forming alpha-aminobutyric from threonine, possibly as a result of the summation of activities 1) and 3).

5) A system forming an unidentified blue fluorescent product by incubation with threonine, but not with any of

a number of related metabolites (serine included).

6) Alpha-ketobutyric decarboxylase, which is activated by cocarboxylase, and has a pH optimum of 5.5.

The threonine deaminase activities of a number of threonineless strains and of a B_6-less strain were compared with those of wild type, using cultures grown under different conditions. The significance of the variability in activity encountered is discussed. Mutant 35423, which requires threonine for growth but is unable to use alpha-ketobutyric or alpha-aminobutyric acid,has the ability of converting threonine into those acids in vitro.

Mutant 44104 cannot utilize alpha-ketobutyric acid in place of alpha-aminobutyric to initiate early growth, but its glutamic-alphaketobutyric transaminase is as active in vitro as that of wild type.

A new scheme of threonine biosynthesis is presented to account for the information available.

An attempt is made to find a common denominator to the mechanisms of the diverse coenzymatic activities of pyridoxal phosphate, and schemes for those mechanisms are proposed.

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I) INTRODUCTION

When, a quarter of a century ago, Kluyver and Donker wrote their paper on "the unity in biochemistry" (1) it seemed possible to them to account for the differences between the diverse types of aerobic and anaerobic bacteria by quantitative differences in the affinity of their proteins towards hydrogen. Microbiologists - Kluyver not excluded (2) - have since turned their attention towards a different order of interpretation of variability, a more successful interpretation: the discreet, particulate one which emerges from the application of the principles of genetics to biochemical variability (3).

Yet while discreetness seems to have the upper hand in the highly organized world of the chromosomes (position effects not withstanding), its rule fades in the territory of physiology and biochemistry. Pleiotropic effects, new metabolic interactions, quantitative differences in enzyme activities, pH and temperature susceptibilities, etc., belong in the sequel of discreet changes, but have to obey the laws of kinetics and of steady state systems, and perhaps other laws not yet understood. We cannot ignore intermediary metabolism, nor fail to use the tools of classical biochemistry if we want to investigate and understand the significance of mutational changes. So the biochemistry of Neurospora acquires a particular significance as a meeting

ground for two different - but in no way conflicting - methods of biological research.

With such thoughts in mind we have undertaken the study of the metabolism of "wild" and "mutant" Neurospora strains in the province of threonine.

II MATERIALS AND METHODS

A) STRAINS

All strains used were derived from the culture collection at the Kerckhoff Biological Laboratories. E-18829-5a is a reisolate (obtained by Mary Emerson) carrying the sulfonamide-requiring gene, first recognized in strain E-13190 (4). It resulted from a cross between E-16561a and E-16104A, which were isolated respectively from crosses 15835a x E-15172A, and C-40a x E-15172A (4) (5). The wild-type strain used throughout is E-5256A (4).

The other strains used - 51504a, 44602, 44104a, 35423a - were obtained by Beadle and Tatum (6) by UV irradiation of wild type conidia.

Cultures were kept at 25° C. on agar slants ("minimal" medium (6), with 1½% agar) supplemented as follows:

no additions for E-5256

2 micrograms pyridoxine HCl for 44602

½ mg/ml DL-threonine for 35423, 44104 and E-18829

½ mg/ml DL-threonine plus ½ mg/ml DL-methionine for 51504

Mutant strains were tested for their ability to grow on

supplemented and unsupplemented media as a check against back-mutation, whenever they were cultured as a source of enzymes.

B) CHEMICALS

Commercial sources were: Eastman Kodak, for DL-alpha-aminobutyric acid; H. M. Chemical Co., for DL-threonine; Nutritional Biochemical Corp. for L-threonine, DL-serine, pyridoxine, pyridoxal, pyridoxamine, and alpha-ketoglutaric acid. Calcium pyridoxal phosphate was kindly supplied by Dr. Karl Folkers, of Merck & Co.

We synthesized alpha-ketobutyric acid by preparing ethyl ethoxalylpropionate (7) and, from there on, following the procedure outlined by Blaikie and Perkin (8). Alpha-keto-butyric acid was collected as the fraction distilling over between 68° and 70°C. (14 mm Hg) and appearing as a light colored oil, which partially solidified after standing a few days in the icebox. The oil (13g) was dissolved in 100 ml of ethanol, and neutralized with alcoholic sodium hydroxide. The precipitate thus obtained was filtered, washed, and re-crystalized from 80% ethanol. Its 2,4-dinitrophenyl hydra-zone, recrystalized from ethyl acetate, melted at 195.5-196° C. (reported in the literature: 198-199° C. (9)). An aliquot containing 20 micromoles of Na alpha-ketobutyrate solution yielded under the action of a preparation of Neurospora alpha-ketobutyric decarboxylase (p. 86) 456 microliters of CO_2, determined as net gas evolution in a

Warburg respirometer. A parallel run, in which the enzyme was incubated without substrate, gave 5 microliters. Difference: 451 microliters. Calculated: 448 microliters of CO_2. The beta-Br derivative was prepared by Dr. H. Garner, and he found it to melt at 59°C (reported in the literature: 60°C (10)).

We are grateful to Dr. H. Garner for supplying us with beta-Br-alpha-ketobutyric acid. From it we prepared ketothreonine (alpha-keto-beta-hydroxybutyric acid) by careful neutralization with sodium hydroxide. The resulting solution was used wherever ketothreonine is indicated (10). Sodium pyruvate was prepared (11) using pyruvic acid manufactured by the Matheson Co.

Cocarboxylase HCl was synthesized after Weijlard (12), who reports a yield of 4 grams. Using one-tenth the amount of ingredients, we obtained 73 mg. The material decomposed at ca 220°C (Weijlard reports 238-240°C.) Two and a half micromoles of our product - containing 0.02 to 0.05 micromoles of inorganic phosphate as impurity - when hydrolized for 7 minutes in 2N HCl yielded 2.4 micromoles of phosphate (determined colorimetrically (38)). The reading was 2.6 micromoles after standing for 1 hour in 60% perchloric acid at 100°C. A third aliquot assayed for 4.6 micromoles of inorganic phosphate after incubation for 6 hours at 118°C in the presence of 10N sulfuric acid.

C) ENZYME PREPARATIONS

Unless otherwise specified, enzymatically active extracts of Neurospora were prepared as follows:

Strain E-5256A was inoculated into a carboy containing 16 liters of sterile "minimal" medium (6), and allowed to grow for 3 to 4½ days, at 25°C, under forced aeration. The mycelium was harvested by filtering in a basket centrifuge, then washed with water or with M/10 pH 7.7 phosphate buffer, and lyophilized. Yields of lyophilizate ranged from 35 to 65 g. The dry material was ground in a mortar and stored in a desiccator over calcium chloride in the icebox. The desiccator was evacuated with a water pump, and kept so during the first day or two after lyophilization, and during long periods of storage. Deaminase activity did not seem to decay over a lapse of 10 months. There were no essential disparities in the enzymatic activities of the different batches prepared in the course of these studies.

Just before using, the required weight of lyophilizate was suspended in cold phosphate buffer (molarity from 0.2M to 1M) so as to have 100 mg of lyophilizate per ml of solution, buffered at pH 7.8 (±0.2). This was homogenized for ca 5 minutes with a motor driven gadget refrigerated with ice water; then centrifuged for 20 minutes - generally in a clinical centrifuge - and the sediment, which occupied 1/3 to 1/2 the volume of the mixture, discarded. The supernatant constitutes our crude enzyme preparation, and such will henceforth be given the name of "Neurospora extract".

Ammonium sulfate fractionations were performed as in the type experiment to be described later on (p. 26), but using Neurospora extract in place of acetone precipitate. All ammonium sulfate precipitations and all dialyses were done in a 3°C cold room. Dialyses were aided by moving the dialysis bags with an electric motor.

D) INCUBATION AND ASSAY METHODS

Where no other particulars are given in the description of experiments, incubations to determine deaminase activity were run as follows:

1 ml "Neurospora extract"
0.2 ml of 0.4 M DL-threonine (0.2 ml of water in the blank)
0.2 ml of other additions (or 0.2 ml of water),

were placed together in a 1/2 dram, screw cap, vial, while kept cold in an ice water bath; then the vial was covered with a rubber cap to avoid loss of ammonia, and incubated in a constant temperature bath. For ammonia determination, 0.1 or 0.2 ml samples could be withdrawn at the required times by means of a hypodermic syringe, and discharged into a drop of ca 1N sulfuric acid placed on a "Conway unit" thus stopping the reaction and precluding ammonia loss. Similarly, samples were withdrawn and discharged into an equal volume of 20% trichloroacetic acid for colorimetric determination of keto acids. When the entire content of a vial was to be assayed, the reaction mixture was brought up to pH 2 or lower by injecting 0.2 ml or more of 1N sulfuric acid through the rubber cap to prevent loss of ammonia. The rubber cap was

then withdrawn and the vial placed in a boiling water bath for 5 to 10 minutes, and the precipitated proteins removed by centrifugation.

Ammonia was determined using "Conway units" (13), and Nesslerizing the trapping solution. Where traces of acetone or acetaldehyde might be present, the acidified aliquots, placed on the "Conway units", were fanned until almost dry before adding the base and sealing the vessel.

Colorimetric keto acid determinations were done following either of Friedman and Haugen's "indirect" methods (14). For method B we used toluene as a solvent and a 540 millimicrons light filter. Alpha-ketoglutaric acid seriously interferes even with the more specific method, the "pyruvate" method; and calculations to correct for this interference are too indirect. Keto threonine also interferes.

We developed a manometric method for the determination of alpha-ketobutyric acid, by means of Neurospora alpha-ketobutyric decarboxylase (p. 86). An aliquot of the solution to be assayed, which had been previously acidified and boiled to stop deaminase activity, was tipped from the side arm of a Warburg respirometer vessel onto a buffered solution of Neurospora lyophilizate, and decarboxylation allowed to go to completion. The enzyme preparation is the supernatant following centrifuging of 30 mg of lyophilizate per ml of M/2 acetate-phosphate buffer, adjusted so that the pH of the reaction mixture would fall between 5.1 and 5.5. Moles of CO_2 evolved, measured as total gas evolution, correspond to moles of alpha-ketobutyric acid in the sample (pp. 3 to 4)

and (p. 86). Pyruvate is also decarboxylated by such preparations; but, since the yields of CO_2 vary in the neighborhood of 50% of the theoretical, it could not be determined quantitatively by this method. Under the conditions of the assay, alpha-ketoglutarate causes a very slow net gas evolution*, so that by the time decarboxylation of alpha-ketobutyrate is completed the error alpha-ketoglutarate could introduce does not exceed 5%, and this can be corrected by extrapolating the CO_2 evolution curve resulting after all the alpha-ketobutyric has been exhausted, if its slope looks significantly different from the blanks.

A 0.2M ketothreonine solution, freshly prepared by hydrolysis of beta-bromo-alpha-ketobutyric acid, was used to see if ketothreonine would interfere with the alpha-ketobutyric assay. Forty micromoles of substrate, which should have yielded 880 microliters of CO_2 if decarboxylated to completion, gave only 45 microliters of CO_2. No O_2 uptake was detected in a duplicate Warburg vessel with KOH in the center well. No inhibition of the decarboxylation of alpha-ketobutyric acid by addition of 0.2 M NaBr solution was found, so there is no reason to believe that NaBr in the

*W.K. Pao (15) reports the presence of alpha-ketoglutaric decarboxylase in Neurospora extracts. The activity of his preparations is distinctly higher than that of those used here, which is not surprising since extracts were made differently. It is noteworthy that Pao only got about 10% yield of CO_2 from ketoglutarate, which is approximately the extent of impurities Dr. H. Garner (personal communication) bioassayed in commercial alpha-ketoglutaric samples. We searched for alpha-ketoglutaric decarboxylate at pHs 4.4, 5.9, and 7.3 and 8.4. The best activity, though low, was at pH 5.9

ketothreonine solution inhibits decarboxylation. It remains to ascertain whether the one-twentieth yield of CO_2 (which was evolved at a much lower rate than CO_2 from alpha-ketobutyric acid) was due to an impurity, or whether ketothreonine was such a small portion of the products of hydrolysis of the bromo derivative. Addition of 2,4-dinitrophenyl hydrazine precipitated a hydrazone in 40% yield (calculated as ketothreonine hydrazone) after standing several days in the ice box. We chromatographed (p. 10) the early crystals and the late ones. The early precipitate gave rise to two spots (Rf, 0.55 and 0.42, while alpha-ketobutyric acid Rf was 0.63); the late precipitate, only one (Rf, 0.42). The two components were in appreciably the same concentration (judging by the visual estimation of the spots) and, since one of them is probably the one identified by Sprinson and Chargaff (10) as ketothreonine, it appears likely that the slight decarboxylation is due to a component other than ketothreonine.

No interference with alpha-ketobutyric manometric determination was found from L or DL-threonine, DL-serine or L-glutamic acid.

The qualitative identification of keto acids by paper chromatography of their 2,4-dinitrophenyl hydrazones has been reported in the literature (30). The systems reported were not satisfactory for our purposes. Among other things, when recrystallized 2,4-dinitrophenyl hydrazones were applied

in high concentrations, double spots were formed at higher pHs, presumably due to the separation of the ionized and unionized acid hydrazones. The p^H of the sample affects the Rfs considerably (Table I).

Best separation of the 2,4-dinitrophenyl hydrazones of alpha-ketobutyric, pyruvic, alpha-ketoglutaric acid, and ketothreonine was obtained using butanol saturated with 2% acetic acid as solvent; and buffering Whatman #1 paper by dipping it in M/10 pH 3.6 acetate buffer, and drying it in air before using (Table II). The yellow spots are visible; they turn reddish when sprayed with alkali (1%), but not more outstanding. Great sensitivity is achieved by observing the untreated spots with an ultraviolet lamp, under which they appear dark.

Quantitative and qualitative amino acid analyses were made by paper chromatography (ascending). Spots were applied in 2 microliter portions, generally applying 2 such portions, and were dried in a current of hot air. The most satisfactory qualitative solvent among those tested was phenol saturated with water. Phenol chromatograms were dried at temperatures below 50°C (31), often at room temperature; and developed by spraying with ¼% ninhydrin in water-saturated n-butanol.

For the quantitative determination of alpha-aminobutyric acid, a solvent consisting of 2 parts n-butanol: 1 part glacial acetic acid: 1 part water was used. It affords a better resolution of alpha-aminobutyric acid and methionine,

TABLE I

Effect of the pH of the sample on Rfs of hydrazones

Paper: Whatman #1. Solvent: 5 parts n-butanol: 4 ethanol: 1 water. Ascending method

2,4-dinitrophenyl hydrazone of	pH of Sample				
	2.5	4.5	6.5	7.9	ca 14
	Rf Values				
alpha-ketoglutaric acid	0.47	0.34	0.26 0.10	0.22 0.05	0.01
alpha-ketobutyric acid	0.57	0.51	0.40	0.40	0.36 0.17 0.05

(Where several values are given, it means that several spots appeared)

TABLE II

Separation of hydrazones by different ascending paper chromatography systems
Paper: Whatman #1
Samples applied as solutions of pure hydrazones in ethyl acetate

2,4-dinitrophenyl-hydrazone of	Solvent	n-butanol sat'd with 3% NH_4OH		5n-butanol: 1.5 ethanol; sat'd with 0.1M boric acid		5n-butanol: 1.5 ethanol; sat'd with 2% acetic acid		2n-propanol: 2 acetic a.		n-butanol saturated with 2% acetic acid		
	Paper buffered with	---	1% NH_4 borate	---	0.1 M boric acid	---	pH5.5 M/20 acetate	---	pH 5.5 M/20 acetate	---	pH 5.5 M/20 acetate	pH3.6 M/10 acetate
		Rf Values										
alpha-ketoglutaric acid		0.07 (0.43)	0.18	0.41	0.34	0.63 0.78	0.42	0.75	0.66	0.39 0.90	0.27	0.28
pyruvic acid												0.39
ketothreonine*		0.44 0.54	0.57 0.70	0.46	0.40	0.55	0.67	0.77	0.87	0.48 0.70	0.33 0.40	0.44
alpha-ketobutyric acid		0.48 0.66	0.68 0.78	0.51 0.65	0.54 0.68	0.77	0.67 0.77	0.89	0.87 0.94	0.67	0.47 0.57	0.52
propionaldehyde												0.92

(Where several values are given, it means that several spots appeared)

*Prepared independently from the 2,4-dinitrophenyl hydrazone reported in page 9. It gave a single spot (crystals were collected overnight) which corresponds to the upper spot of page 9. Differences in Rfs in both runs may be due to uncontrolled factors (e.g., temperature, age of solvent.)

and saves the pains necessary to prevent destruction of amino acids in the process of drying phenol chromatograms (32) (33). Whatman #1 paper gives variable ninhydrin blanks (34). After some trials (Table III) it was decided to wash it by boiling twice with sodium hydroxide, leaving it overnight in the same solution, and then washing with distilled water until neutral to universal indicator.

Chromatograms were run overnight and then heated in an over for 30 minutes at 100° to 110° C so that the amino acid spots could be located under the ultra violet lamp by the fluorescence produced during heating (33). We found no appreciable destruction of alpha-aminobutyric acid in the course of one hour of heating (Table IV), while the intensity of the fluorescence continued to increase during more prolonged heating.

Paper squares containing the located spots were cut out, placed in test tubes, and assayed for amino acids following Moore and Stein's procedure (35), modified by using only 1 gram of ninhydrin (Dougherty Chemicals product, not recrystallized) per 500 ml of methylcellosolve, and adding 2 ml of ninhydrin reagent per test tube. After the spots were cut out, the residual paper was sprayed with qualitative ninhydrin reagent to make sure that edges or streaks of spots were not left behind. Color was read in a Beckman spectrophotometer at 570 millimicrons, and standards were run simultaneously with the assay in all cases.

TABLE III

Ninhydrin readings given by 1 inch square of Whatman #1 paper, treated and untreated.

(Color developed as described in p. 13)

Readings in Klett colorimeter; filter: 530 millimicrons.

Blank without paper set at zero reading.

Treatment of the paper	Reading
Untreated	40 61 87
Boiled once in 1% NaOH, washed until neutral	33 41
Boiled three times in 1% Na OH, washed until neutral	23 34 42
Left overnight in 1% NaOH, washed until neutral	15 19

TABLE IV

Recovery of amino acid spots heated on filter paper impregnated with 2 butanol: 1 acetic acid: 1 water

Concentration of the alpha-aminobutyric acid solution from which 2 microliter spots were applied	Heating Time			
	No Heating	10 min.	40 min.	60 min.
	Ninhydrin readings (optical density) in a Coleman Junior spectorphotometer; 570 millimicrons. (Paper blanks subtracted).			
0.01M	0.120 0.080	0.110 0.088	0.098 0.100	0.141 0.149
0.08M	0.502 0.492	0.500 0.478	0.500 0.500	0.485 0.489

III) THREONINE AND SERINE DEAMINASES

A) A REVIEW

A convention on nomenclature first: we shall designate the above mentioned deaminase(s) as two distinct enzymes, according to the substrate deaminated. In keeping with this convention we shall not imply any judgment about their identity.

Serine and threonine can be deaminated by oxidative enzyme systems, and by non-oxidative ones. The first systems are given the name of oxidases (amino acid oxidase, for instance), or dehydrogenases in the terminology of Lardy *et al* (16). Their natural hydrogen acceptor is oxygen, and thus one can find preparations which deaminate serine only aerobically (17) (18), giving rise to beta-hydroxypyruvic acid (19). Also threonine can be attacked by oxidative deaminases (18). On the other hand, the names serine deaminase and threonine deaminase are reserved for systems deaminating the corresponding amino acids without the need of oxygen or added hydrogen acceptors. More specifically, for the systems breaking serine down to pyruvic acid and ammonia; and threonine, to alpha-keto-butyric acid and ammonia*.

The first systematic study of serine deamination is

*Binkley (20) prefers to use the name serine dehydrase, in place of serine deaminase. We shall stick to "serine deaminase" because it implies no commitment as to the reaction mechanism.

that of Gale and Stevenson, in 1938 (21), using resting E. coli cells. In 1943 Chargaff and Sprinson (22) demonstrated pyruvic acid and alpha-ketobutyric acid to be the end products of anaerobic deamination of serine and threonine respectively. Deaminase preparations consisted of resting bacterial cells (E. coli, Pseudomonas pycocyanea, Proteus X-19, Clostridium welchii) and, for serine, of cell free extracts of mouse, rat, and rabbit liver. Both deaminases were also found in B. cadaveris (23). In 1949, Wood and Gunsalus (24) purified serine and threonine deaminases from E. coli by precipitation with ammonium sulfate and adsorption on a calcium phosphate gel.

There is no general agreement as to the nature of the prosthetic group of the deaminases in question.

Gale and Stevenson's cell suspensions (21) lost activity by the hour standing in water. At 37°C decay was prevented by M/100 phosphate, adenosine-5-phosphate, glutathione, and other reducing agents. At 0°C, only adenosine-5-phosphate was effective. After decay had set in by aging at 0°C, mixtures of phosphate plus reducing agents were capable of reversing decay. Addition of adenosine-5-phosphate would block that recovery.

Chargaff and Sprinson (22) found it necessary to add magnesium ions to their liver preparations in order to get deaminase activity. Binkley (20) states that the

serine deaminase activity of bacterial cell-free extracts is restored after dialysis by the addition of zinc, magnesium or manganese ions.

Lichstein et al (23) (25) (26) (27) (28) (29) have encountered a biotin effect in bacterial cells aged for one hour at pH 4 in phosphate buffer. Such cells would show higher deaminase activity when biotin was added to the reaction mixture. Adenosine-5-phosphate, in much greater concentrations, had a similar effect. A biotin containing fraction of yeast extract, separated chromatographically, had an activity similar to that of biotin towards serine deaminase of resting cells, and was capable of reactivating cell free preparations which failed to respond to free biotin. Thus, the suggestion was put forth that the combined biotin of yeast extract is the coenzyme of serine deaminase, or a compound closely related to it. This biotin containing fraction also reactivated the deaminases of threonine and aspartic acid, and the decarboxylases of oxaloacetic and succinic acid; as if it also were the coenzyme for those enzymes.

Wood and Gunsalus (24), using their purified preparations, failed to find any yeast extract effect. Instead, they confirmed the finding that glutathione and adenosine-5-phosphate activate the deaminases of serine and threonine. From a series of heavy metal binders, only sodium sulfide and sodium cyanide had partial activity in lieu of

glutathione, suggesting that the role of glutathione might be that of preserving the enzyme's sulfhydryl groups. Adenosine-5-phosphate prevented inactivation of the preparations stored at 0°C.

The following inhibitors have been reported: 10^{-3}M fluoride, 10^{-3}M cyanide (after reactivation of dialyzate with zinc) (20); 10^{-5}M mercuric, silver on cupric ions (24).

B) EXTRACTION AND PURIFICATION

(1) Extraction

Before deciding on the extraction method described in the experimental part, some tests were run (Tables V and VI).

By altering the concentration of lyophilizate in the extracting solution, we found that the solution was not saturated with serine deaminase activity at 50 mg of lyopholizate per ml.

(2) Precipitation by organic solvents

In a preliminary experiment, serine deaminase was precipitated at -16°C from Neurospora extract (made in 1 M, pH 8.1 phosphate buffer) by adding ethanol up to 78%, and also by adding acetone up to the same concentration. Both precipitates, tested for serine deaminase, had about the same activity. Since the acetone precipitate showed about one-half the pyruvic decarboxylase activity* of the alcohol precipitate,

*Studies on pyruvic decarboxylase are not described in this thesis because they are still too incomplete. That enzyme is active in the range of pH 4 to pH 7. Working at pH 7.3 or above, no destruction of pyruvate was noticed in one experiment; some, in another. On the whole it is preferable to get rid of pyruvic decarboxylase if one is to determine deaminase activity by assaying for pyruvate.

TABLE V

Extractability of serine deaminase

Enzyme: 100 mg lyophilizate per ml in 1 M phosphate buffer, pH 7.6. Shaken by hand for 5 minutes, then centrifuged for 15 at ca. 2000RPM, or not centrifuged at all. Reaction mixture: 0.5 ml enzyme solution, and 0.2 ml 0.2M DL-serine or water, and 0.3 ml water. Incubated at 30°C in vials exposed to the air.

Enzyme preparation		Uncentrifuged		Supernatant after centrifugation
Substrate		Water	Serine	Serine
micromoles of pyruvate per ml	after 1½ hrs. incubation	0.5	5.5	4.0
	after 4½ hrs. incubation	0.4	11	10

TABLE VI

Efficiency of different methods for serine deaminase extraction

Enzyme: 50 mg lyophilizate per ml in citrate-phosphate buffer, pH 7.8, shaken by hand for 5 minutes, treated as indicated, then centrifuged and used the supernatant, and 0.2 ml 0.2M DL-serine. Incubated 3 hrs. at 30°C.

Treatment of the lyophilizate suspension	Micromoles of pyruvate per ml
Left standing 1 hr. at 0°C	4.1
Left standing 1 hr. at room temperature	3.5
Left standing 40 min., then shaken in vibrator 20 min., all at room temperature	4.1
Left standing 40 min., then homogenized for 10 min., and shaken in vibrator other 10; all at room temperature	4.3

acetone was preferred for further work.

The pH of the solution from which the deaminases are precipitated by acetone, has a marked effect on the yield (Fig. 1, curve A, and Fig. 2). Curve B (Fig. 1) is also included in fig. 1 because it indicates that the observed pH effect may be specific for the deaminases, rather than a general effect on protein precipitation, thus suggesting an approach to the problem of purification of the deaminases.

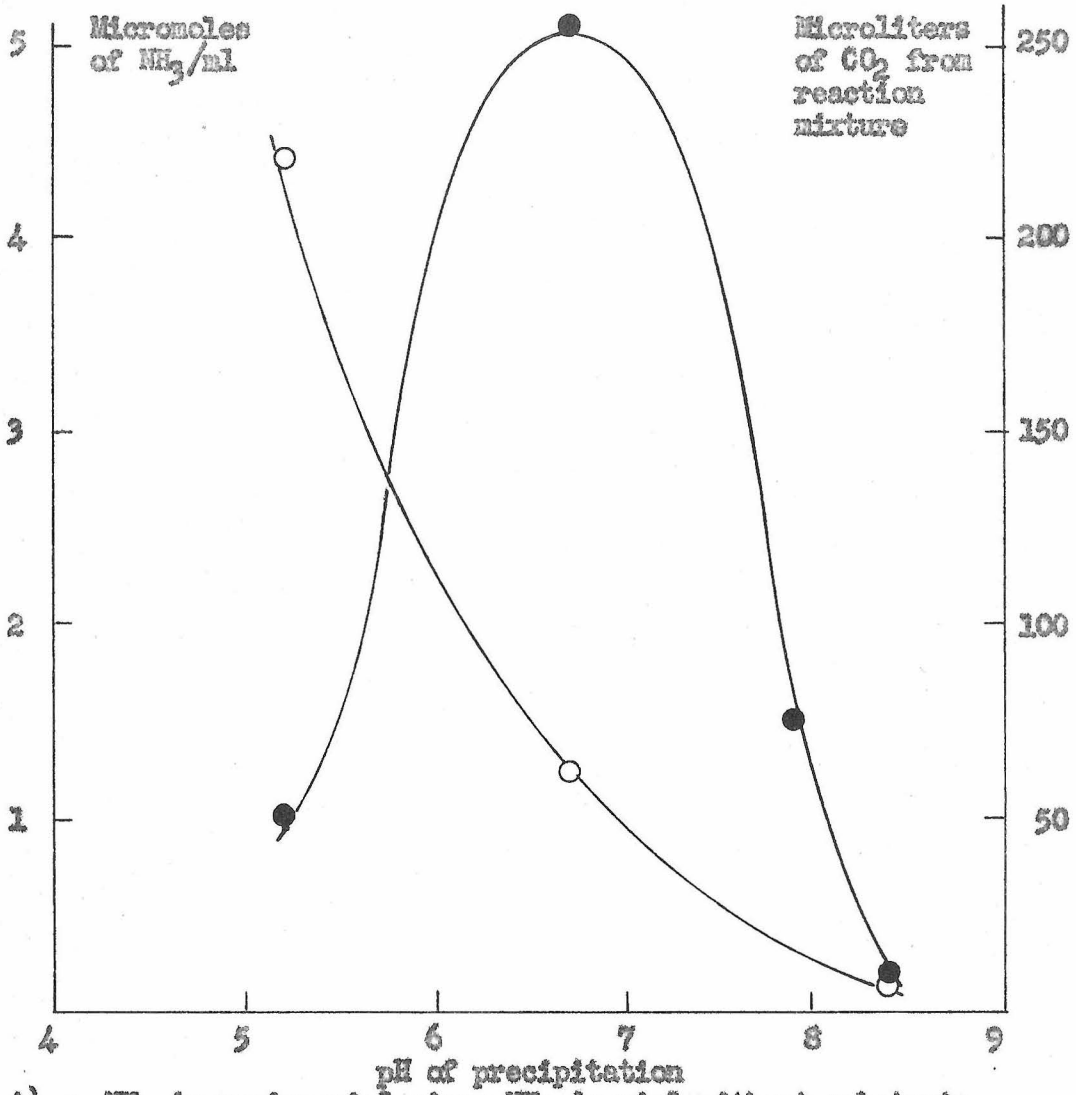
While the agreement between curves A of figures 1 and 2 is not as good as one might wish, they both show the same trend. Partial disagreement may be due to somewhat different experimental conditions, as specified in the legends, or to other uncontrolled factors (e.g., shaking and stirring of the precipitating solutions).

The effect of the acetone concentration on the serine deaminase activity of the precipitate is illustrated in figure 3.

A batch of 70% acetone precipitate was prepared from a Neurospora extract (pH 6.7, in M/20 borate), thoroughly dried <u>in vacuo</u>, and stored in a desiccator over calcium chloride in the ice box. After 4 months its threonine deaminase activity was of the same order of magnitude as that of the fresh powder; after 11 months it was practically nil.

Figure 1

Effect of pH on precipitation by acetone



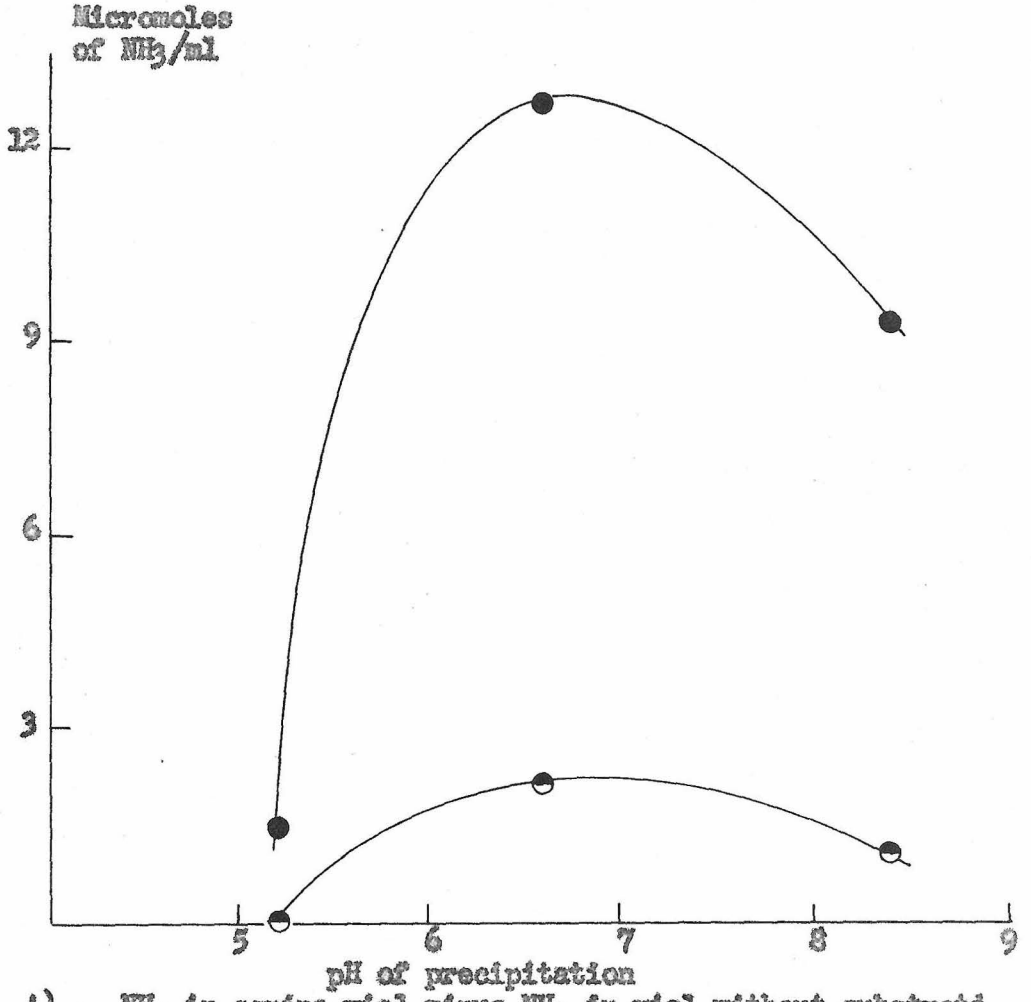
A) ● NH_3 in serine vial minus NH_3 in vial without substrate

B) ○ CO_2 produced from pyruvate

Enzyme preparations: 3 ml of Neurospora extract, plus 5 ml of:
McIlvaine buffer (pH 5.0) ------ resulting pH, 5.2
M/20 borate ---------- " pH, 6.7
1 M phosphate buffer ---------- " pH, 7.9
M/20 borate ---------- " pH, 8.4 ; plus
acetone up to 80% (at -16°C). After 1 hour, centrifuged, and took up precipitate in 3.6 ml M/5 phosphate buffer. Resulting pH ranged from 6.7 to 7.8
Incubated to test for serine deaminase in the usual manner, 5 hrs. at 30°.

Pyruvic decarboxylase activity was determined in a Warburg respirometer, at 35°C, using the same enzyme preparations after titrating them to pH 5.1 to 6.0 (Within this range, decarboxylase activity varies little). Recorded in this figure: gas evolved in the first 90 minutes.

Figure 2
Effect of pH on precipitation by acetone



A) ● NH_3 in serine vial minus NH_3 in vial without substrate

B) ◒ NH_3 in threonine vial minus NH_3 in vial without substrate

Enzyme preparations: 5 ml of Neurospora extract plus 5 ml of the following buffers:

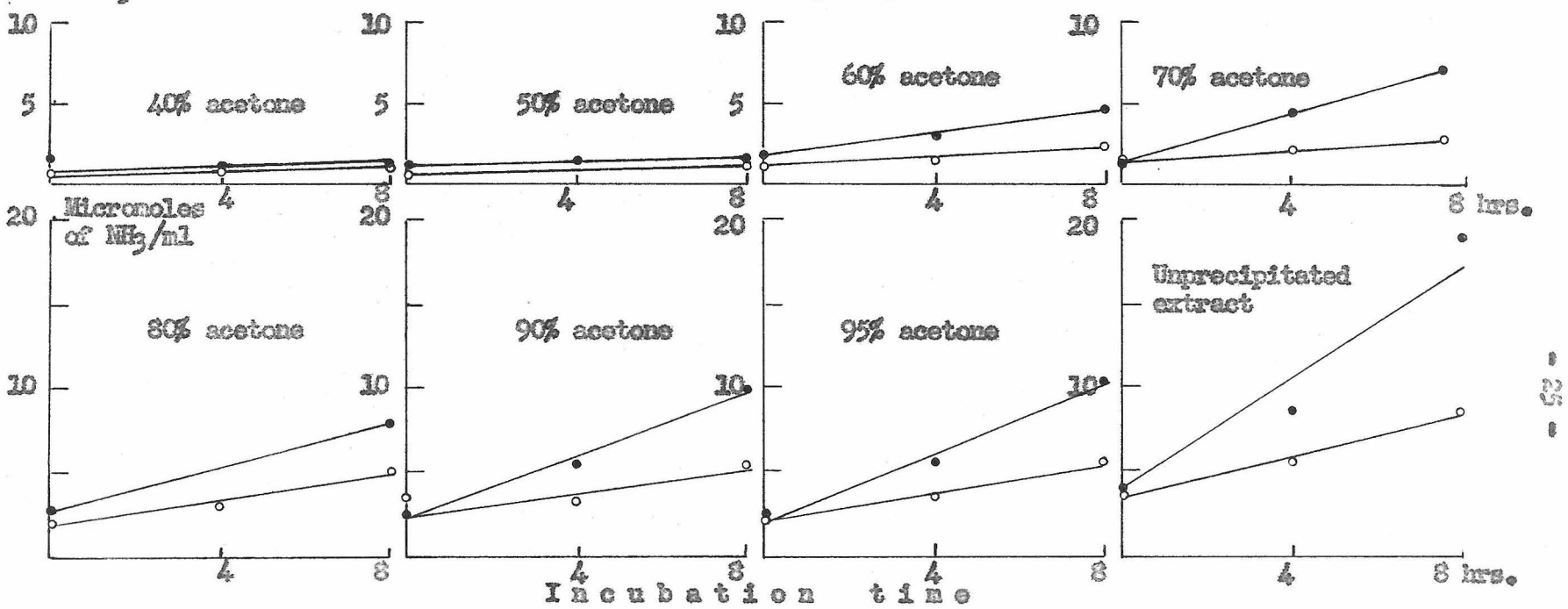
M/5 citrate——resulting pH, 5.2
M/20 borate—— " pH, 6.6
M/20 " —— " pH, 8.4 ; plus

acetone up to 80% (at -16°C). After ½ hour, centrifuged, and took up precipitate in 5 ml M/10 phosphate buffer. Resulting pH ranged from 7.5 to 7.8

Incubated in the usual manner, for 4 hours at 37°C.

NOTE: The shape of a curve passing through three points cannot have much significance. This figure is mainly intended for comparison with figure 1.

Figure 3
Effect of acetone concentration on yield of serine deaminase in the precipitate



• NH_3 in serine vials ° NH_3 in vials without substrate

Enzyme preparations: 2 ml aliquots from a Neurospora extract (pH 6.7, in phosphate buffer), made up to 40 ml by addition (at -16°C) of the required water-acetone mixtures. Centrifuged after one hour, dried the precipitate by means of a water pump for one hour, and took up in 2 ml distilled water.
Incubated at 30°C
Reaction mixtures: 0.8 ml enzyme plus 0.2 ml 1 M phosphate buffer (pH 8.1)
0.2 ml 0.2M DL-serine , or 0.2 ml water
PH of the reaction mixtures ranged from 7.5 to 7.8, except for unprecipitated extract which gave 7.2 .

(3) Precipitation by ammonium sulfate

The acetone powder mentioned above was used to find out what yields of deaminase could be obtained by precipitation with different ammonium sulfate concentrations. It was dissolved in distilled water so as to make it twice as concentrated as the original Neurospora extract. Four volumes of ammonium sulfate solution of the required concentration were added dropwise to aliquots of that solution, from a burette, with stirring. The stock saturated ammonium sulfate solution had 0.01M of ammonium hydroxide added per liter to neutralize the acidity of the salt (36). After standing for 12 hours, the precipitates were centrifuged off (in this experiment using the clinical centrifuge; in all others using a Sorval; both for 20 to 30 minutes), dissolved in M/100 ammonium hydroxide (36), and dialyzed for 20 hours against M/10 pH 7.6 phosphate buffer. Incubations, with and without serine, were run for 3 hours at 30°C. (Table VII).

The precipitate obtained with 30% saturated ammonium sulfate, was conspicuously less bulky than the others.

Pyruvic decarboxylase activities of the same preparations used in the experiment of table VII are shown in table VIII.

C) EFFECT OF PH AND TEMPERATURE ON THE RATE OF DEAMINATION

Four grams of lyophilizate were suspended in M/4 pH

TABLE VII

% saturation of ammonium sulfate during precipitation		30%	50%	70%	90%	Untreated acetone powder*
Micromoles of ammonia per ml	From blank vial	0.9	1.0	1.3	2.0	3.8
	From serine vial	1.2	3.4	5.0	5.5	7.0
	Difference	0.3	2.4	3.7	3.5	3.2

TABLE VIII

% saturation of ammonium sulfate during precipitation	30%	50%	70%	90%	Untreated acetone powder*
Microliters of CO_2 produced in half an hour (Cocarboxylase and Manganous ions added)	12	55	75	103	500

* By extrapolation, from another experiment run under similar conditions

8.3 phosphate buffer, making a total volume of 60 ml, and homogenized and centrifuged as usually. The supernatant was dialyzed for 21 hours against M/10 pH 7.7 phosphate buffer in order to reduce the ammonia blanks. After adding calcium pyridoxal phosphate up to 3 micrograms per ml, aliquots were taken and titrated to the desired pH with citric acid and sodium hydroxide, effecting in each case a 1.25 fold dilution. Those solutions constitute the enzyme preparations used in the experiment described in figures 4 and 5.

While the p^H optimum for serine deaminase lies about 7.8, that for threonine deaminase is above 9. The temperature curves show a shift in the optimum for serine according to the time at which ammonia was determined, as if inactivation of the enzyme had become a factor of consideration at the time of the late readings. The deamination of threonine does not show such shift in temperature optimum.

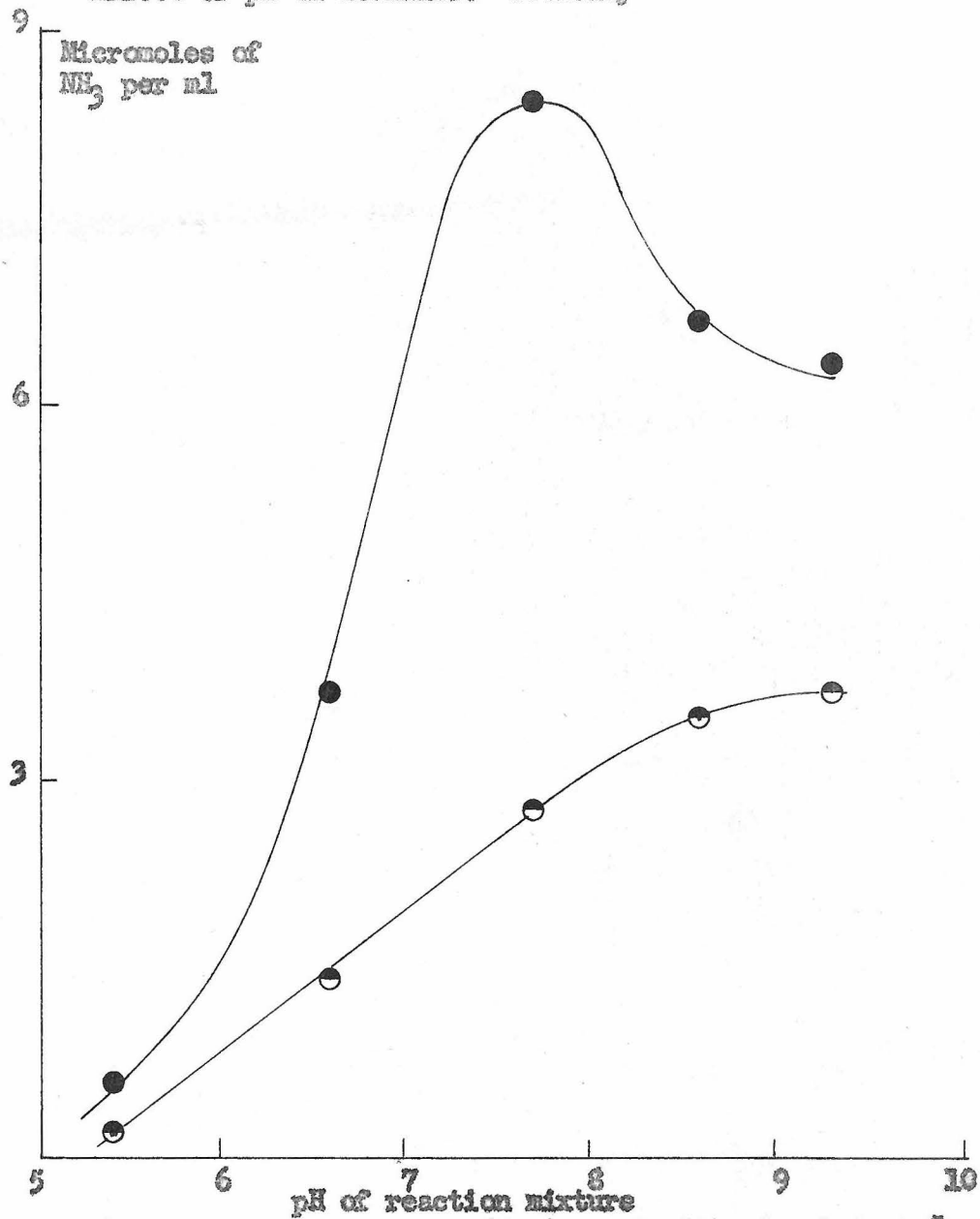
D) ACTIVATORS

As a sequel of the studies on the reactivation by pyridoxal phosphate of the system catalyzing the transformation of threonine in alpha-aminobutyric acid (p.62), we found that serine and threonine deaminases were also activated by that compound.

A number of different enzyme preparations, treated and

incubated in diverse fashions, were used in an attempt to magnify the pyridoxal phosphate effect. Much of the resulting data is presented in tables IX to XVII.

Figure 4
Effect of pH on deaminase activity

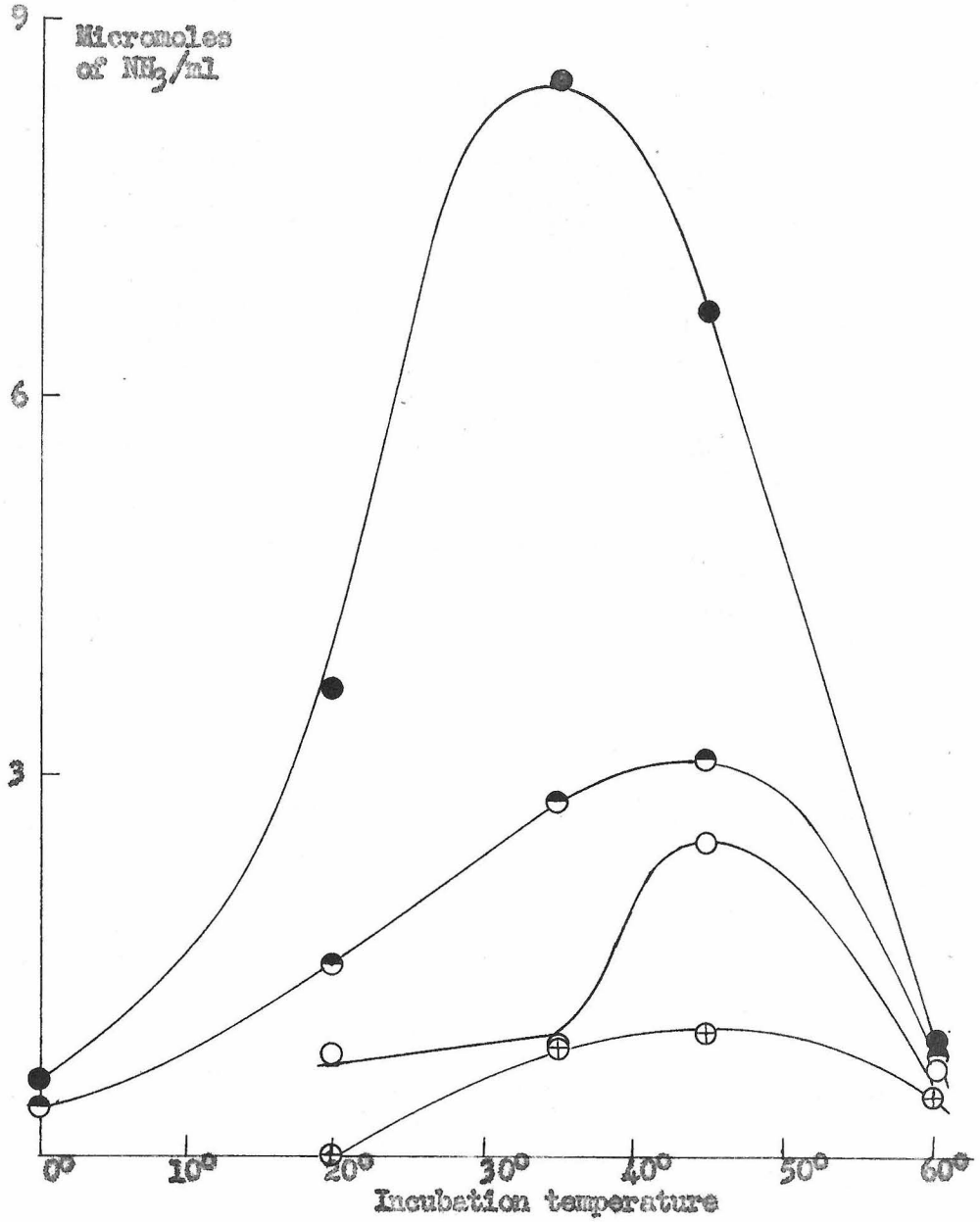


Incubated for 4 hours at 35°C, in the usual manner

* Readings in blank ranged from 0.4 to 0.8 micromoles/ ml

Figure 5

Effect of temperature on deaminase activity



● NH_3 in serine vial, minus ammonia in vial without substrate*, after 4 hours incubation.

○ NH_3 in serine vial, minus ammonia in vial without substrate*, after 1 hour incubation.

⊖ NH_3 in threonine vial, minus NH_3 in vial without substrate*, after 4 hours incubation.

⊕ NH_3 in threonine vial, minus NH_3 in vial without substrate*, after 1 hour incubation.

Incubated at pH 7.7 , in the usual manner.

* Readings in blanks ranged from 0.4 to 0.8 micromoles/ml.

TABLE IX

Activation of threonine deaminase by B_6

Enzyme preparation: ammonium sulfate precipitated Neurospora extract, dialyzed against M/10 pH 7.8 phosphate buffer for 16 hours, and stored at -16°C for 3 days
Incubation: for 1½ hours. First at 40°C; and, after about 1 hour, at temperatures gradually rising up to 90°C
Reaction mixture: 0.5 ml enzyme, and 0.1 ml substrate or water, and 0.2 ml cofactor or water

Enzyme precipitated by	Substrate	Cofactor			R***
		No Cofactor	10 micrograms B_6al-Ph**	Boiled Neurospora extract	
90% saturated ammonium sulfate		Micromoles of ammonia/ml			
	No substrate	0.8		2.4	
	0.4M DL-threonine	3.0 2.4*	4.3* 4.5* 4.5	5.2	
	Minus blank	(av.) 1.9	3.6	2.8	1.9
50% saturated ammonium sulfate	No substrate	0.6		3.3	
	0.4M DL-threonine	3.3* 2.3	5.4 5.9* 5.3*	5.3	
	Minus blank	(av.) 2.2	5.0	2.0	2.3

*Vessels had other vitamins added, but we treat them here as duplicates

**B_6al-Ph will henceforth be used as abbreviation for the calcium salt of pyridoxal phosphate

***By "R" we shall denote the ratio of activity with B_6al-Ph added, to activity without B_6al-Ph

TABLE X

Activation of the deamination of L- and DL-threonine by B_6

Enzyme preparation: Neurospora extract, precipitated with 50% ammonium sulfate, dialyzed for 12 hours against M/10 pH 7.8 phosphate buffer
Incubation; for 5 hours at 40°C.
Reaction mixture: 0.8 ml enzyme; 0.2 ml substrate or water; 0.1 ml cofactor or water

Substrate	Cofactor								R	
	No cofactor		10 micrograms of B_6 al-Ph		Pyridoxal HCl*		CaCl2*	Cocarboxylase HCl*		
	NH3/ ml	alpha-k.but/ ml	NH3/ ml	alpha-k.but/ ml	NH3/ ml	alpha-k.but/ ml	NH3/ ml	NH3/ ml	NH3	alpha-k.but
No substrate	0.7 0.7	0.4	1.7 0.5	1.1	0.7	1.3	0.7	0.8		
0.4M DL-threonine	8.0 7.0	7.1	9.8 9.3	8.1						
Minus blank (av)	6.8	6.7	8.5	7.0					1.3	1.1
0.2M L-threonine	10.1		15.5 13.8	14.0	10.1	8.5	10.0	10.0		
Minus blank (av)	9.4		13.6	12.9	9.4	7.2	9.3	9.2	1.5	1.8

Ammonia recovery, after incubation as above: 98%; and 105% with pyridoxal phosphate
Alpha-ketobutyric acid recovery, after incubation as above: 102%; and 95% with pyridoxal phosphate

*In concentrations equivalent to that of B_6 al-Ph

TABLE XI

Activation of the deamination of L- and DL-threonine by B_6

Enzyme preparation: same as in experiment of table X, stored at -16°C for 3 days.
Incubation: 6 hours at 37° C
Reaction mixture: 0.5 ml enzyme
0.2 ml substrate or water
0.1 ml cofactor or water

Substrate	Cofactor		
	no cofactor	10 micrograms B_6al-Ph	
	Micromoles of NH_3/ml		
No substrate	0.0	0.0	R
0.1M DL-threonine	2.5	5.0	2.0
0.05M L-threonine	2.0	4.2	2.1
0.4M DL-threonine	5.0	6.8	1.4
0.2M L-threonine	6.5	11.5	1.8

TABLE XII

Temperature dependence of the B_6 effect on the deaminases

Enzyme: crude Neurospora extract
Incubation: 3 hours
Reaction mixture: 1 ml enzyme
0.2 ml substrate or water
0.2 ml cofactor or water

		Cofactor		
	Substrate	No Cofactor	60 micrograms B6al-Ph	
		Micromoles NH3/ml		
Incubated at 35°C	No substrate	4.0	5.8	
	0.4M DL-threonine	12.8	17.6	R
	Minus blank	8.8	11.8	1.3
	0.4M DL-serine	12.2	17.6	
	Minus blank	8.2	11.8	1.4
Incubated at 40°C	No substrate	5.0	5.1	
	0.4M DL-threonine	12.2	20.7	
	Minus blank	7.2	15.6	2.2
	0.4M DL-serine	11.9	21.0	
	Minus blank	6.9	15.9	2.3
S/T*	35°	0.9	1.0	
	40°	1.0	1.0	

*By S/T we shall henceforth denote the ratio of serine deaminase activity to threonine deaminase activity

TABLE XIII

B_6 effect on threonine deaminase after prolonged dialysis

Enzyme preparation: Neurospora extract (from a lyophilizate of a 7 day old wild type culture) dialyzed against indicated buffers, which were changed in 3 liter batches after 12, 24, 50 and 108 hours.
Incubation: 5 hours at 37° C
Reaction mixture: 1 ml enzyme
0.2 ml substrate or water
0.2 ml cofactor or water

Duration of dialysis	Buffer used for dialysis	Substrate	Cofactor: No cofactor	Cofactor: B6al-Ph 10 microgram	Cofactor: Boiled Neurospora extract	R
			Micromoles of ammonia/ml			
84 hours	M/10 pH 7.6 phosphate	No substrate	0.0	0.0	0.5	
		0.4M DL-threonine	2.5	3.3	3.4	
		Minus blank	2.5	3.3	2.9	1.3
	M/10 pH8.6 borate	No substrate	0.8	0.8	1.3	
		0.4M DL-threonine	2.1	2.5	2.8	
		Minus blank	1.3	1.7	1.5	1.3
132 hours	M/10 pH 7.6 phosphate	No substrate	0.2	0.1	0.9	
		0.4M DL-threonine	1.0*	1.5	2.0	
		Minus blank	0.8	1.4	1.1	1.8

*This value was arrived at by correcting in a somewhat subjective way the colorimeter reading, which was too high because of turbidity in the Nessler developed solution. It may be in error up to plus-minus o.2

TABLE XIV

B_6 effect on threonine deaminase, with cyanide additions

Incubation: 3 hours at 40°C
Reaction mixture: 0.5 ml Neurospora extract (in M/4 phosphate)
0.1 ml substrate or water
0.1 ml cofactor or water
0.1 ml cyanide or water

Cyanide additions	Substrate	Cofactor		
		No Cofactor	30 micrograms B6al-Ph	
		Micromoles of NH3/ml		
No cyanide	No substrate	4.0		
	0.4M DL-threonine	7.7	18.8	R
	Minus blank	3.7	14.8	4.0
10^{-6} moles cyanide	No substrate	4.5		
	0.4M DL-threonine	8.0	18.0	
	Minus blank	3.5	13.5	3.9
10^{-5} moles cyanide	No substrate	5.6		
	0.4M DL-threonine	7.5	19.6	
	Minus blank	1.9	14.0	7.4

TABLE XV

More on B_6 effect

Enzyme preparation: Neurospora extract (in M/4 phosphate) made with the same lyophilizate used for experiment of table XIV. Lyophilizate was fresh when used in the former experiment, and had been stored for 4 days in the icebox - in an unevacuated desiccator over $CaCl_2$ - before using for the present one.
Incubation: 3 hours at 40°C
Reaction mixture: 1 ml enzyme
0.2 ml substrate or water
0.2 ml cofactor or water

	Cofactor		
Substrate	No cofactor	30 micrograms B6al-Ph	
	Micromoles NH3/ml		
No substrate	6.0	6.0	
0.4M DL-threonine	13.4	21.2	R
Minus blank	7.4	15.2	2.1
0.4M DL-serine	10.6	17.6	
Minus blank	4.6	11.6	2.5
S/T	0.6	0.8	

TABLE XVI

Effect of preincubation of enzyme preparation on the B_6 activation of serine and threonine deaminases

Incubation: 3 hours at 35° C
Reaction mixture: 1 ml enzyme
0.2 ml substrate or water
0.2 ml cofactor or water

Enzyme preparation #1: Neurospora extract (same used for experiment of table XIV) dialyzed for 20 hours against M/10 pH 7.6 phosphate buffer. Diluted with 1/10 volume of water

	Cofactor		
Substrate	No cofactor	60 micrograms B6al-Ph	
	Micromoles of NH3/ml		
No substrate	0.5	0.7	
0.4M DL-threonine	1.3	2.5	R
Minus blank	0.8	1.8	2.2
0.4M DL-serine	6.9	13.0	
Minus blank	6.4	12.3	1.9
S/T	8.0	6.8	

TABLE XVI (continued)

Enzyme preparation #2: preparation #1 was covered with toluene and incubated for 2 hours at 45°C. Before using for deaminase test, it was cooled.

	Cofactor		
Substrate	No cofactor	60 micrograms B6al-Ph	
	Micromoles of NH3/ml		
No substrate	0.4	0.6	
0.4M DL-threonine	1.2	1.2	R
Minus blank	0.8	0.6	0.8
0.4M DL-serine	2.1	5.0	
Minus blank	1.7	4.4	2.6
S/T	1.9	7.3	

Enzyme preparation #3: preparation #1 was covered with toluene and incubated for 4 hours at 45°C. Before using for deaminase test, it was cooled.

	Cofactor		
Substrate	No cofactor	60 micrograms B6al-Ph	
	Micromoles of NH3/ml		
No substrate	0.8	1.0	
0.4M DL-threonine	1.0	1.0	R
Minus blank	0.2	0.0	-
0.4M DL-serine	1.3	3.2	
Minus blank	0.5	2.2	4.4
S/T	2.5	-	

TABLE XVI (continued)

Enzyme preparation #4: Dialyzate used in preparation #1 was diluted with 1/10 volume of phosphate preparation (0.2 mg/ml intenstinal phosphatase - see text -in pH 8.6 buffer; M/10 in borate, M/50 in $MgSO_4$); was then covered with toluene and incubated for 4 hours at 45°C. Before using for deaminase test, it was cooled.

	Cofactor		
Substrate	No cofactor	60 micrograms B6al-Ph	
	Micromoles NH3/ml		
No substrate	2.4	2.2	
0.4M DL-threonine	2.7	2.7	R
Minus blank	0.3	0.5	1.7
0.4M DL-serine	3.0	4.7	
Minus blank	0.6	2.5	4.2
S/T	2.0	5.0	

TABLE XVII

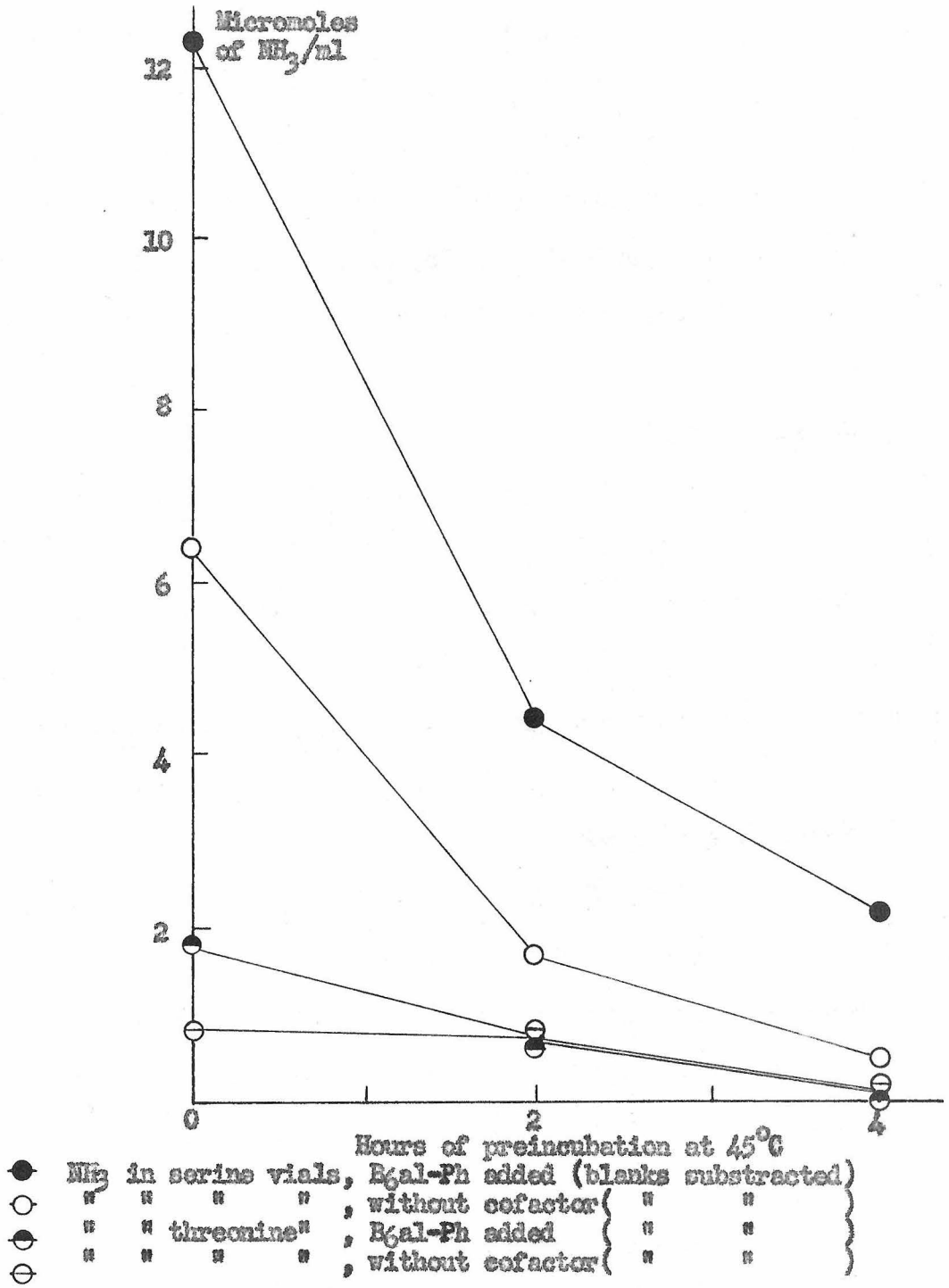
B_6 activation of the deaminases from mycelium kept frozen for 33 days

Enzyme preparation: E-5256A was grown for 4 days in 2 Fernbach flasks, with 1 liter of "minimal" medium in each, at 25° C. After harvesting and drying by suction and blotting, the mold was kept for 14 days at -16° C, and for 19 more days at -10° C. It was then lyophilized and immediately used to make a Neurospora extract in the usual way (in M/4 phosphate buffer).
Incubation: 3 hours at 40°C
Reaction mixture: 0.5 ml enzyme
0.1 ml substrate or water
0.1 ml cofactor or water

	Cofactor		
Substrate	No cofactor	30 micrograms B6al-Ph	
	Micromoles of NH_3/ml		
No substrate	9.4	8.2	
0.2M L-threonine	10.3 10.8	16.6	R
Minus blank (aver.)	1.1	8.4	7.6
0.4M DL-serine	14.0 15.9	23.0 23.9	
Minus blank (aver.)	5.6	15.2	2.7
S/T	5.1	1.8	

Duplicates are duplicate ammonia determinations

Figure 6
Plot of the results listed on table XVI



The tables show activation of threonine and serine deaminases by pyridoxal phosphate, in almost all cases, causing an increase in activity up to 7.6 fold. Boiled Neurospora extract also causes activation, but in all cases lower than that due to pyridoxal phosphate (Tables IX and XIII). Neither calcium chloride, nor pyridoxal, nor cocarboxylase could substitute for pyridoxal phosphate. There is a parallel increase in the yields of ammonia and of alpha-ketobutyric acid when pyridoxal phosphate is added (Table X).

Tables X and XI are consistent in showing that reaction mixtures with L-threonine respond to pyridoxal phosphate better than those with DL-threonine as substrate. Table XII reveals that on raising the temperature from 35°C to 40°C, activation by B_6 is magnified.

Dialysis did not prove a successful method to obtain coenzyme-free preparations (Table XIII).

In view of the fact that cyanide ions behave as inhibitors for pyridoxal phosphate enzymes, presumably in their capacity of carbonyl reagents (37), and since we observed that M/100 KCN destroys the fluorescence and the yellow color of a solution of 30 micrograms of pyridoxal phosphate per ml, we tested the effect of cyanide ions on threonine deaminase (Table XIV). An inhibition of deamination appears to have taken place at the highest cyanide concentration in the vial without added pyridoxal phosphate.

Since there is considerable variation in the blanks, this apparent effect of cyanide might be an artifact.

The enzyme preparation used in the above experiment was unusually sensitive to B_6 activation in the cyanideless reaction mixtures. Using the same lyophilizate, we attempted to reproduce the magnitude of that effect (Table XV), but did not succeed. The only important recognizable difference between both experiments lies in the fact that for the one reported on table XIV a fresh lyopholizate was used, while the lyopholizate used for the other experiment had stood in the icebox, in contact with air, for four days.

A commercial purified preparation of intestinal phosphatase (alkaline), put out by Armour and kindly supplied by Mr. George Ellman, was tested for its ability to hydrolyze pyridoxal phosphate. In the course of half an hour incubation at 37°C a solution of 1 mg of purified phosphatase in 4 ml of M/15 pH 8.6 borate buffer (0.008 molar in magnesium sulfate) and containing 0.24 micromoles of calcium pyridoxal phosphate at the beginning of incubation, liberated 0.15 micromoles of inorganic phosphate (determined colorimetrically by Berenblum and Chain's method (38)). Using one-tenth the amount of phosphatase, under identical conditions, 0.10 micromoles of phosphate were liberated. In the course of both incubations the yellow color of pyridoxal phosphate was bleached, and its fluorescence, as observed under a UV lamp, turned from its typical green

to a blue shade like the one of pyridoxal. The possibilities, (1) that this phosphatase might destroy the deaminase-coenzyme activity of Neurospora extract, and (2) that by preincubation of the Neurospora extract its own battery of enzymes might inactivate the deaminase-coenzyme, were tested in the experiment described in table XVI and figure 6. Preincubation did indeed enhance the effect of addition of pyridoxal phosphate to the reaction mixture where serine was deaminated. In the case of threonine deaminase, the results are inconsistent and obscured by the experimental errors resulting from high blanks and low activity. The addition of phosphatase does not seem to enhance the pyridoxal phosphate effect of the Neurospora extract.

The above experiment also indicates that preincubation at 45°C, particularly in the tests where no cofactor was added, decreases the S/T ratio, as if serine deaminase were inactivated (by coenzyme dissociation?) more rapidly than threonine deaminase. Variability of the ratio of serine to threonine deaminase activity (S/T) can be noticed by inspection of tables XII, and XV to XVII.

The most successful way of resolving threonine deaminase from its pyridoxal phosphate-like component was aging the harvested mold in a frozen condition (Table XVII, compare with table XV or part of table XIV, making allowance for the fact that L-threonine was used in one case, and DL- in the others). Notice that serine deaminase was

was not resolved by such storage beyond the stage of resolution found in our ordinary Neurospora extracts.

The search for greater values of R (activity with B_6 over activity without), led us to test other Neurospora preparations.

An acetone precipitated powder which after 11 months in the icebox had lost most of its threonine deaminase activity (p. 22) failed to respond to pyridoxal phosphate.

Addition of pyridoxal phosphate in tests for threonine deaminase activity in precipitates of Neurospora extract thrown down by acetone at different pHs failed to alter the shape of curve B in figure 2.

Extracts of a mutant strain requiring either pyridoxine or a "minimal" medium of pH 7 (or higher) for growth (39) were found to have little deaminase activity when grown at pH 7 (p. 73). Their threonine deaminase activity, with and without B_6, is compared with that of strain E-18829 (grown in "minimal" medium, with threonine added), the closest "control" available, in table XVII.

TABLE XVIII

Threonine deaminase activities of strains 44602 and E-18829

Enzyme preparation: Used the same lyophilizate described in table XXIII, but kept for 6 months in a desiccator in the icebox. Extracts were prepared as usually, but containing 70 mg of lyophilizate per ml, and further treated as follows to decrease the ammonia blanks: incubated at 30°C for 45 minutes, then left standing for 10 minutes in Thunberg tubes, evacuated with a water pump, and finally flooded with nitrogen.
Incubation: 5 hours at 40°C
Reaction mixture: 0.5 ml enzyme
0.1 ml substrate or water
0.1 ml cofactor or water

		Cofactor	
Enzyme source	Substrate	No cofactor	10 micrograms B_6al-Ph
		Micromoles of NH_3/ml	
strain 44602	No substrate	9.0	9.0
	0.4M DL-threonine	14.5	13.2
	Minus blank	5.5	4.2
strain 18829 (grown with added threonine)	No substrate	7.2	8.0
	0.4M DL-threonine	21.7	23.0
	Minus blank	14.5	15.0

It is apparent that pyridoxal phosphate deficiency is not the cause of the lower activity of extracts from the B_6-less strain.

The following compounds were added to deaminase reaction mixtures, and failed to show any effect, either as activators or inhibitors of the reaction.

a) Tested in addition to B_6al-Ph:

2×10^{-2}M $Na_2S_2O_4$ (Freshly made solution)

10^{-3}M $CuSO_4$

0.04 micrograms biocytin*/ml

A vitamin mixture* added so that 1 ml of reaction would contain:

0.15 mg l-quinic acid
10^{-4} mg folic acid
10^{-5} mg B_{12}
0.002 mg riboflavin
10^{-4} mg folinic acid

M/700 sulfanilamide

M/700 para-aminobenzoic acid

b) Tested by itself:

0.04 micrograms biocytin/ml

The vitamin mixture described above

Layering with toluene (tested for serine deaminase)

---

*We are grateful to Dr. Jean Mauron for these solutions

E) REACTION BALANCES - PRODUCTS, SIDE PRODUCTS AND POSSIBLE INTERMEDIATES

(1) Deamination of threonine and serine

The course of the deamination of threonine is followed in table XIX and figure 7. The enzyme solution used in that experiment was prepared as follows: a Neurospora extract, made up in M/4 pH 9.7 phosphate buffer (the pH of the resulting extract was ca. 7.8) had ammonium sulfate added up to 60% saturation, and the precipitate obtained was treated in the usual way, dialyzed against M/10 pH 7.8 phosphate buffer for 15 hours, and diluted with one volume of the same buffer containing pyridoxal phosphate. Final concentration of pyridoxal phosphate was 10 micrograms per ml of the enzyme preparation: The reaction mixtures consisted of:

2 ml enzyme preparation ;
0.5 ml substrate A, or water;
0.5 ml substrate B, or water.

They were incubated in 1½ dram vials, covered with rubber caps, at 40°C. A different vial was used to take readings at each specified time; when 0.4 ml of 1N sulfuric acid were added, the vials were placed in a boiling water bath, the coagulum centrifuged off, and the supernatant stored for a couple of days at -16°C while the assays were being performed.

TABLE XIX - part 1

		Time zero					Time 1½ hours				
		Quantitative In micromoles/ml		Semi-quantitative (spot intensities)			Quantitative in micromoles/ml		Semi-quantitative (spot intensities		
Substrate A	Substrate B	NH3	Alpha-keto-butyric acid	Glut-amic acid	Threo nine	Alpha-amino butyric acid	NH3	Alpha-keto-butyric acid	Glut-amic acid	Threo-nine	Alpha amino butyric acid
---	---	0.25		-	-	-	0.3		-	-	-
0.2M L-thre onine	---	0.75		-	#6	-	5.65 6.25	6.0	-	#6	-
0.2M DL-Na alpha-keto-butyrate	---	0.0	28.5	-	-	-					
0.2M L-threonine	0.2 Na alpha-keto-glutarate						6.25 5.8	6.0	-	#6	-
0.1M $(NH_4)_2SO_4$	0.2M Na alpha-keto glutarate	24.5		-	-	-	27.5		-	-	-
0.04M DL-alpha-amino butyric a.	---	0.25 0.5	0.5	-	-	#4					
0.04M Na L-glutamate	---	0.5	0.0	#4	-	-					

Where 0.2M solutions were added, the calculated concentration in the reaction mixture is 29.4 micromoles per ml; where 0.04, it is 5.88 micromoles per ml.

Duplicates correspond to duplicate assays of the same reaction mixture.

Intensity of chromatographic spots, estimated visually, is rated with symbols ranging from #- to #6. No spot is indicated by -. Only spots corresponding to the 3 amino acids listed appeared in the chromatograms

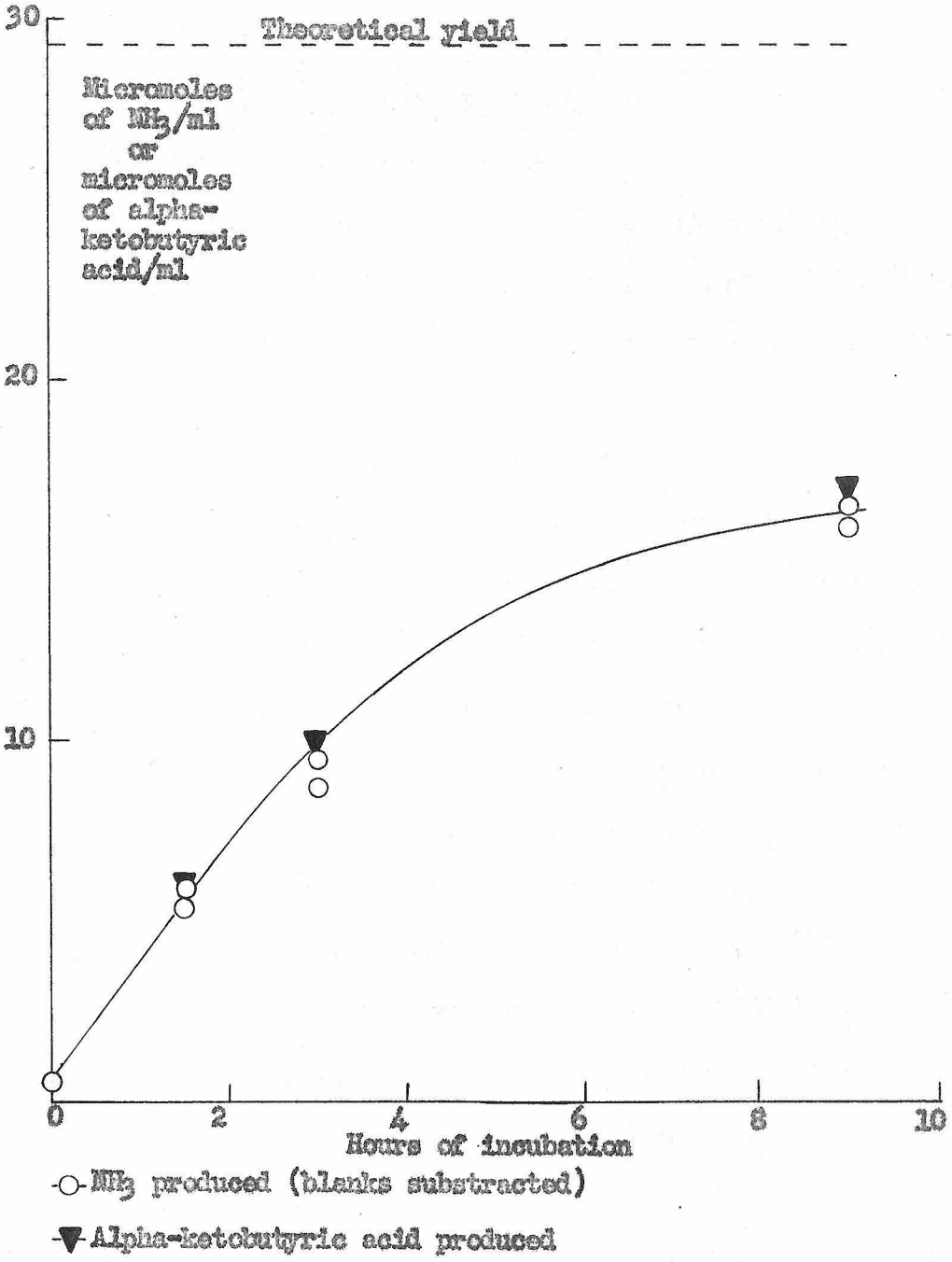
Where no figures or symbols are recorded it means that no determination was made.

TABLE XIX - part 2

		Time 3 hours					Time 9 hours					
		Quantitave micromoles ml		Semi-quantitative (spot intensities			Quantitative micromoles/ml			Semi-quantitative (spot intensities)		
Substrate A	Substrate B	NH3	Alpha-keto-butyr-ic a.	Glut-amic acid	Threo-nine	Alpha-amino buty-ric a.	NH3	Alpha-keto-buty-ric a.	Alpha amino buty-ric a.	Glut amic acid	Threo nine	Alpha-amino buty-ric a.
---	---	0.3		-	-	-	0.5 0.7	0.0*	1.2 0.0	#	-	-
0.2M L-threonine	---	9.0 9.65	10	-	#6	-	16.4 17.0	16	2.8 2.3 2.5	-	#5	#
0.2M DL-Na alpha keto butyrate	---						0.0	28	2.5 2.6 2.7	#	-	#
0.2M L-threonine	0.2M Na alpha-keto glutarate	10.3 10.5	9.5	-	#6	-	17.6 18.0	15.5		#-	#5	-
0.1M $(NH_4)_2SO_4$	0.2M Na alpha-keto glutarate	26.0		#	-	-	28.0 26.0	0.5		#2	-	-
0.04M DL-alpha-amino butyric a.	---						1.5 2.0	1.4	3.8	#-	-	#4
0.04M Na L-glutamate	---						2.0			#4	-	-

*All values of alpha keto butyric acid given in this table were corrected so that the one indicated by the asterisk would be zero.

Figure 7
The course of the deamination of threonine
(A plot of data from table XIX)



The following facts emerge from table XIX:

Alpha-aminobutyric acid is formed by incubation of threonine, and of alpha-ketobutyric acid (this process will be dealt with later on). Its production is not of such magnitude that it would significantly alter the yields of alpha-ketobutyric acid.

Production of ammonia and alpha-ketobutyric acid from threonine parallel each other quite well.

No transamination between threonine and alpha-ketoglutaric acid can be detected.

The addition of alpha-ketoglutaric acid does not affect the yields of ammonia or alpha-ketobutyric acid.

Alpha-aminobutyric acid is deaminated at such a slow rate that it must be ruled out as possible intermediate in the deamination of threonine.

Addition of an aliquot from the 9 hour vial to which threonine had been added as the only substrate, to a solution of 2,4-dinitrophenyl hydrazine, resulted in the formation of a yellow precipitate which, recrystallized once from ethyl acetate, melted at 190°- 192°C (corrected). A sample of the 2,4-dinitrophenyl hydrazone of alpha-ketobutyric acid melted at 195°C (corrected) in a simultaneous run.

Under the same conditions employed to obtain the above hydrazone precipitate, an aliquot from the 9 hour vial to which no substrate had been added produced no

precipitate when added to the 2,4-dinitrophenyl hydrazone solution.

Oxygen uptake in the course of the deamination of threonine was measured in the Warburg respirometer, under air (Table XX). While both alpha-aminobutyric and threonine (or their by-products) are oxidized by the enzyme preparation used, the rate of oxidation is too low to account for the deamination of threonine. A perhaps significant decrease in the rate of oxygen uptake by threonine in the vessel to which pyridoxal phosphate was added may be noticed. It is evident again in this experiment that the deamination of alpha-aminobutyric acid is not a step in the deamination of threonine, and that it could not be responsible for the increase in the yields of ammonia when pyridoxal phosphate is added.

TABLE XX

Oxygen uptake and deamination of threonine and alpha-aminobutyric acid

Enzyme preparation: ammonium sulfate precipitates used in experiment of table IX, combined and diluted with 1/10 volume of M/10 pH 7.6 phosphate buffer
Incubation: in Warburg respirometer, at 35°C, for 6 hours. All center wells had 0.2 ml of 4 N KOH.
Reaction mixture: 0.7 ml enzyme
0.2 ml substrate or M/10 pH 7.6 phosphate
0.2 ml cofactor or M/10 pH 7.6 phosphate

Substrate	Cofactor				
	No cofactor		20 micrograms B_6al-Ph		
	Micromoles of NH_3/ml	Micromoles of O_2 taken up/ml	Micromoles of NH_3/ml	Micromoles of O_2 taken up/ml	
No substrate			0.9	0.0	
0.4M DL-threonine	2.8	0.7	3.9	0.4	R
Minus blank	1.9	0.7	3.0	0.4	1.6
0.4M DL-alpha-amino-butyric acid	1.0	0.3	1.1	0.4	
Minus blank	0.1	0.3	0.2	0.4	

The anaerobic character of the deamination of threonine is again put in evidence by the results reported in table XXI, which indicate that it can take place in a nitrogen atmosphere. No deamination, nor other side reactions, took place in the boiled controls. Chromatographic keto acid analysis reveals that ketobutyric is by far the main keto acid produced. Slight , somewhat questionable production of additional keto acids could be due to transamination of alpha-ketobutyric acid with glutamic acid and alanine (p. 79). Ketothreonine can be excluded as an important product of deamination.

L- and DL-threonine* are deaminated at the same rate according to table XXI, but deamination of the L form was faster in the experiment described in table X.

In order to understand the nature of the B_6 effect, it became imperative to ascertain whether the increments in the keto acid assay where B_6 was added were due to increased alpha-ketobutyric production, or whether they resulted from the generation of some different keto acid in the presence of pyridoxal phosphate. We added 2,4-dinitrophenyl hydrazine to aliquots from four vials in which threonine, with and without added B_6, had undergone deamination in the course of the experiments described in tables XIV (vials without cyanide) and XVI #1. The precipitates were distinctly bulkier in the cases where B_6 had been an ingredient of the reaction mixture.

* The concentration of L-threonine being equal.

TABLE XXI

On reaction balances

Enzyme: prepared as for table XIX, but dialyzate was used undiluted, with no additions.
Incubation: in Thunberg tubes. Before mixing enzyme and substrate, tubes were evacuated with a water pump, flooded with nitrogen, reevacuated, and reflooded with nitrogen. Then, contents were mixed and incubated at 40°C for 4 hours, at the end of which period 0.1 ml 2N H_2SO_4 were added to the contents of the tube, boiled and centrifuged.
Reaction mixture: 2 ml enzyme (fresh or pre-boiled); 0.1 ml containing 10 micrograms B_6al-Ph; 0.2 ml M/10 pH 7.8 phosphate buffer; 0.2 ml substrate or named buffer.

	Substrate	Micromoles alpha keto but. a./ml		Keto acid hydrazone spots*			Amino acid chromatograms**		
		Found	Minus blank	Rf. 2.5 to 3.3	Rf. 3.5 to 3.8	Rf. 5.2 to 5.8	Glutamic a.	Threonine	alpha-amino-but. a.
Fresh enzyme	No substrate	1.2	0.0	-	-	-	#2	#-	-
	0.4 M DL-threonine	11.8	10.6	#-	-	#	#2	#5	#
	0.2M L-threonine	11.4	10.2	-	#-?	#	#2	#3	#3
	0.2 M Na alpha-keto-butyrate	17.0	15.8	#-?	-	#	#2	-	#3
Boiled enzyme	0.4 M DL-threonine	0.5		-	-	-	-	#5	-
	0.2 M Na alpha-keto-butyrate	16.4		-	-	#	-	-	-

Calculated substrate concentration in the reaction mixture (of the L form where racemic mixtures used) is 15.4 micromoles/ml.

*The keto acid hydrazones were obtained by adding aliquots of test solutions to a 2,4-dinitrophenyl hydrazine solution, extracting with ethyl acetate, reextracting with

TABLE XXI (continued)

sodium carbonate, acidifying and extracting back with ethyl acetate. Spots were applied on the paper so that they would all represent the same volume of test solution. The lowest Rf corresponds to that of alpha-ketoglutaric acid; the middle one, either to pyruvate of ketothreonine, and the upper spot to alpha-ketobutyric acid. Spot intensities were rated either #- or #. The question mark indicates doubt of whether the spot exists at all.

** Spot intensities were rated from #- to #5

But the nature of the precipitates was qualitative identical in all four cases: chromatographic analysis revealed a single spot for each, corresponding to the hydrazone of alpha-ketobutyric acid; and all four washed crude precipitates decomposed at 187°C. The solutions remaining after filtering off the hydrazone precipitates were extracted with ethyl acetate; the organic phase then extracted with Na_2CO_3, which after acidifying was again extracted with ethyl acetate. Chromatography of the latter solution resulted in the same pattern of spots whether the hydrazones were derived from the incubation of threonine with or without B_6 addition.

We analysed in a similar fashion four mixtures resulting from serine deamination (table XVI, #1 and #3). In only one vial, a B_6 containing one, did a hydrazone precipitate come down. It analysed chromatographically as the 2,4-dinitrophenyl hydrazone of pyruvate (with a secondary hazy spot of Rf. about 0.8). The extracted hydrazones gave rise in all cases to the same pattern of spots, with the spot corresponding to pyruvate as the main component (except in the case where the pyruvate hydrazone had precipitated out). In the course of the extraction of acid hydrazones, the color of the Na_2CO_3 layer was redder where we were dealing with the products of deamination accomplished in the presence of B_6. All these observations tend to prove that pyridoxal phosphate affected keto acid production from serine and threonine quantitatively but not qualitatively.

TABLE XXII

Keto acid production from threonine in the presence of added ketobutyrate

Enzyme preparation: Neurospora extract, dialyzed for 22 hours against a M/10 pH 7.6 phosphate buffer. Ten micrograms of B_6al-Ph per ml added.
Incubation: at 40°C
Reaction mixture: 1.3 ml enzyme; 0.2 ml substrate A or water; 0.2 ml substrate B or water

Substrate A	Substrate B	Incubation time					
		1 hour			4 hours		
		Micromoles NH_3/ml	Micromoles alpha-keto-but./ml		Micromoles NH_3/ml	Micromoles alpha-keto-but./ml	
			Manometric	Colorimetric		Manometric	Colorimetric
---	---	0.7	1.1	0	1.0	0.4	0
0.4M DL-threonine	---	2.0	2.2	1.6	4.1	3.3	2.5
Minus blank		1.3	1.1	1.6	3.1	2.9	2.5
Ketobutyrate formed			1.1	1.6		2.9	2.5
0.4M DL-threonine	0.2M alpha-ketobut. a.	1.6	20.4	25.5	3.5	22.9	24.5
Minus blank		0.9	19.3	25.5	2.5	22.5	24.5
Ketobutyrate formed			1.0	2.5		3.1	3.1
---	0.2M alpha-ketobut. a.	0.6	19.3	23.0	0.9	19.8	21.4
Minus blank		-0.1	18.2	23.0	-0.1	19.4	21.4

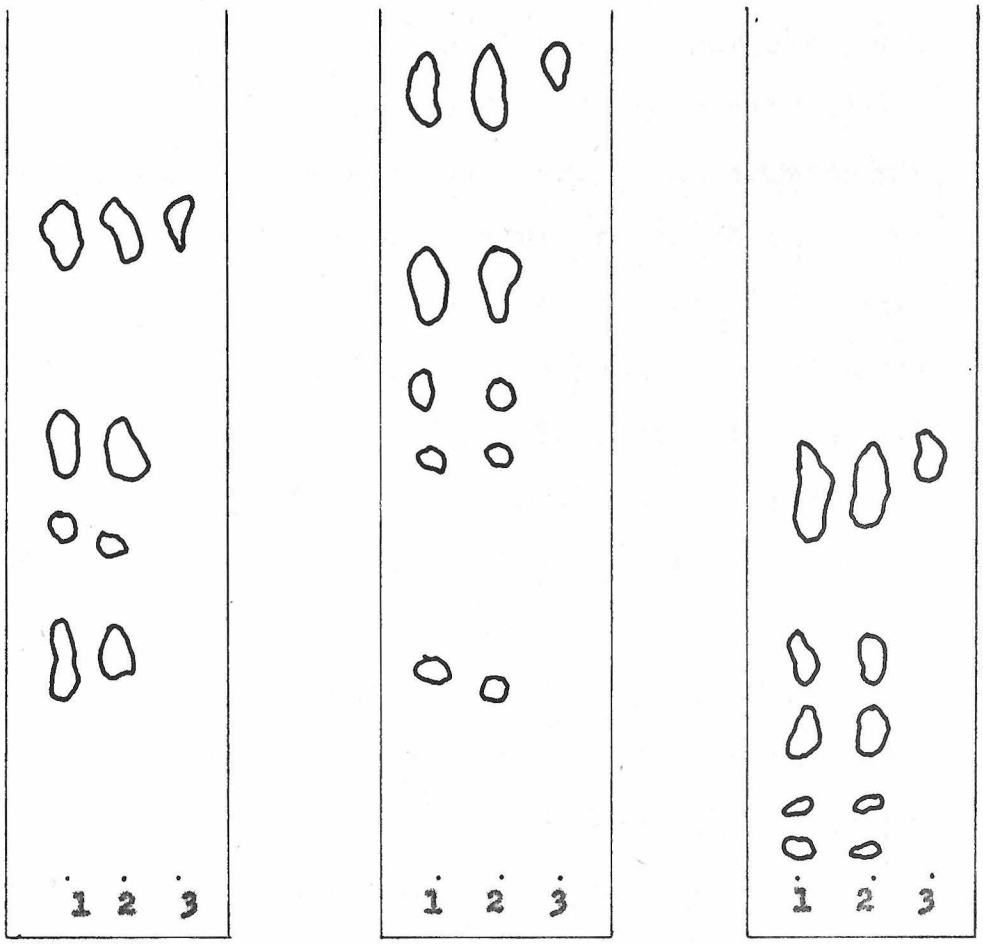
Colorimetric keto acid determination was performed by Friedman and Haugen's (14) "total keto acids" method, using a calibration curve corresponding to alpha-ketobutyric acid. This curve was not linear (as if extraction of the keto acid were a limiting process) and thus the accuracy of the determination suffered.

It seemed possible that deamination of threonine might yield some deaminated intermediate different from alpha-ketobutyric acid and in equilibrium with it. That intermediate could, for instance, be alpha-hydroxybutyric acid, as suggested by Mr. Bruce Ames. If that were the case, it would be feasible to depress the yields of alpha-ketobutyric acid in relation to those of ammonia, by adding alpha-ketobutyric acid initially to the reaction mixture. Such was tried in the experiment reported in table XXII, with results which fail to demonstrate the existence of the postulated intermediate.

2) The formation of alpha-aminobutyric acid from threonine

We have already mentioned this process in connection with tables XIX and XXI, which show alpha-aminobutyric acid formation by incubation of L- or DL-threonine with purified enzyme preparations. The same preparations, however, convert alpha-ketobutyric acid into alpha-aminobutyric acid. At the time we realized this fact, knowing that alpha-ketobutyric is produced by deamination of threonine; the problem of alpha-aminobutyric formation by incubation of threonine, became the problem of its formation from alpha-ketobutyric which we shall discuss in a latter section (Chap. IV). Still, it may be worthwhile to report briefly on the investigations undertaken while we thought that alpha-aminobutyrate arose more directly from threonine.

Figure 8
Chromatographic identification of alpha-aminobutyric acid produced by enzymatic incubation of threonine



1- Reaction mixture in which threonine was incubated with Neurospora extract.

3 - Alpha-aminobutyric acid sample
2 - Mixture of 1 and 3

(There was no spot corresponding with alpha-aminobutyric acid in a sample of a reaction mixture in which Neurospora extract was incubated without the addition of substrate)

The identity of the "alpha-aminobutyric acid" chromatographic spot was established with a fair degree of likelihood by comparison of its Rf on paper using four different solvents. The results with three of them are depicted in figure 8. Water saturated phenol produced a similar picture.

In a rough semi-quantitative way, by visual estimation of the intensity of the alpha-aminobutyric spots on paper chromatograms, we found that the yields were better (1) when the reaction was carried under anaerobic conditions, (2) when the temperature of incubation was increased from 25° to 35°, and from 35° to 40°; (3) when methionine was added to the culture medium in which the mycelium for extracts was grown. Extracts of pads grown for three days (in 125 ml Ehrlenmeyer flasks with 20 ml of medium) with constant shaking, were incapable of giving rise to alpha-aminobutyric acid when incubated with threonine. These extracts, chromatographed after incubation without added substrate, were quite poor in free amino acids (glutamic acid, aspartic acid, alanine) as compared with those derived from unshaken cultures.

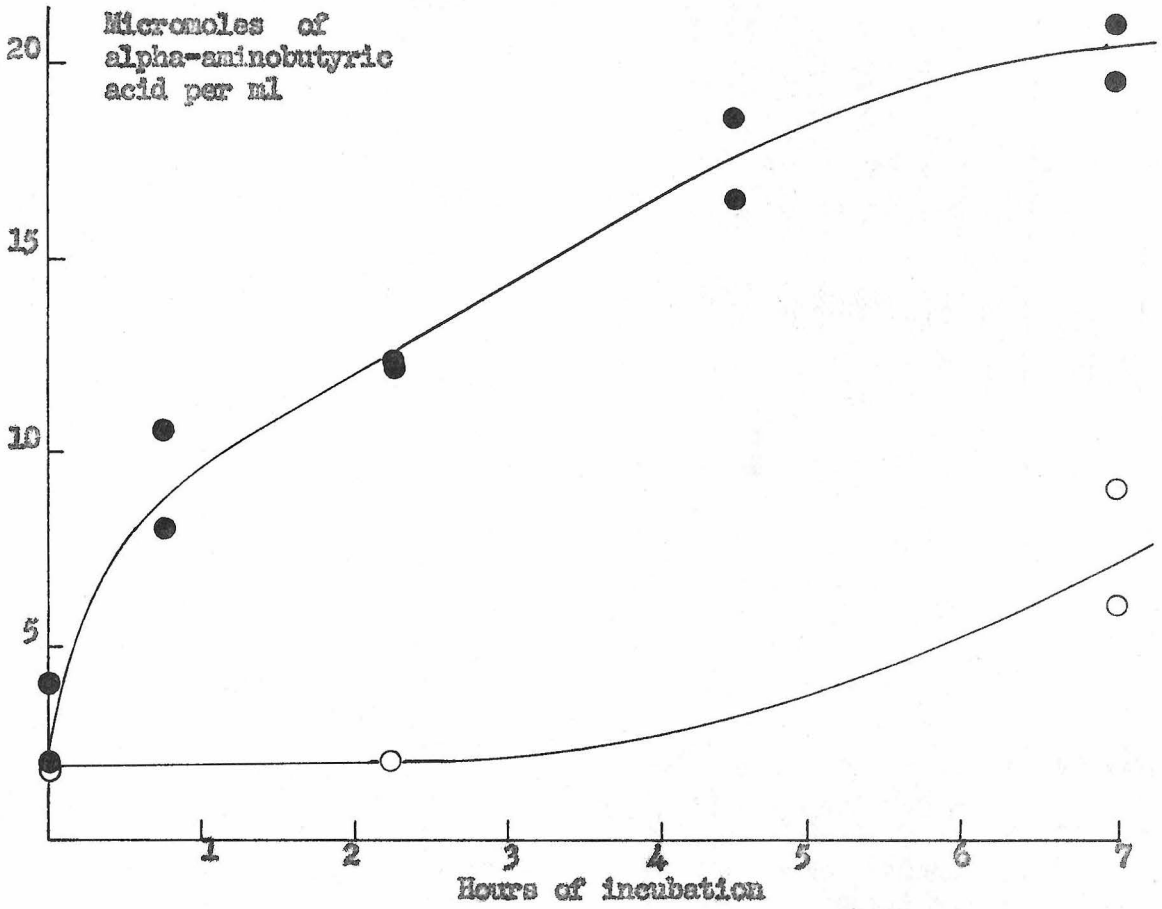
The course of the reaction under consideration and its pH dependence are depicted in figures 9 and 10. Notice the rate of reaction slows down during the first hour. Notice also the abrupt fall in the pH curve between pHs 7.4 and 7.0.

Alpha-aminobutyric acid forming activity was lost by storing Neurospora extracts overnight in the icebox. It could be recovered by adding a freshly prepared boiled extract or pyridoxal phosphate. Three micromoles of pyridoxal phosphate per ml were enough to induce recovery, but activity was boosted more when 30 micrograms were added. Equivalent concentrations of pyridoxal, pyridoxine hydrochloride and pyridoxamine dihydrochloride had no effect on the system.

Since threonine has to be reduced in order to become alpha-aminobutyric acid, it is clear that threonine must either undergo dismutation or that the process must be linked with some oxidative transformation. The low yields obtained when purified preparations were used and the shape of the rate curve supported the second idea. A number of substrate combinations were thus incubated with Neurospora extract in an attempt to recognize all the components of the aminobutyric generating system, to wit:

DL-threonine and ascorbic acid
" " " DL-homoserine
" " " DL-methionine
" " " DL-homocystine
" " " L-cystine
" " " glutathione
" " " L-malic acid
" " " L-glutamic acid
" " " DL-alanine
" " " succinic acid
DL-alpha-aminobutyric acid and DL-homoserine
DL-alpha-aminobutyric acid
DL-threonine
DL-homoserine
DL-methionine
DL-alanine
L- glutamic acid

Figure 9
The course of the formation of alpha-aminobutyric acid by incubation of threonine with Neurospora extract



● Alpha-aminobutyric in threonine vial.

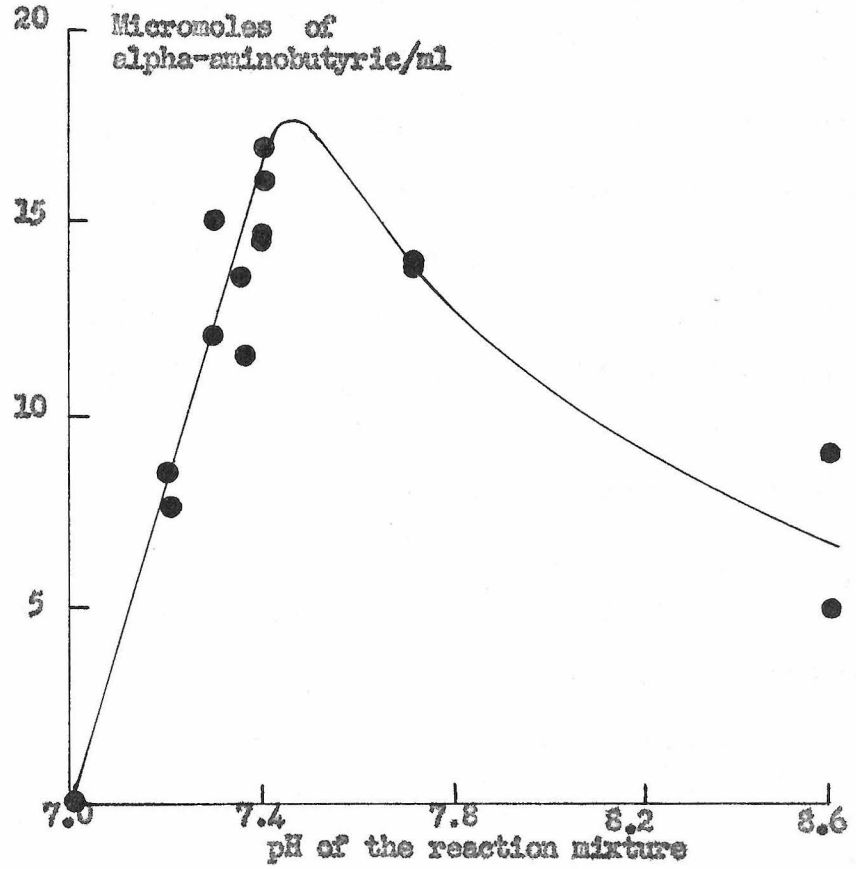
○ " " " vial without substrate

Reaction mixture: 1 ml Neurospora extract
0.2 ml 0.4 M DL-threonine, or water
Incubated at 40°C, in open vials, in a nitrogen-filled chamber.

(Duplicates represent duplicate amino acid determinations).

Figure 10

PH dependence of the formation of alpha-aminobutyric acid from threonine



● Alpha-aminobutyric at the end of incubation, minus
" " " " beginning of incubation.

Reaction mixture: 0.5 ml Neurospora extract
0.5 ml buffer (M/4 or M/2 phosphate)
0.2 ml 0.4M DL-threonine
Incubated at 40°C, under nitrogen.

The above list combines the results from different experiments. Alpha-aminobutyric acid was formed wherever threonine was added. The only addition which increased the yields was glutamic acid. No other amino-acid interconversion was detected by qualitative chromatographic analysis of the reaction mixtures.

Formation of alanine from serine was also observed. Since Neurospora extracts contain a fair amount of free alanine and purified preparations generate free alanine endogenously, we found it awkward to study this system in a quantitative or semi-quantitative fashion.

3) Development of blue fluorescence

Casual observation under an ultra-violet lamp (3650 peak emission) of vials in which Neurospora extract had been incubated with and without threonine, revealed a striking difference in their appearance: those incubated with threonine appeared blue; the others, yellow. This phenomenon has been observed many times. However, we have not yet come to the point of studying it systematically. We were not able to reproduce it every time we attempted to, which indicates that we have not learned how to control some of the essential conditions for the development of blue fluorescence.

Blue fluorescence (which we shall abbreviate BF) appears associated with the protein coagulum. Sometimes,

after acidifying, boiling and centrifuging the contents of the vials as we do in most of the routine incubations, the BF can be observed as a bright fluorescent layer in the bottom part of the sediment. Other times it appears more dispersed. BF has not been observed unless vials had been previously acidified and placed in a boiling water bath. We were unable to extract BF with organic solvents.

The yellow fluorescence present in the blank vials is acid and alkali labile. BF is not, and so we could ascertain that BF was absent in the blank vials. Sodium hydrosulfite ($Na_2S_2O_4$) destroys both fluorescences, and hydrogen peroxide restores them.

The BF phenomenon was most remarkable in experiments where the source of lyophilizate was a wild type strain grown at 25°C under forced aereation for 7 days on 9 liters of "minimal" medium supplemented with threonine and alpha-aminobutyric acid. Dialyzed preparations failed to show differences between threonine and threonine-free reaction mixtures when observed under the UV lamp.

In the experiment reported in table XVIII, only the vials where 18829 extracts were incubated with threonine possessed BF.

A series of substrates were tested for their ability to induce BF formation by incubation for 4 hours at 40°C with an extract from a 7-day old threonine and alpha-aminobutyric acid supplement culture. Incubation with

alpha-ketobutyrate, DL-alpha-aminobutyric acid, DL-serine, pyruvate, DL-alanine, L-glutamate, alpha-keto-glutarate, or with no additions, failed to produce BF; while DL- and L-threonine gave positive results. Controls where each of the named substrates was incubated with the boiled extract, and which were acidified, boiled and centrifuged like the other reaction mixtures at the end of the incubation, showed no BF.

F) DEAMINASE ACTIVITIES OF DIFFERENT STRAINS

The threonine-requiring mutants of Neurospora crassa have been studied by Teas et al (40) (41) (42) (43), Emerson (44), Fling and Horowitz (45), and more recently by Harold Garner (46). As a result of these investigations, the growth requirements of a number of mutants have been established. Strain 44105 (or 46003, which may carry an identical gene mutation) will grow on "minimal" medium after a lag phase of several days, but will grow at a normal rate if the medium is supplemented with 20 to 100 micrograms per 20 ml of either alpha-aminobutyric, alpha-ketobutyric acid, isoleucine canavanine, or homoserine; or if larger amounts of threonine or possibly keto-threonine are added*. Strain 51504 requires addition of either L-homoserine or a mixture of threonine and methionine for growth. Cystathionine,

*Keto-isoleucine is also active

homocysteine, or larger concentrations of D-homoserine or gamma-hydroxythreonine, can substitute for methionine. Large amounts of threonine can do it, too, but in a very sluggish way. The threonine requirement can be only partially spared by alpha-aminobutyric, alpha-keto-butyric acid or isoleucine, and possibly by ketothreonine. Mutant 44104 grows well in a medium supplemented with threonine or alpha-aminobutyric acid, and less well if alpha-ketobutyric acid or isoleucine are added instead*. Methionine is able to spare threonine some. Cultures of 35423 show a highly specific threonine requirement. The above information can perhaps be better visualized by reference to the schemes suggested in pp.121 and 122.

Bacterial mutants blocked in the threonine region, studied by Teas (47) and by Umbarger and Mueller (48), fall in classes not too different from those corresponding to the Neurospora mutants. Except for some E. coli strains (e.g., JHM544) which unlike any Neurospora ones, cannot use L-threonine for growth but are exacting towards alpha-aminobutyric, alpha-ketobutyric acid, ketothreonine or D-threonine. In none of the mutants of Neurospora can D-threonine act as a growth substrate.

Other Neurospora mutants used in connection with our research are E-18829 and 44602. Zalokar (49) (50) found that E-18829, originally identified as a temperature-sensitive strain requiring sulfanilamide for growth at 35°C, will also grow at that temperature in the absence of

* Keto-isoleucine is also active

sulfonamides if any of the following conditions is met: 1) if the para-aminobenzoic acid available to the strain is limited by introducing the para-aminobenzoic-less gene and supplementing the medium with very low concentrations of that vitamin; 2) if the available methionine is limited by a similar method; 3) by adding threonine (valine, isoleucine, alpha-ketobutyric, β-methylcrotonic, or alpha-aminobutyric acid in place of threonine stimulate growth, but are not as effective); 4) if the pH is raised, and thus free ammonia is made available to the mold.

Mutant 44602 is a B_6 requiring strain (39) (50), which will grow in the absence of B_6 if free ammonia is made available. Its ammonia requirement, and the fact that when grown in the absence of B_6 it is sensitive to inhibition by methionine which can be reversed by threonine or sulfanilamide, relates 44602 to E18829. Methionine-threonine antagonisms are encountered also in the typical threonine-less mutants, but there the inhibition mechanism appears to be different (44).

It seemed that the metabolic derangement which causes the reviewed mutants to require threonine in order to grow or to antagonize methionine inhibition, might in some case involve threonine deaminase. To test this possibility, five Fernbach flasks, each containing 500 ml of minimal medium supplemented as indicated, were inoculated with different mutants. After $3\frac{1}{2}$ days of growth at 25°C, they were harvested, lyophilized, and tested for threonine

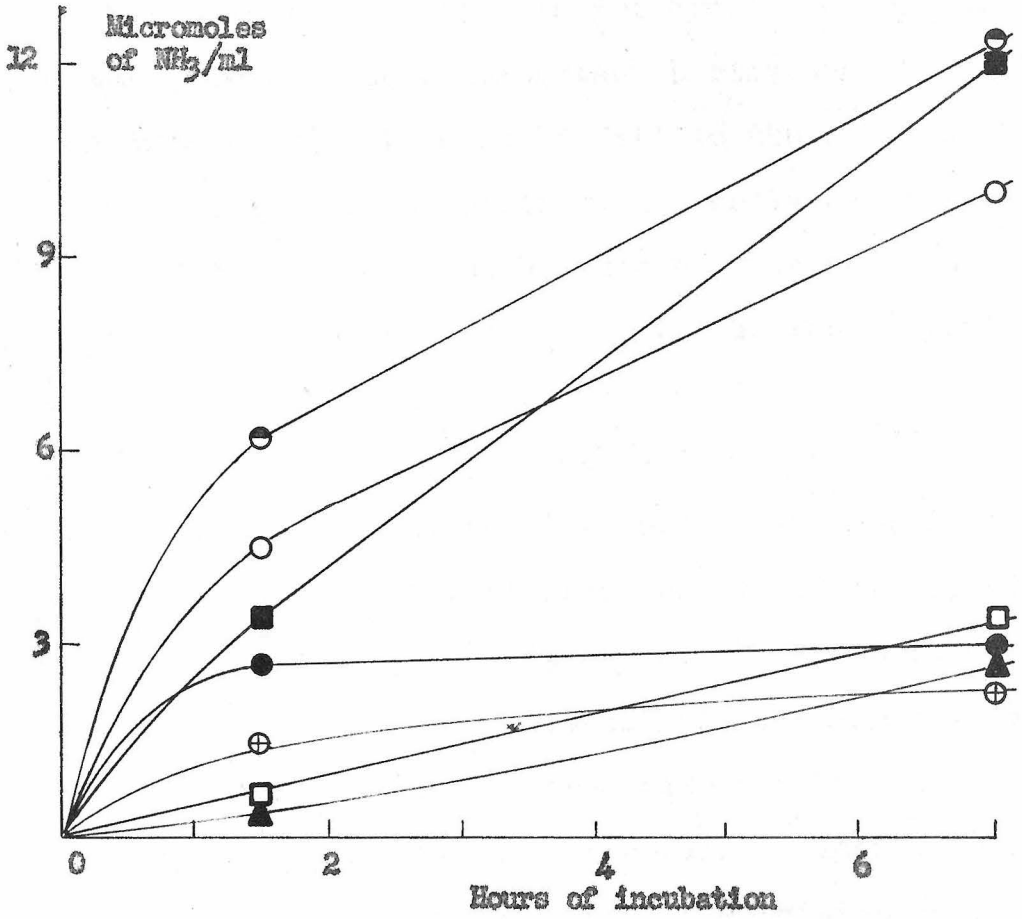
TABLE XXIII

Deaminase activities of different strains

Reaction mixture: 50 mg lyophilizate
0.5 ml M/2 pH 8.1 phosphate buffer
1 ml 0.04M DL-threonine or water
Incubation: at 30°C

On enzyme sources			On deaminase test			
Lyophilizate from strain	Strain grown on "minimal" plus	Weight of lyophilizate obtained (mg)	Substrate	Reading at time zero	Reading after 1½ hrs.	Reading after 7 hrs.
				Micromoles of NH3/ml		
51504	500 mg DL-methionine & 500 mg DL-threonine	700	None		5.2	8.0
			Threonine	2.8	8.5	20
			Minus blank	0	3.3	12
44104	500 mg DL-threonine	530	None		2.9	5.5
			Threonine	1.2	3.5	8.6
			Minus blank	0	0.6	3.1
35423	500 mg DL-threonine	450	None		2.2	6.5
			Threonine	1.5	3.8	9.0
			Minus blank	0	1.6	2.5
E-18829	---	420	None		2.2	7.0
			Threonine	1.1	6.7	17
			Minus blank	0	4.5	10
E-18829	500 mg DL-threonine	580	None		2.2	5.7
			Threonine	1.5	8.4	18
			Minus blank	0	6.2	12
E-18829	M/50 Na_2HPO_4 to bring pH to 7	500	None		4.0	12
			Threonine	2.3	6.7	15
			Minus blank	0	2.7	3
44602	M/50 Na_2HPO_4 to bring pH to 7	500	None		4.2	8.0
			Threonine	2.2	4.5	11
			Minus blank	0	0.3	3

Figure 11
Threonine deaminase in different strains
(A plot of the data in table XXIII)



Enzyme source:

- ■ Strain 51504
- ○ " E-18829 (grown on "minimal" medium, at 25°C)
- ● " E-18829 (grown on pH 7 "minimal" medium, at 25°C)
- ⊖ " E-18829 (grown on "minimal" medium plus threonine, at 25°C)
- ▲ " 44602
- □ " 44104
- ⊕ " 35423

Plotted: readings minus blanks.

deaminase activity with the results reported on table XXIII and figure 11. All strains seem to possess deaminase activity, but that of 35423, 44104, 44602, and of E-18829 (grown at pH 7) are lower than the rest. Although a wild type control is not available in this experiment, results to be reported shortly suggest that the activity of wild type would fall in the class of the more active strains. Chromatographic analyses of the reaction mixtures showed alpha-aminobutyric acid formation from threonine in presence of the enzyme preparations from 51504, 35423, and E-18829. Absence of visible aminobutyric spot in the 44104 and 44602 vials may indicate a quantitative difference only. In latter experiments, using different crops of lyophilizate, 44104 was fully active, and 44602 (grown at pH 7 with no additions) showed a low but unequivocal alpha-aminobutyric acid forming activity as compared with a wild type control. However, the original batch of lyophilized 44602, when tested again under the most favorable conditions showed once no detectable activity, while a lyophilizate of the same age (E-18829, grown on threonine) gave rise to a very noticeable aminobutyric spot after incubation with threonine. The paper chromatograms indicated that 44602 extracts had more free glutamic acid, and more free amino acids in general, than those of E-18829.

It appeared important to ascertain whether the deaminase of 35423 broke down threonine to the same end

products as the one of wild type. A 35423 extract was incubated with threonine for 6 hours at 40°, simultaneously with a boiled control. Aliquots of each reaction mixture (acidified, placed 5 minutes in a boiling water bath and centrifuged at the end of the incubation) were added to aliquots of a 2,4-dinitrophenyl hydrazine solution. No precipitate formed from the control solution. The test solution gave rise to a yellow precipitate which was identified chromatographically as the 2,4-dinitrophenyl hydrazone of alpha-ketobutyric acid.

The behaviour of E-18829 as seen in table XXIII suggests a relation between pH of the culture medium and deaminase activity. Table XXIV corroborates this pH effect for the in vitro deaminase activity of E-18829 and 44602, but shows that there is no such effect in the case of the wild type strain. Using dialyzed preparations, as can be seen in table XXV, the activities of E-18829 grown at different pHs are less dissimilar. This last experiment, however, was not designed to compare quantitatively the activities of different cultures because dialysis of small volumes introduces uncontrollable dilution and loses which may result in errors as high as plus-minus 20%. The ratios of serine to threonine deaminase activities, however, are not subject to such errors. It is difficult to decide if the variations observed in S/T values should be considered significant or not.

TABLE XXIV

Deaminase activity and culture conditions

Growth of cultures: 1 liter of "phosphate minimal" medium (39) titrated to the desired pH with KOH and supplemented with 1 g of NH_4NO_3, contained in a Fernbach flask, was inoculated with the indicated strain. The cultures were harvested after 3 (or 4?) days at 25°C, and lyophilized.
Reaction mixture: 30 mg lyophilizate
0.5 ml M/2 pH 8.3 phosphate
0.1 ml with 10 micrograms B_6al-Ph
0.1 ml 0.4M DL-threonine or water
Incubation: 6 hours at 37°C

On enzyme source				On deaminase test	
Lyophilizate from strain	Strain grown on NH_4NO_3 minimal	Weight of lyophilizate obtained (mg)	Conidiation	Substrate	Micromoles of NH_3/ml
E-5256	pH 5.7 and no addition	790	---	None	5.5
				Threonine	20.8
				Minus blank	15.3
	pH 7.0 and no addition	900	---	None	7.5
				Threonine	21.0
				Minus blank	13.5
E-18829	pH 5.7 $5x10^{-5}$ M sulfanilamide added	540	---	None	6.5
				Threonine	24.8
				Minus blank	18.3
	pH 7.0 and same addition	340	---	None	10.3
				Threonine	13.6
				Minus blank	3.3
	pH 7.0 and no addition	250	#	None	6.7
				Threonine	12.7
				Minus blank	6.0
44602	pH 5.7 and 0.1 mg pyridoxine added	470	#	None	5.1
				Threonine	20.0
				Minus blank	14.9
	pH 7.0 and same ADDITION	420	?	None	7.5
				Threonine	16.3
				Minus blank	8.8
	pH 7.0 and no addition	290	#	None	7.5
				Threonine	14.5
				Minus blank	7.0

TABLE XXV

S/T ratio and culture conditions

Enzyme preparations: same lyophilizates described in table XXIV were used to make extracts in the standard way, but twice as diluted. The extracts were dialyzed against M/10 pH 7.6 phosphate buffer for 6 hours.
Reaction mixtures: 1 ml enzyme
0.1 ml with 30 micromoles B_6al-Ph
0.2 ml 0.4 M substrate or water
Incubation: 3 hours at 35°C

Enzyme source	Substrate	Micromoles of NH3 per ml	
E-5256 grown at pH 5.7	No substrate	1.3	
	DL-threonine	3.7	S/T
	Minus blank	2.4	
	DL-serine	5.9	
	Minus blank	4.6	1.9
E-5256 grown at pH 7.0	No substrate	1.6	
	DL-threonine	3.4	
	Minus blank	1.8	
	DL-serine	6.3	
	Minus blank	4.7	2.6
E-18829 grown at pH 5.7 with sulfanil-amide	No substrate	1.3	
	DL-threonine	3.5	
	Minus blank	2.2	
	DL-serine	4.7	
	Minus blank	3.4	1.5
E-18829 grown at pH 7.0 with sulfanil-amide	No substrate	1.7	
	DL-threonine	2.8	
	Minus blank	1.1	
	DL-serine	4.2	
	Minus blank	2.5	2.3

IV GLUTAMIC-ALPHAKETOBUTYRIC TRANSAMINASE*

In 1938 Braunstein and Kristman (52) reported the occurrence in minced pigeon muscle of transamination between glutamic and alpha-ketobutyric acid, and between alpha-aminobutyric and alpha-ketoglutaric acid, along with transaminations involving a large number of other amino acids. Ambiguities inherent to the methods employed, which were pointed out by other workers in the field, caused Braunstein (53) to recognize that several of the transaminations reported were only artifacts, but he did not question the reality of the glutamic-alpha-ketobutyric system. Cohen (54) reports "slight" formation of glutamic acid from alpha-aminobutyric and alpha-ketoglutaric acid, and destruction of it when added to alpha-ketobutyric acid, in the presence of pigeon muscle. Since the transamination reaction had appeared to Cohen as one of a very restricted scope (Glutamic, aspartic acid, and their corresponding keto acids being the only noteworthy participants), and since alpha-aminobutyric acid was thought to be a physiologically rare compound, it is not surprising that he conceived glutamic-alpha-ketobutyric transamination as the product of some sort of illigitimate union between alpha-aminobutyric acid and the system normally catalyzing alanine transamination. The results of Green, Leloir and Nocito (55) are consistent with this idea: glutamic-alphaketobutyric transaminase

*We have adopted Cohen's terminology (56)

activity accompanies the glutamic-pyruvic purified transaminase (the degree of purification of this system is discussed by Cohen (56)).

Cohen's early reluctance to consider the possibility that alpha-aminobutyric might be transaminated by an enzyme system of its own, appears now unjustified. The occurrence of free alpha-aminobutyric acid in blood, urine, extract of yeast (57) (58), and in other natural products (59) (60) has been demonstrated. The biological role of this amino acid is further substantiated by its ability to support growth of Neurospora and E. coli exacting strains. Besides, Cammarata and Cohen (61), Feldman and Gunsalus (62), and Stumpf (63) have recently demonstrated, in animal, bacterial and higher plant tissues, respectively, the occurrence of a wide variety of transaminases. Again Cammarata and Cohen, and Stumpf, report transamination involving alpha-aminobutyric and alpha-ketoglutaric acids.

Roswell (63a) reports transamination between ketobutyric and a number of aminoacids in the presence of supernatants from rat liver homogenates.

Strauss (39) (51) and Fincham (64) have demonstrated the presence of glutamic-pyruvic and glutamic-oxaloacetic transaminases in Neurospora extracts, and their activation by pyridoxal phosphate. Glutamic-ketobutyric transaminase activity can also be demonstrated as shown by table XXVI and figure 12. The possible identity of the latter system with the former ones has not been investigated.

TABLE XXVI

Glutamic-alphaketobutyric transaminase

	Substrate		Cofactor						
			None			B_{6}al-Ph			
			Assays, in micromoles per ml						
	Substrate A	Substrate B	alpha-ketobutyric a.	alpha-aminobutyric a.	Glutamic acid	alpha-ketobutyric a.	alpha-aminobutyric a.	Glutamic acid	
Active enzyme	---	---				0.3	0.0	21	Uncorrected
						0.0	0.0	0.0	Corrected for reading in substrate free tube
	0.1 M alpha-ketobutyrate	---	18.7	0.0	0.0	18.7	0.3	0.6	
	0.1 M alpha-ketobutyrate	0.1 M L-glutamate	17.7	1.7 1.9	ca13	15.2	2.3 3.3	ca15	
	---	0.1 M L-glutamate		0.0	ca11		0.0	ca15	
	0.1 M alpha ketoglutarate	---				0.5	0.0	0.2 0.3	
	0.1 M alpha ketoglutarate	0.2 M DL-alpha aminobut.				2.9		1.7 0.9	
	---	0.2 M DL-alpha aminobutyrate				0.7		0.6	
Boiled enzyme	---	---					2.0*	0.0	
	0.1 M alpha ketobutyrate	0.1 M L-glutamate				19.7	0.0	ca12	

Enzyme preparation: Neurospora extract, dialyzed for 20 hours against M/10 pH 7.7 phosphate buffer. Part of it was boiled in a water bath for 5 minutes.
Reaction mixture: 0.5 ml enzyme, fresh or boiled; 0.1 ml cofactor (30 micrograms of B_{6}al-Ph) or water; 0.2 ml substrate A; 0.2 ml substrate B (continued)

TABLE XXVI (Continued)

Incubation: in open 10x75 mm test tubes, at 40°C for one hour. At the end of the incubation, 0.1 ml 1N H_2SO_4 were added, the tubes placed for 5 minutes in a boiling water bath, and centrifuged, using the supernatant for the determinations.

- - - - -

Duplicate values represent duplicate assays. Figure indicated with an asterisk is probably in error due to some contamination, since no corresponding spot could be seen in the qualitative chromatogram (figure 12). Figures preceded by "ca" are inaccurate because they correspond to amino acid concentrations beyond the range within which the method is reliable.

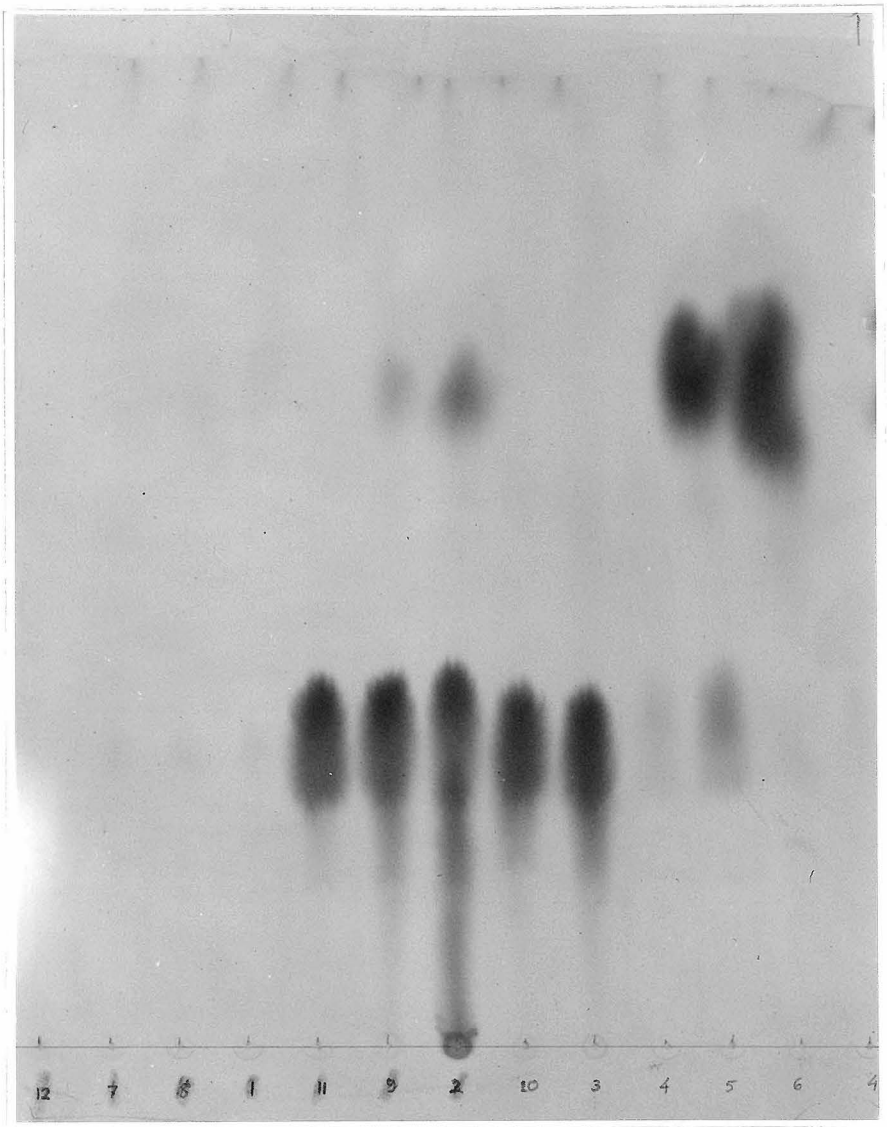
All substrates (counting only the L- form where DL- is used) found themselves at a concentration of 18.2 micromoles per ml in the initial reaction mixture.

The true transaminating nature of the system under consideration is indicated by the observation that alpha-ketobutyric acid disappears only when incubated in the presence of glutamic, and that its disappearance corresponds with the appearance of alpha-aminobutyric acid within the accuracy of our analytical methods. Similarly, more alpha-ketobutyric acid is formed from alpha-aminobutyric when alpha-ketoglutaric is added. Notice also the activation of the system by calcium pyridoxal phosphate and its heat-lability.

We have mentioned the fact that mutant 44104 grew better on alpha-aminobutyric acid than on alpha-ketobutyric acid, unlike other threonine-less mutants which use both equally well. From alpha-ketobutyric acid supplemented cultures one has to wait about 7 days to get the same dry weight of 44104 mycelium that an equivalent concentration of alpha-aminobutyric acid will allow to develop in 3 days. Pyridoxine addition to the culture medium will stimulate growth of 44104 on threonine and ketobutyric mixtures, but not on threonine alone (Harold Garner, personal communication). We investigated the <u>in vitro</u> alpha-ketobutyric to alpha-aminobutyric conversion as catalyzed by 44104 extracts, and compared it with that catalyzed by wild type extracts. For that purpose we used lyophilizate from four different cultures of each strain, grown on "minimal" medium with

Figure 12
Glutamic-alphaketobutyric transaminase

The reaction mixtures described in table XXVI were chromatographed (applying 16 microliters per spot) with water-saturated phenol as solvent. After developing with ninhydrin solution, the following pattern was seen:



Substrates	none	none	ketobutyric	ketobutyric	ketobutyric & glutamic	ketobutyric & glutamic	ketobutyric & glutamic	glutamic	glutamic	ketoglutaric	ketoglutaric & aminobutyric	aminobutyric
B_6al-Ph	x	x	-	x	x	-	x	-	x	x	x	x
Enzyme: fresh (f)or boiled (b)	b	f	f	f	b	f	f	f	f	f	f	f

each of the following additions: threonine, threonine and alpha-aminobutyric acid, threonine and alpha-ketobutyric acid, threonine and alpha-ketobutyric acid and pyridoxine. Extracts were incubated with glutamic acid in the blanks, and with glutamic and alpha-keto-butyric acid in the test mixtures. Transaminase activity we determined by disappearance of Ketobutyric and appearance of aminobutyric, quantitatively. There were no significant differences between the activities of any of the cultures. We noticed that wild type extracts grown on aminobutyric gave rise to a much stronger aminobutyric spot in the qualitative chromatogram of the blank than that found in the corresponding 44104 chromatogram.

V) ALPHA-KETOBUTYRIC DECARBOXYLASE

Neuberg and Kerb (65) found in 1912 that live yeast and Lebedew juice were both able to catalyze the decarboxylation of alpha-ketobutyric acid as well as that of pyruvate. They identified propionaldehyde as a product of the former reaction. Peters (66), using washed dry yeast, obtained a 20-fold increase in alpha-ketobutyric decarboxylase activity by adding cocarboxylase. Hägglund and Ringbom (67) determined the pH dependence of that activity.

We have found that Neurospora extracts are able to decarboxylate alpha-ketobutyric acid at a rate quite similar to that of pyruvic acid decarboxylation. Conditions under which the reaction can take place, and yields of CO_2, have already been reported in the section on materials and methods (pp3+7).

Propionaldehyde can be recognized as a product of keto-butyrate decarboxylation by its characteristic smell. The distillate from a reaction mixture in which alpha-ketobutyric has been decarboxylated, was collected in a 2,4-dinitrophenyl hydrazine solution. A precipitate came down which, after filtering and washing, could not be distinguished from a known sample of the 2,4-dinitrophenyl hydrazone of propionaldehyde when chromatographed using the method we adopted for keto acid analysis. In a last minute experiment, the same precipitate was recrystallized

from ethyl acetate and its melting point found to be 136°-140° C. (Reported for the propionaldehyde derivative: 155°C).

By dialyzing overnight a Neurospora extract against pH 8.2 phosphate buffer, a cocarboxylase effect could be demonstrated (fig. 13). The alpha-ketobutyric decarboxylase activity of the preparation had dropped to about one-fourth its original value after dialyzing and adding cocarboxylase and manganous ions. When we attempted to remove all the cocarboxylase from the preparation by dialyzing for longer time and/or at higher pH*, we lost all of the decarboxylase activity and did not recover it by adding cocarboxylase and manganous ions.

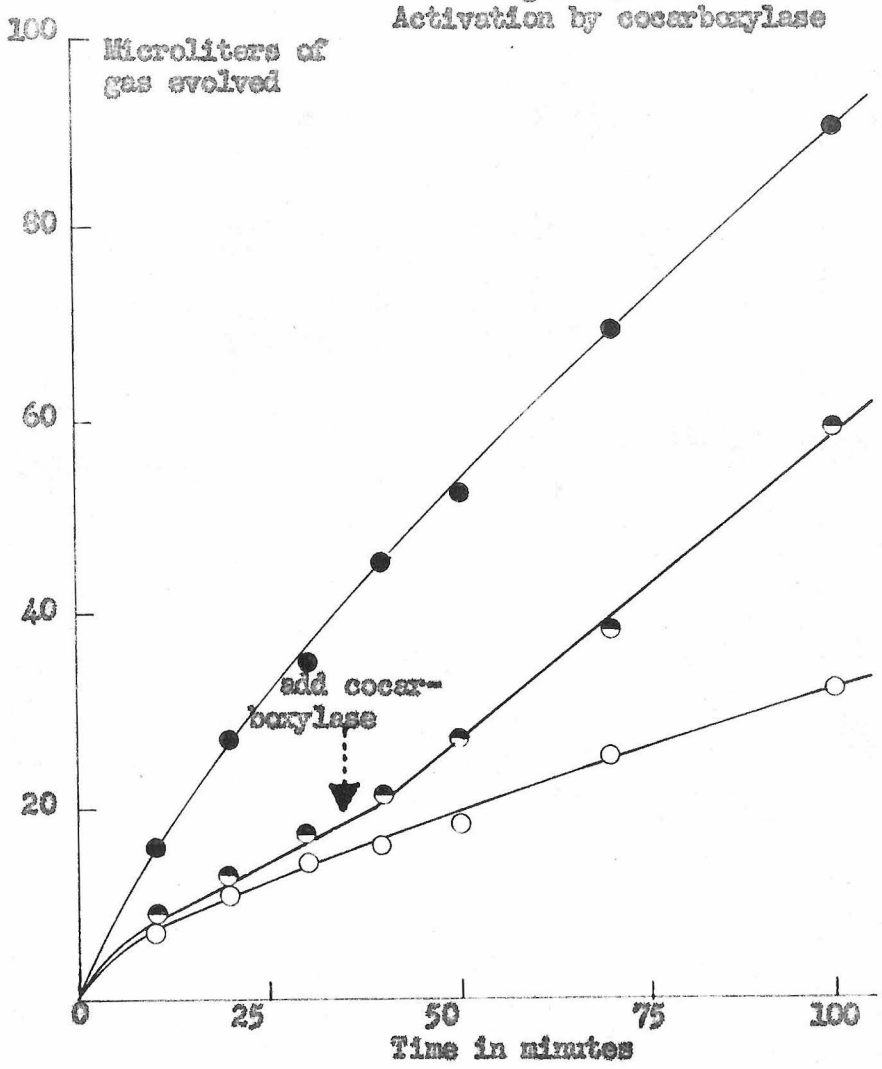
We used the same dialyzed preparation which cocarboxylase addition activated, in order to establish the effect of pH on the rate of decarboxylation (fig. 14). Such a drop in decarboxylase activity from pH 5.5 to pH 5.1 as the one found in this experiment, has not been encountered in other runs in which undialyzed preparations were used.

In connection with our attempts to elucidate why the in vivo metabolism of alpha-ketobutyric acid differs in in 44104 from that in other strains, we tested the eight extracts of the cultures of wild type and 44104 mentioned

*We tried dialyzing against pH 7.6 M/10 phosphate for 84 hours, and against pH 8.6 M/10 borate for 26, 77, and 84 hours. Buffer was renewed every 12 to 24 hours.

in pages 83-85 for ketobutyric decarboxylase activity, adding cocarboxylase and manganese ions. No essential differences in activity were observed.

Figure 13
Activation by cocarboxylase



A) ● Manganous ions and cocarboxylase added initially.
B) ◐ " " added initially; cocarboxylase added after 32 minutes.
C) ○ No additions.

Enzyme preparation: Neurospora extract (half as concentrated as in standard procedure), dialyzed for 12 hours against M/10 pH 8.2 borate. PH of dialyzate was adjusted to 5.5 with citric acid (M/5) effecting a 1.4 fold dilution.

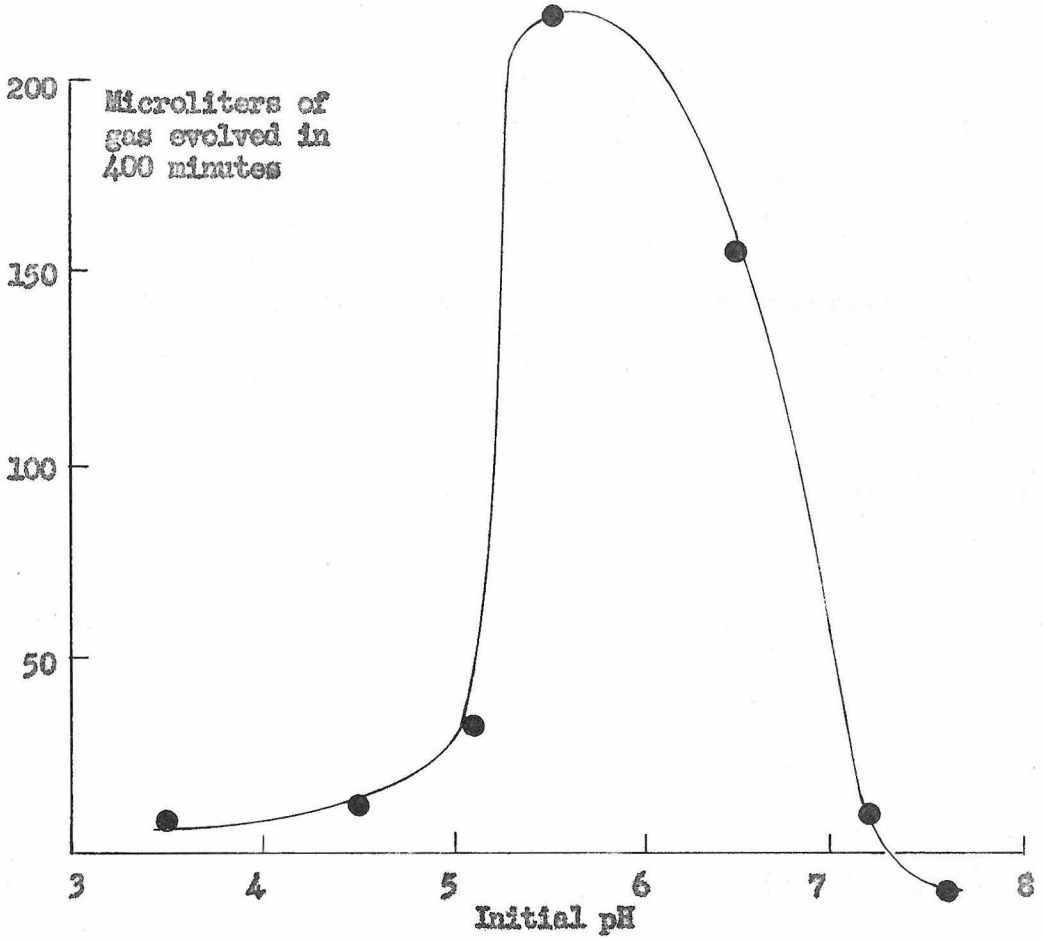
Reaction mixture: 0.7 ml enzyme
(main vessel) 0.2 ml containing 20 micograms cocarboxylase HCl or water
0.1 ml containing 100 micrograms manganous sulfater or water

Incubation: in a Warburg respirometer, at 35°C. At time zero, 0.3 ml 0.1M alpha-ketobutyrate was added from a side arm. To vessel B, from a second side arm, 0.2 ml cocarboxylase solution were added at the indicated time. After this last addition, the reaction mixture in vessel B resulted more diluted than in the other vessels.

Before time zero (time when substrate was added) gas exchange was practically nil in all vessels.

Figure 14

pH dependence of alpha-ketobutyric decarboxylase



This test was run under the same conditions and with the same ingredients specified for curve A, figure 13, but using aliquots of the enzyme preparation titrated to the desired pH. Four hundred minutes after the addition of the substrate from a side arm, 0.1 ml of 1 N H_2SO_4 was added to the vessels run at pH 6.5, 7.2 and 7.6 in order to displace the combined CO_2. To a blank of pH 7.2, the same acid was added immediately before adding the substrate at time zero, and this treatment resulted in the evolution of 10 microliters of gas. This volume was substracted from the values resulting after acid addition in vessels of pH 6.5 , 7.2 and 7.6 to obtain the values plotted.

VI) DISCUSSION

A) RE IN VITRO METABOLISM

(1) Summary of results making up the background of the discussion

We have encountered in Neurospora extracts enzymatic activities describable as serine deaminase, threonine deaminase (chapter III), glutamic-alphaketobutyric transaminase (chapter IV), alpha-ketobutyric decarboxylase (chapter V), pyruvic decarboxylase (ft. note, p. 19), systems leading to the formation of alpha-aminobutyric acid from threonine (Chap. III-E-2) and of alanine from serine (p.68), and a system which generates an unidentified blue fluorescence by incubation with threonine (chap. III-E-3).

Alpha-ketobutyric decarboxylase preparations, after dialysis at pH 8.2, are active within the range of ca. pH 5 to ca. pH 7.2 (fig. 14). The reaction is speeded up by adding cocarboxylase (fig. 13).

Pyridoxal phosphate activates glutamic-alphaketobutyric transaminase (table XXVI, fig. 12). Reaction balances of this system were analyzed (p.83).

The formation of alpha-aminobutyric acid by incubations of Neurospora extracts with threonine, requires addition of pyridoxal phosphate when aged extracts are used. The plot of this activity versus pH falls sharply between pH 7.4 and pH 7.0 (fig. 10).

Preparations able to yield alpha-aminobutyric upon incubation with threonine were, in all cases tested, able

to do so when incubated with alpha-ketobutyric as well (tables XIX and XXI).

Pyridoxal phosphate activates the deaminases of serine and threonine (R values* in tables IX to XVII and XX).

R values are larger when incubation is carried at 40°C instead of 35°C (table XII). They are not increased significantly by dialysis (table XIII) or by addition of phosphatase (table XVI). Cyanide addition resulted in an increase in R (table XIV), but the significance of this effect is doubtful. Preincubation for a few hours at 45°C results in larger values of R for serine deaminase (table XVI) and storing the mold in a frozen condition for a month increases the R ratio for threonine deaminase (table XVII).

Extracts of the pyridoxineless strain 44602, while deaminating threonine at a subnormal rate, were not activated by pyridoxal phosphate addition (table XVIII).

The deamination of threonine proceeds with the formation of equimolar amounts of ammonia and alpha-ketobutyric acid (table XIX and p.54). Addition of a pyridoxal phosphate resulted in increased alpha-ketobutyric acid formation from threonine and increased pyruvic acid formation from serine, as well as increased ammonia formation; and did not alter qualitatively the nature of

* We have defined R as the ratio of deaminase activity with added pyridoxal phosphate to activity without pyridoxal phosphate.

the keto acids resulting as products or side products of the reaction (p 60); notes on the specificity of the ketobutyric assay, pp. 8 and 9; and table X.

Addition of alpha-ketoglutaric acid did not affect the course of the deamination of threonine.

Alpha-aminobutyric and glutamic acid could be ruled out as intermediates in the deamination of threonine, either in the presence or in the absence of added pyridoxal phosphate, in the light of the results described in tables XIX and XX.

We failed to find any evidence for the existence of a nitrogen-free alpha-ketobutyric acid precursor in the deamination of threonine (table XXII).

Incubation of threonine with some Neurospora extracts resulted in the production of blue fluorescence (BF), while incubation of a number of other substrates listed on page 70 did not. BF appears to be associated with the protein coagulum, and is destroyed by reduction with sodium hydrosulfite (chapter III-E-3).

The ratio S/T (serine to threonine deaminase activity) varied in the different experiments within the extreme values of 0.6 (table XV) and 8 (table XVI) (and fig. 2, pH 8.4). Crude extracts gave values close to unity in two experiments (tables XII and XV), and higher where they came from mycelium stored frozen for a month (table XVII). Dialyzed preparations exhibited in all cases S/T values

higher than unity. S/T oscillated from 1.5 to 3.0 with changing pH and temperature of incubation (calculated from figs. 4 and 5). It decreased from 8.0 to 2.0 by preincubation at 45°C (table XVI). Figure 5 shows a decrease in S/T on raising the temperature from 35° to 45°C (late readings). There are no outstanding discrepancies in the ratios of serine to threonine deaminase activity of the strains tested on table XXV, but just a doubtful suggestion of an effect of the pH of the medium. The yields of serine and threonine deaminase parallel each other fairly well after acetone precipitation at different pHs (fig. 2).

The course of the deamination of serine and threonine in the presence of ammonium sulfate precipitated preparations, follows in the first few hours a fairly straight line when plotted against time.

(2) Alpha-ketobutyric decarboxylase

While we were able to demonstrate some activation of this system by synthetic cocarboxylase- and this fact, supported by much more impressive demonstrations recorded in the literature of the role of cocarboxylase in this reaction, is a good indication that cocarboxylase is an essential constituent of the system - the effect demonstrated is rather feeble. Inactivation in the course of more prologued dialysis may be due to protein

denaturation, or to loss of some unrecognized cofactor, but it may be helpful for further work to also consider the possibility that dialysis has caused a dissociation of cocarboxylase which is difficult to reverse. That this may be the case is suggested by the work of Kubowitz and Lütgens (70), who found that pyruvic decarboxylase (yeast) which had lost its activity by standing for half an hour at pH 8.1, could regain it by precipitation with ammonium sulfate followed by dialysis at pH 6.2 and addition of 20 times concentration of cocarboxylase and magnesium found in the original preparation.

One should also keep in mind the possibility that the pH curve obtained in our experiment, besides representing the effect of pH and ionic strength on the rate of reaction, may be affected by the pH dependence of the reaction leading to the reassociation of apo-decarboxylase with cocarboxylase.

(3) Glutamic-ketobutyric transaminase

We pointed out that the reaction balances were those which correspond to a transaminating system, rather than to a system able to deaminate the amino-acid and aminate the keto acid in question. This observation does not furnish an air-tight proof of the nature of the system. Amination coupled to deamination would bring about the same results. By the same token, running controls with the keto acid and ammonia is not enough to prove that

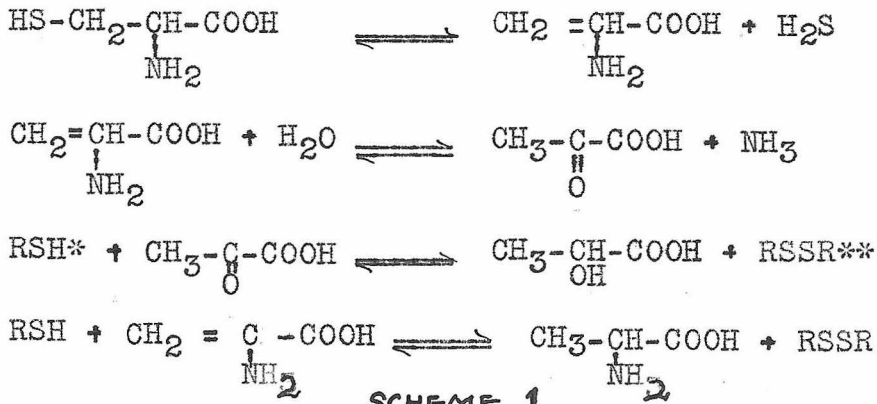
transamination does not operate via free ammonia. One would have to set up transaminase incubations in the presence of labeled ammonia, in order to decide between both alternatives. Such experiments have been run (71) with other systems and thus the direct transfer of amino groups in transamination was established. Our system has in common with those the property of being activated by pyridoxal phosphate. This property, and the nature of the balances, amount to pretty strong evidence in favor of considering the system investigated as a true transaminating system.

(4) The formation of alpha-aminobutyric acid from threonine

All the observations presented concerning this transformation can be interpreted by assuming that threonine is first deaminated to give rise to alpha-ketobutyric acid, which in turn generates alpha-aminobutyric by transamination with glutamic acid. Other amino acids may also contribute their amino groups to this process via transamination with alpha-ketoglutaric (or by direct transamination? (63a)). Proteins and peptides may do it also by virtue of the proteolytic activities present in the preparations used. Such an interpretation finds further support in the following facts: 1) aminobutyric formation is not encountered at the pH range where ketobutyric decarboxylase becomes active, 2) pyridoxal

phosphate is a cofactor in the deamination of threonine, the transamination giving rise to aminobutyric, and in the formation of aminobutyric from threonine, 3) the yield of aminobutyric decreased sharply by purification of the system.

It must be kept in mind however, that the possibility of a direct transformation has not been altogether excluded. Such a transformation - if it occurs at all - might be conceived to take place along the lines of the conversion of cysteine into alanine. This latter reaction was recognized by Fromageot et al (72) (73), who found that preparations of "cysteine desulfhydase" which liberate less ammonia than hydrogen sulfide when incubated with cysteine, also give rise to cystine, lactic acid and alanine besides pyruvate. Although the reported balances are somewhat irregular due to other side reactions, it appears likely that such results are due to the concomitance of the following reactions (74):



SCHEME 1

* abbreviation for cysteine
**abbreviation for cystine

The intermediary role of alpha-aminoacrylic acid is hypothetical, but it appears almost unavoidable to postulate it, at least at the "paper chemistry degree of approximation", if one is to unify relevant observations. Binkley and Okeson (75) have obtained purified preparation of "cysteine desulfhydrase" which liberate H_2S, but no ammonia, from cysteine. Fromageot et al (73) on the other hand, were able to favor the reaction leading to pyruvate plus ammonia and hydrogen sulfide over the others, by treating their enzyme preparation with chloroform. This appears at first sight analogous to what befalls to our Neurospora preparations, which in the course of purification lose most of the alpha-aminobutyric forming ability. It is noteworthy that Fromageot has not reported running controls to which pyruvate was added, to see if alanine would be formed. In his case, however, the existence of systems yielding alanine, cystine, and hydrogen sulfide from cysteine (74) gives strong support to the kind of interpretation outlined in scheme 1.

(5) Path of deamination and B_6 effect

The observation that pyridoxal phosphate activates the deamination of serine and threonine, puts under suspicion the interpretation of these processes as direct deaminations. One is reminded of the experiments of Braunstein and Asarkh (76) in which the oxidative deamination of certain amino acids in the presence of ground

ox kidney was made to proceed via transamination with alpha-ketoglutaric acid and deamination of the resulting glutamic acid. However, an intermediary role of transamination appears to be ruled out in the systems we studied since: 1) addition of alpha-ketoglutaric is without effect on either rates or yields; 2)alpha-ketobutyric acid is the only keto acid produced in the deamination of threonine in yields comparable to those of ammonia; 3) B_6 increases this yield of alpha-ketobutyric as well as that of pyruvate from serine, without altering the pattern of keto acids found in the reaction mixture.

One might prima facie try to explain the pyridoxal phosphate effect on threonine deaminase as a function of the activation of the conversion of threonine to alpha-aminobutyric by the same cofactor. Such an explanation would involve the assumptions that: 1) the threonine to alpha-aminobutyric conversion occurs directly, 2) pyridoxal phosphate is a coenzyme for that step, and 3) threonine deamination, or the increased deamination encountered when B_6 is added, results via deamination of alpha-aminobutyric acid. The latter possibility, however, is ruled out by our data.

One is thus lead to postulate that pyridoxal phosphate, or a compound closely related to it, is the prosthetic group of threonine and serine deaminases. If this is true, the reported role of phosphorylating agents such as

adenosine-5-phosphate or mixtures of inorganic phosphate plus reducing agents as activators of the bacterial deaminases, may become intelligible.

The difficulties encountered in obtaining a good resolution of the deaminases and pyridoxal phosphate are not uncommon among pyridoxal phosphate systems. Dialysis was found to be an ineffectual method for resolving lysine, tyrosine and arginine decarboxylase (77), glutamic-oxaloacetic transaminase (78), and kynureninase (79). In some cases (77) aging has proved to be a better method. In Neurospora, threonine deaminase is resolved to a fair degree by aging the harvested mycelium for a month in a frozen condition, a behaviour parallel to that of the "tryptophane enzyme" which after a similar treatment shows a 6.7 fold increase in activity when pyridoxal phosphate is added (80).

The occurrence of only a doubtful cyanide effect may be explained away by assuming either that the apoenzymes of the deaminases protect the carbonyl group, or that cyanide is destroyed at a fast rate, or that pyridoxal phosphate is not itself the coenzyme but is able to give rise to a cyanide stable coenzyme. Similar subterfuges may be used to harmonize the fact that phosphatase fails to destroy the co-deaminase activity of Neurospora preparations with the postulate that pyridoxal phosphate is the co-deaminase. One may also assume that

the vulnerable pyridoxal phosphate present in the extracts is already saturated by Neurospora phosphatase, so that addition of foreign enzyme has no effect.

The temperature dependence of the R ratio suggests that the rates of the reaction of deamination and of that leading to coenzyme association may have a different temperature coefficient. If that is the case it may become feasible to study both reactions somewhat independently. It is also possible that the effect of temperature on R reflects the temperature dependence of the dissociation constant of the coenzyme.

There is only partial parallelism between the behaviour of threonine deaminase and glutamic-pyruvic transaminase (as studied by Bernard Strauss (39)) of mutant 44602 grown at pH 7, in the absence of added B_6. Both systems are present in such cultures in lower activities than in wild type. But while the transaminase can be made as active as in wild type (or more) by addition of pyridoxal phosphate, the deaminase fails to respond to B_6. This discrepancy may reflect an essential difference in the apoenzyme coenzyme relations, but it may also result from differences in preparative methods. Besides, it is possible that the low deaminase activity has no direct relation to the pyridoxine-less character of the mutant.

(6) A unifying conception of the mechanism of pyridoxal phosphate catalysis

The coenzymatic capacities of B_6 were first recognized in its role as activator of tyrosine decarboxylase (81) (82)*. Pyridoxal phosphate has since been shown to act as a cofactor for all bacterial and plant decarboxylases, and also for some animal ones (83) (84).

The participation of pyridoxal phosphate in enzymatic transamination has been established in all such systems tested (84) (56). A third type of reaction in which B_6 takes part is concerned with the synthesis and breakdown of tryptophane. Tryptophanase breaks tryptophane down to indole, pyruvic acid and ammonia. It was resolved by Wood et al (85) (86) using E. coli as enzyme source, and shown to require pyridoxal phosphate. An enzyme system found in Neurospora extracts, different from tryptophanase (86) (we shall call it "tryptophane enzyme"), catalyzes the synthesis of tryptophane from serine and indole (87) and is also activated by pyridoxal phosphate (80).

Braunstein (79) was able to demonstrate a 2-fold increase in the in vitro activity of kynureninase (system breaking kynurenine down to anthranilic acid and

*Pyridoxal phosphate appears to be the coenzyme form of B_6 in the reactions thus far investigated. The activity exhibited by other forms as substitutes for pyridoxal phosphate has been interpreted as a function of the capacity of those forms to generate pyridoxal phosphate, since it is generally possible to find preparations which would be activated only by pyridoxal phosphate.

alanine) by addition of pyridoxal phosphate to extracts of B_6 deficient rats incubated with kynurenine. Snell and Wright (88) point out that if pyridoxal phosphate was implicated in the conversion of 3-hydroxykynurenine to 3-hydroxyanthranilic acid, a step analogous to the one studied by Braunstein, the relations between B_6 and nicotinic acid formation as established by *in vivo* experiments could be understood. The same could be said for the *in vivo* effect of B_6 on xanthurenic acid formation (89).

The deaminations of serine and threonine are examples of a fifth type of reaction mediated by pyridoxal phosphate. Analogies in the postulated mechanisms of action of serine deaminase and cysteine desulfydrase, and in the nature of the substrates, prompted Glenn Fischer to test the effect of pyridoxal phosphate on cysteine desulfhydrase of Neurospora. Preliminary results (personal communication) indicate a significant increase in the rate of H_2S production from cysteine when pyridoxal phosphate is present.

Very recently Binkley and Christensen (90) found that pyridoxal phosphate activates the enzymatic clevage of djenkolic acid, as determined by the cysteine produced.

Vitamin B_6 has also been implicated in the racemization of aminoacids (88) (91) (92), and in the metabolism of unsaturated fatty acids (93), but the specific reactions through which these effects are exerted have not been identified.

It seems to us possible, at least on paper, to find a common denominator to the coenzyme functions of pyridoxal phosphate by assuming that the initial step is in all cases the formation of a Schiff base between pyridoxal phosphate (bound to the apoenzyme) and the substrate. The explanatory merits of such a hypothesis will be discussed in the following paragraphs in relation to each type of reaction.

That transamination is mediated by the formation of Schiff bases between amino acids and keto acids - or in the thinking of later times between pyridoxal phosphate and amino acids, and pyridoxamine phosphate and keto acids - has been the assumption underlying all speculations on the mechanism of transamination (53) (56). In its simpler form, the hypothesis can be represented by the equations:

$$\text{pyridoxal phosphate} + \text{amino acid}_{(1)} \rightleftharpoons \text{pyridoxamine phosphate} + \text{keto acid}_{(1)}$$

$$\text{pyridoxamine phosphate} + \text{keto acid}_{(2)} \rightleftharpoons \text{pyridoxal phosphate} + \text{amino acid}_{(2)}$$

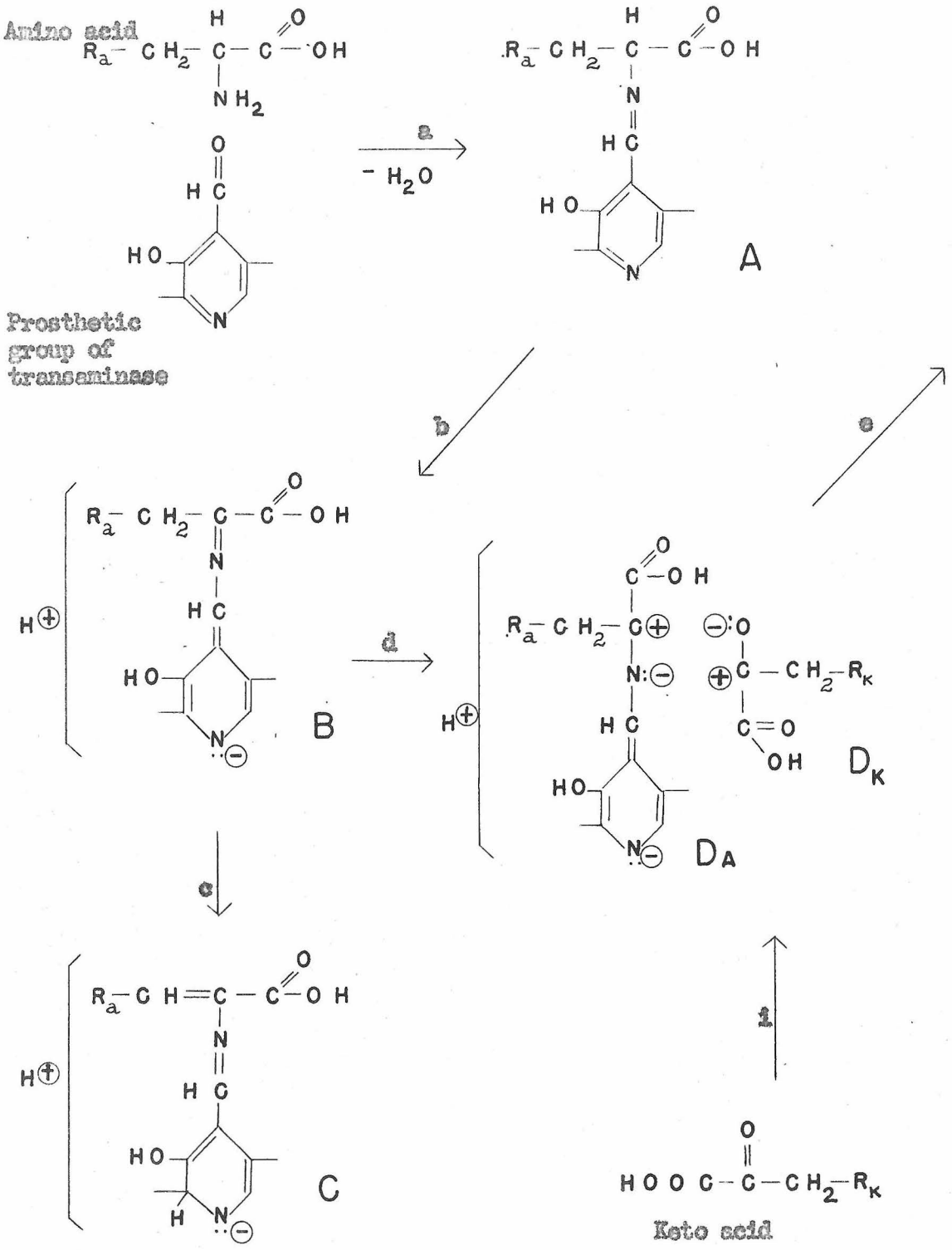
each of them including the corresponding Schiff base intermediate (94). It is an essential part of this hypothesis that pyridoxal phosphate and pyridoxamine phosphate should be equivalent coenzyme forms. The finding of a purified transaminase preparation which could be activated by pyridoxal phosphate but not by pyridoxamine phosphate (95) has brought to a close speculation along

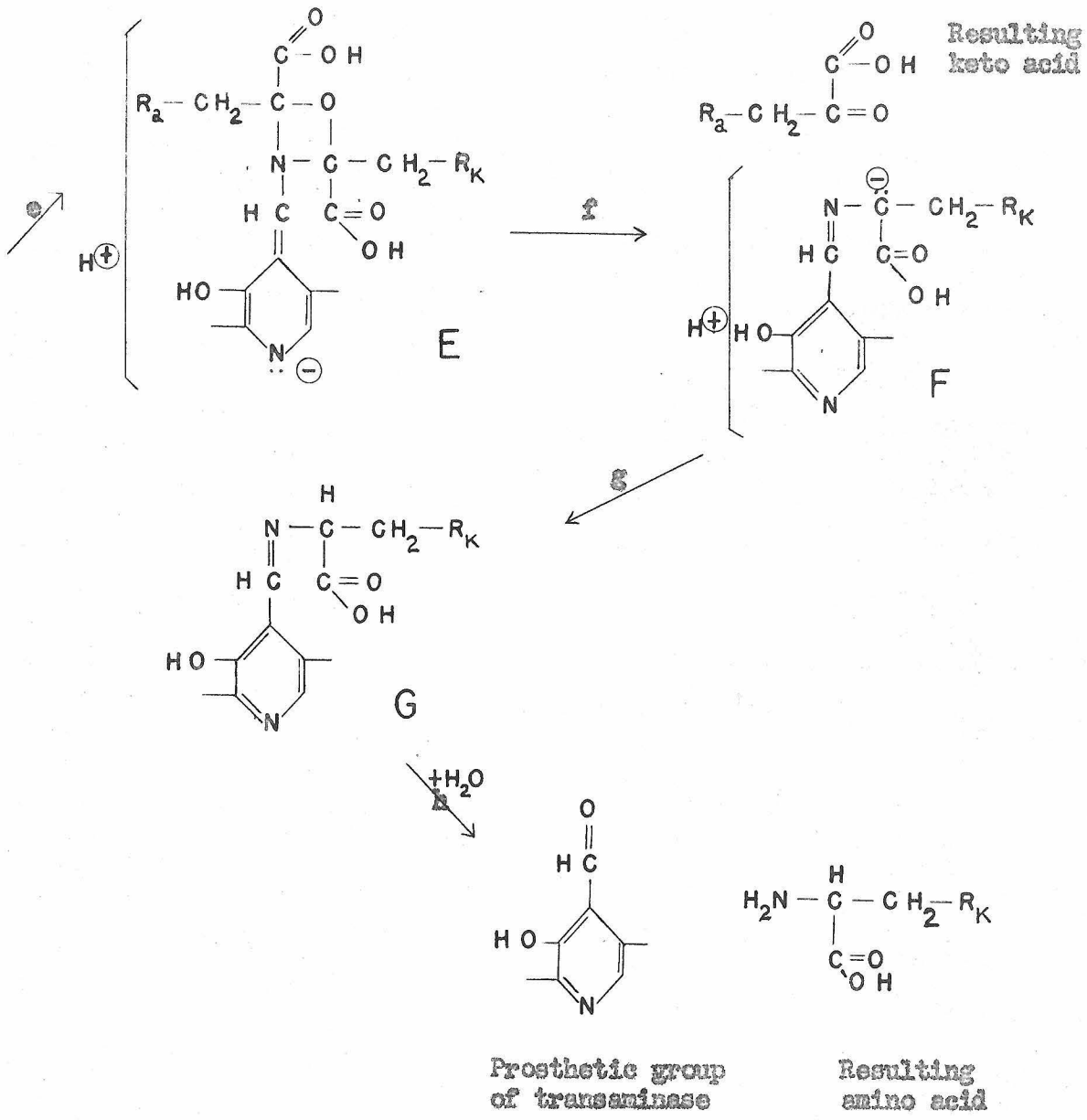
such lines. This is in accordance with the unwritten rules of scientific speculation which are not uhlike those of the soccer game, where the player is allowed to by-pass his opponents while in the middle of the field but cannot do it in the close proximity of the goal. Here we are only trying to speculate from afar, and so the reader may allow us to try to circumvent the observation of the inactivity of pyridoxamine while preserving the idea that Schiff base formation between pyridoxal and the amino acid is the first step in transamination. One could do so by assuming that the inactivity of pyridoxamine is simply due to inability of this form to attach itself to the apoenzyme. An alternative based on less ambiguous concepts can be found in a hypothesis in which amino group exchange is thought to take place through the formation of a pyridoxal coenzyme-amino acid-keto acid complex which is formed and broken down in such a way as to never liberate the pyridoxamine form of the coenzyme (scheme 2).

The acidic character of the alpha position in the amino acid moiety, made manifest in step b, has been demonstrated by Konikova et al (96) by incubating alpha-deuterio amino acids with purified glutamic-pyruvic transaminase preparations. Under such conditions, alpha-deuterioglutamic acid exchanges deuterium with the aqueous medium. The alpha-deuterium of alanine under the same conditions, does not become labile unless alpha-

SCHEME 2

On the mechanism of transamination




e
f
g
h
+H2O
E
F
G
Resulting keto acid
Prosthetic group of transaminase
Resulting amino acid

ketoglutaric or pyruvic acids are added. While a finished explanation of this latter phenomenon appears premature in the light of present knowledge, a situation like that found in scheme 2 may be in the background of such observations. We can conceive that addition of keto acids able to combine with structure Da may increase the probability of the occurrence of intermediate forms like B. The experiments of the Russian workers also show that boiled transaminase preparations, thus devoid of transaminase activity, are able to labilize the alpha position of amino acids, thus suggesting that a heat stable catalyst is involved in steps a and b (and d?). Pyridoxal phosphate has no alpha-position labilizing-activity incubating for 2 hours at 38°C, indicating that steps a and/or b involve apoenzyme catalysis.

A model for step c is found in the experiments of Herbst and Rittenberg (97), who found that when non enzymatic transamination between an amino acid and a keto acid is realized in a heavy water medium, both the alpha and the beta position of the amino acid resulting by transamination are labeled with deuterium.

****

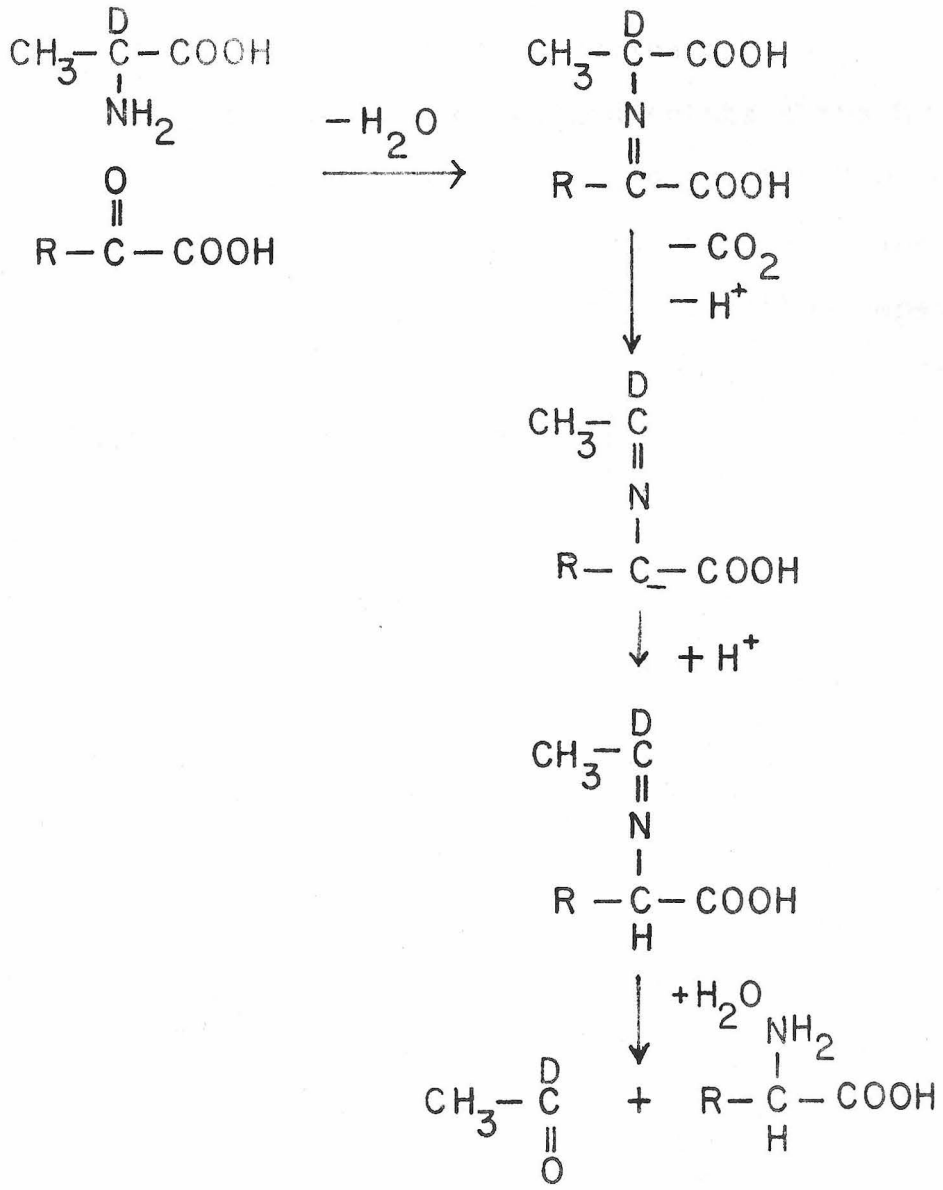
A number of carbonyl compounds are able to cause (non enzymatically) the deamination of amino acids, presumably via Schiff base formation and transamination. Among them are to be found (98) keto acids, alpha-dicarbonyls like methylglyoxal and phenylglyoxal, ninhydrin,

alloxan, isatin, o-quinones, pyridoxal. (Notice that the grouping -X=C-C=O is present in all cases). In the cases of keto acids and pyridoxal, transamination is made evident by the recovery of a new amino acid and of pyridoxamine respectively. With the exception of the pyridoxal mediated reaction, all the other types of deaminations listed lead to simultaneous decarboxylation of the added amino acid. The mechanism of amino acid-keto acid non enzymatic transamination was studied by Herbst and collaborators (97) (98) using alpha-deuterioalanine. The deuterium was not labilized in the course of the reaction, which lead the authors to postulate that after Schiff base formation the shift of the double bond which is thought to initiate amino group transfer was made possible by decarboxylation rather than by prototropic movement away from the alpha carbon of the original amino acid (scheme 3). Amino acid-keto acid chemical transamination can be made to occur without concommitant decarboxylation if the carboxyls are esterified and a non aqueous medium is used (99). This latter type of transamination is inhibited by acidifying, while the decarboxylative one requires acid conditions.

Blashko (110) has suggested that enzymatic decarboxylation is mediated by the formation of a Schiff base between the amino acid substrate and pyridoxal phosphate, on the basis of the observation that N-methyl amino acids cannot be attacked by decarboxylases.

SCHEME 3

The mechanism of non-enzymatic deamination and decarboxylation of amino acids, under the action of keto acids, as inferred from experiments with deuterium labeled amino acids (After Herbst and Rittenberg (97)).



Enzymatic decarboxylation can be thought to occur along the lines of scheme 3, but with the methyleneazomethine bridge cleaving finally in such a way that no transamination would result. As in the amino acid-keto acid model, the enzymatic outcome might be either prototropic shift and transamination, or decarboxylation, depending upon the relative stability conferred to the alpha hydrogen and the carboxyl group. Supplementary bonding between enzyme and substrate could accomplish this. The similarity between enzymes and models is strengthened by the fact that enzymatic decarboxylation requires acidic pHs while enzymatic transamination occurs only in basic media (100).

In the above conception of the mechanism transamination and decarboxylation, the bond shift in the methylene azomethine bridge, prompted by conjugation with double bonds in the rest of the molecule, becomes the driving force for initiating the reactions. It is not difficult to see how the same shift can result in deamination of serine, threonine and cysteine in the proper catalytic environment. The three deaminations have been assumed to occur via dehydration or desulfhydration to alpha-aminoacrylic acid or, in the case of threonine, to alpha-aminocrotonic acid (101) (102).

In the case of cysteine, the existence of enzyme preparations which liberate hydrogen sulfide but not

ammonia, and the absence of preparations causing deamination without concommitant desulfydration, constitutes strong evidence in favor of the idea that desulfhydration is a prerequisite for deamination.

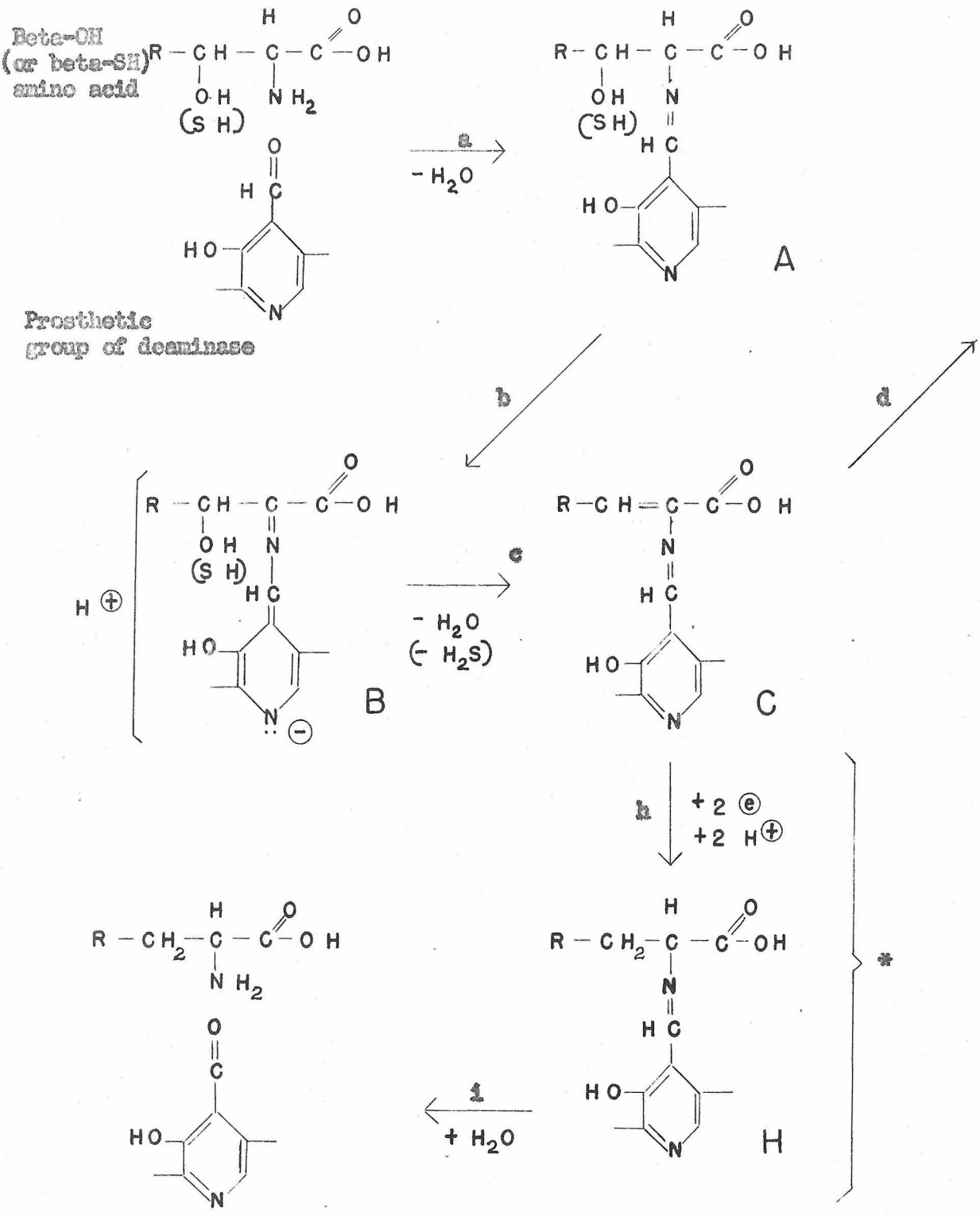
Already in 1925, Bergman et al (103) (104) suggested that alpha-aminoacrylic acid lies in the crossroads of the biological interconversion of serine, pyruvate and alanine, studying as model for those processes the non enzymatic transformations glycylserine underwent. Because of the inactivity of serine deaminase towards serine esterified via its hydroxyl group, Chargaff and Sprinson (102) suggested the intermediacy of alpha-aminoacrylic acid.

A possible path for deamination of serine, cysteine and threonine is pictured in scheme 4. Steps a, b, and c may be reversible, but if so only very slowly, as indicated by the experiments of Smythe and Halliday (105). They incubated cysteine and cysteine desulfhydrase in the presence of labeled hydrogen sulfide. When deamination had progressed about one fourth of the way, the remaining cysteine was isolated. It was found that the heavy sulfur content of cysteine was about 1% of that expected if it had reached equilibrium with the pool. The 1% incorporation might be due to slow reversal, or to side reactions.

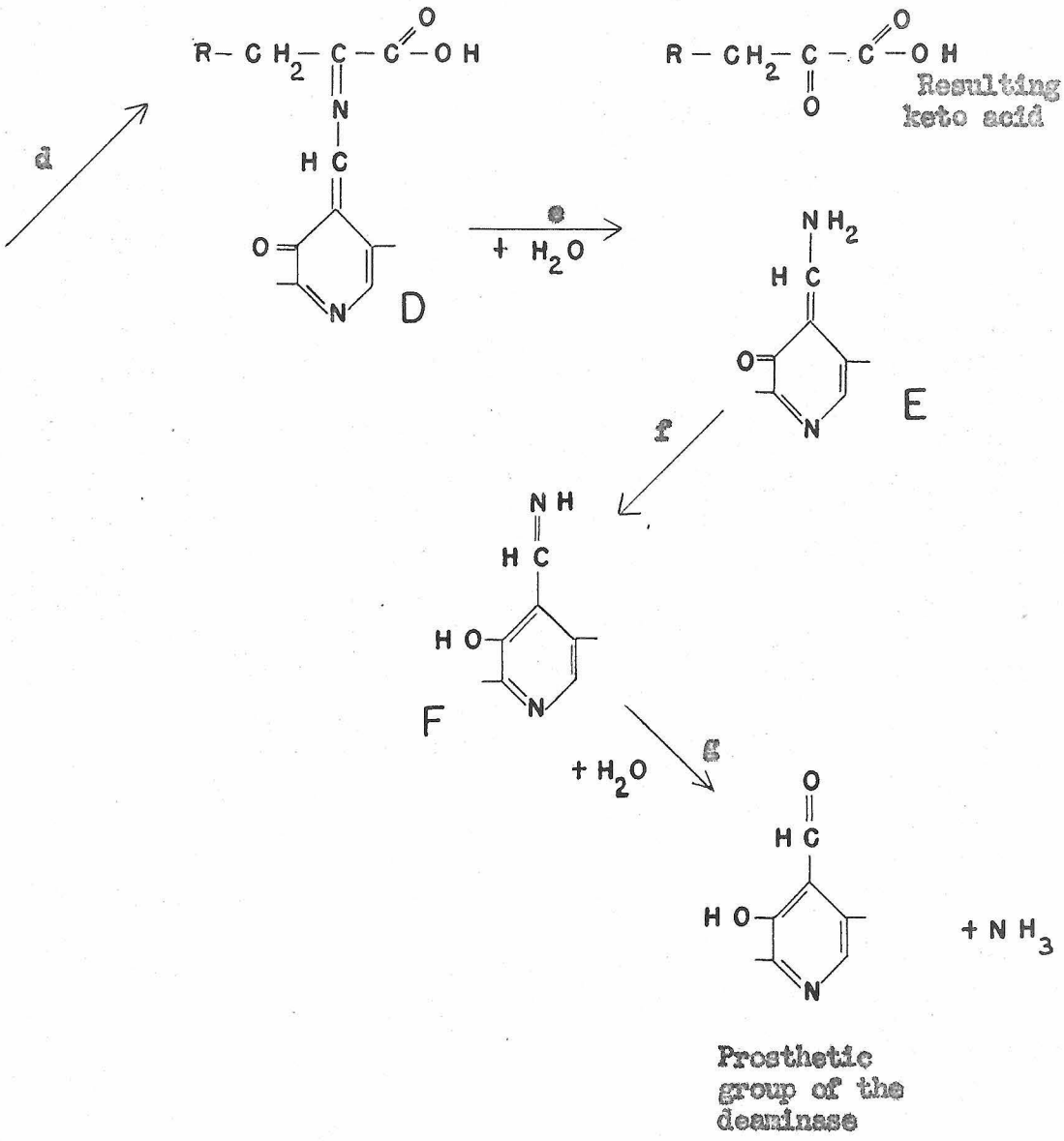
The breakdown of tryptophane to indole, pyruvate and ammonia can be described by the same scheme, substituting

SCHEME 4

On the mechanism of beta-OH and beta-SH amino acid deamination



*Steps in the postulated cysteine-alanine conversion path. May involve an apoenzyme different from cysteine desulfhydrase.


R – CH2 – C – C – OH
N
HC
O
N
D
d
e
+ H2O
R – CH2 – C – C – OH
O
Resulting keto acid
NH2
HC
O
N
E
f
NH
HC
HO
N
F
+ H2O
g
HC
O
HO
N
+ NH3
Prosthetic group of the deaminase

the indole nucleus for the hydroxyl group. The kynureninase reaction can also be reduced to the same scheme if we assume that it takes place in two stages analogous to those postulated for the cysteine-alanine conversion: 1) split of kynurenine to give o-aminobenzaldehyde and alpha-aminoacrylic acid (the latter, in the form of a Schiff base with the pyridoxal prosthetic group), 2) oxidation and reduction yielding o-aminobenzoic acid (anthranilic acid) and alanine.

The synthesis of tryptophane would be accomplished also along the lines of scheme 4, the role of serine being that of generating the alpha-aminoacrylic complex onto which indole can be attached. Pyruvate and ammonia cannot be coupled with indole by that enzyme system. On the other hand, tryptophane breakdown does not go through serine (nor through any of many related compounds tested) (86). This would indicate that steps d and/or e are irreversible or at least that d to g prevails over e to a (cf. scheme 4). This is consistent with the results of Smythe and Halliday mentioned above. Of course, conclusions drawn by pooling observations concerning the same prosthetic group attached to different enzymes, constitute only a very rough approximation. Some inkling as to the differences in the path of reaction due to different apoenzymes could be obtained by separating the

"tryptophane enzyme" from serine deaminase and determining whether there is deamination of serine specifically associated with the working of the "tryptophane enzyme".

By analogy with the cases reviewed, one is led to suspect that pyridoxal phosphate may be involved in the formation of cystathionine from homocysteine and serine (106), a possibility not yet investigated to our knowledge. Fromageot (74) discusses the possibility that alpha-aminoacrylic acid could be an intermediate in that condensation, and concludes that it could not because a mixture of pyruvate and ammonia, which one expects to be in equilibrium with alpha-aminoacrylic acid, was inactive in place of serine. The argument is not relevant to our scheme, because the postulated intermediate is not alpha-aminoacrylic itself but the product of its condensation with the pyridoxal coenzyme.

By the same token, one suspects pyridoxal phosphate to be involved in the breakdown of cystathionine by Neurospora*. Nutritional data indicates that this reaction gives rise to homocysteine plus an unknown product(s) different from serine (107), and analogy with the tryptophanase reaction suggests that pyruvate and ammonia may make up the balance of the reaction products.

An explanation of the mode of action of pyridoxal phosphate in the clevage of djenkolic acid to give

*Glenn Fischer is investigating this point

cysteine, cannot be achieved without removing ourselves a step further from better known relations, and we should rather not venture into that territory. The suspected racemizing role of B_6 enzymes, on the other hand, may be the result of steps a and b (scheme 2), and their reversal, being catalyzed by an enzyme devoid of optical specificity.

7) The production of blue fluorescence

As a working hypothesis - which admittedly has not reached the stage where it may try to be convincing - we propose to consider BF as the result of a transformation undergone by the coenzyme form of B_6 in the course of threonine deamination. The hypothesis is consistent with the following facts:

1) In most natural materials, B_6 is found in a bound form (protein bound presumably) (108).

2) When proteins are coagulated, codecarboxylase (pyridoxal phosphate) is found associated with the protein coagulum (55).

3) Pyridoxal phosphate solutions fluoresce green; pyridoxal and pyridoxamine, blue.

4) Sodium hydrosulfite destroys both BF and B_6 fluorescence, and hydrogen peroxide restores them.

5) In comparing the activities of strains E-18829 and 44602, BF forming activity accompanied threonine deaminase activity.

Our hypothesis is complicated by the observation that preparations incubated with serine do not give rise to BF.

8) Serine and threonine deaminases: one or two enzymes?

Wood and Gunsalus (24), using purified E. coli preparations possessing serine and threonine deaminase activities, observed that incubation with serine lead to eventual loss of the deaminase activity of the preparation towards both substrates. This and other analogies in the behaviour of both activities, in the words of Wood and Gunsalus "suggests the identity of the two enzymes". Our data on the Neurospora deaminases, although still kinetically crude and incomplete, suggest that serine and threonine are deaminated by distinct enzymes.

Comparison of the S/T ratio of the different preparations reveals variability which exceeds by far the experimental errors involved. Since the course of deamination appears to be approximately linear, variability of S/T might be due either to the existence of two distinct enzymes in different ratios, or to the presence of variable concentrations of inhibitors affecting unequally the two activities of the same enzyme. The latter alternative, however, is unappealingly complicated, and is unable to explain the different response of serine and threonine deaminase to activation by pyridoxal phosphate.

We have already indicated that different B_6 enzymes exhibit characteristic responses to pyridoxal phosphate. Cammarata and Cohen (61) have encountered this phenomenon in their study of animal transaminases and have taken it as presumptive evidence that the enzymes in question are different.

On trying to divine some pattern in the different effect of pyridoxal phosphate on serine and threonine deaminase, the following can be stated: storing the frozen mycelium or dialysing extracts overnight in the cold (which might represent the effect of simple storage in the cold) results in better response of threonine deaminase to pyridoxal phosphate addition (more extensive threonine coenzyme dissociation?); preincubation at 45°C gives rise to larger pyridoxal phosphate effect for serine deaminase (more extensive serine coenzyme dissociation?). Without pretreatment, both enzymes respond similarly to pyridoxal phosphate. The significance of the above observations is however doubtful, since they are based on only a few experiments which were not designed to test the point in question.

On the other hand, the similar behaviour of serine and threonine deaminase in acetone precipitation, indicates that both enzymes must have physical properties in common.

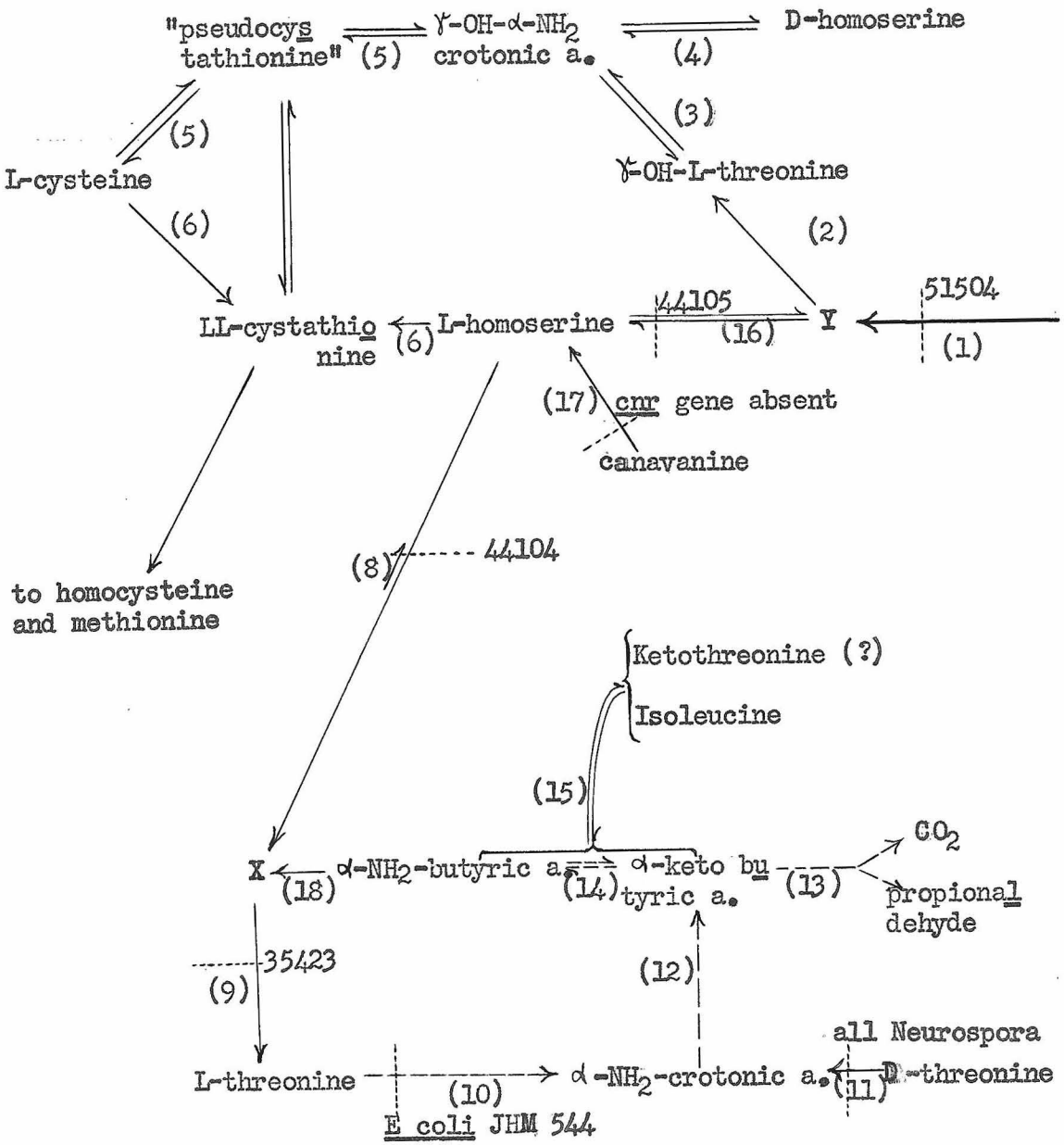
B) RE IN VIVO METABOLISM

The set of threonineless mutants of Neurospora is one of that kind which cannot be arranged in a consistent scheme without making assumptions which go beyond the simple assignation of a biochemical block to each mutant gene. It is indeed not surprising that such complications would arise in the study of biochemical mutation. But it is still baffling to have to choose on paper among an indefinite number of more or less complicated assumptions, each of which is able to legitimize a different scheme of mutant blocks. Such an arbitrary choice, justified because it has the nature of the working hypothesis and also because it provides a convenient framework of reference for the discussion, is involved in the alternate schemes presented*.

Scheme 5 is a modification of that of Emerson and Teas (44). We have altered the hypothesis of Horowitz, which assumed homoserine to be involved in the X to threonine reaction, so as to make it consistent with the amendment suggested by Teas. The activity of canavanine for strain 46003-cnr has been discussed by Teas (40) (47), and it appears from nutritional data that the cnr gene induces resistance to canavanine by making the step canavanine to homoserine possible. Low activity of gamma-hydroxythreonine and D-homoserine is indicated by

* For a description of the requirements of the mutants in question, refer back to chapter III-F

SCHEME 5

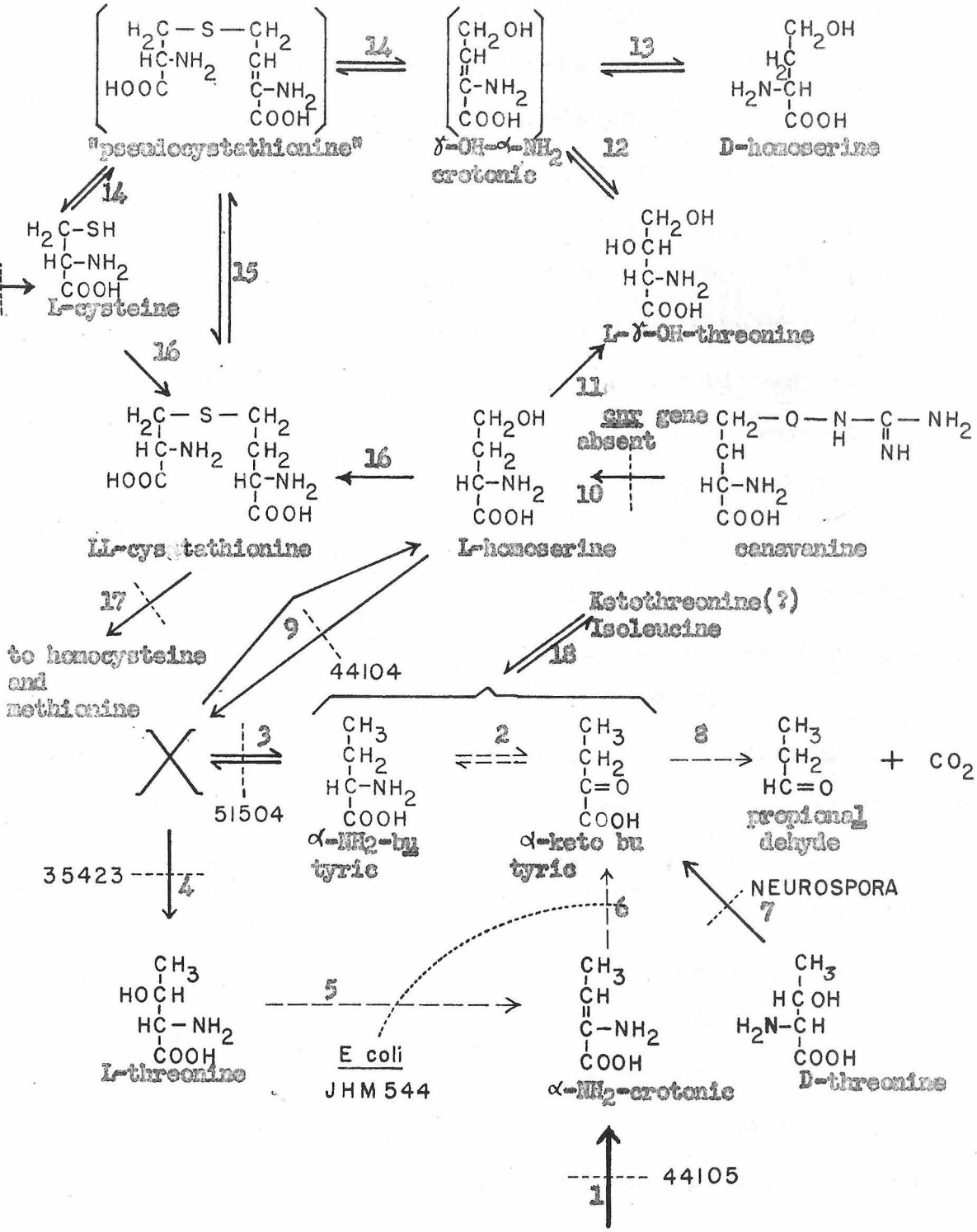


This scheme is consistent with the known facts, provided that:
(I) Either Y, γ-OH-threonine, γ-OH-α-NH_2-crotonic, D-homoserine, "pseudocystathionine" or cystathionine are required for step (9) to take place. For instance, steps (3) and (9) might be linked.
(II) Steps (5) and/or (6) are slow.
(III) Steps (10) and/or (12) are slow *in vivo*.

(For structural formulae consult scheme 6)

Broken arrows indicate enzymatically demonstrated steps. Dotted lines, represent genetic blocks.

SCHEME 6



With the following specifications:

(I) Step 5 is slow in vivo.

(II) Steps 14 and/or 15 are slow

(III) The "one way block" of step 9 by mutant 44104 represents a derrangement of the equilibrium of that step, such that incoming metabolites are drained towards homoserine and further.

Broken arrows represent enzymatically demonstrated steps. Dotted lines, blocks.

slowness of steps 5 and/or 6. Those compounds are active as methionine sources, but not as threonine source, thus steps 2 and 6 are assumed irreversible. Inactivity of cystathionine as threonine substitute (mutant 51504) also necessitates that 6 be irreversible, and the low activity of threonine as a substitute of methionine is indicated by the sluggish reversibility of 8. The relative positions of alpha-aminobutyric and alpha-ketobutyric, isoleucine and ketothreonine are somewhat arbitrary (however, see page 130). The lower activity of L-threonine as compared with amino or ketobutyric for 44105 could be due to the *in vivo* slowness of the deaminase.

While scheme 5 required additional assumptions to explain the behaviour of mutant 51504, scheme 6 is built around the requirements of 51504, 35423, and 44105, showing less respect for the properties of strain 44104. This choice is based on the fact that 44104 does behave in an unorthodox fashion, as recently shown by Garner (46). Double mutants of 44104 with strains blocked in the methionine pathway accumulate substances which feed 44104 and other threonineless mutants, while the participation of gene 51504 in a double mutant stops the accumulation of threonine-like substances. We have assumed that 44104 experiences a threonine deficiency because step 9 (scheme 6) prevails over step 4 by being very active in the direction X to homoserine and draining its supply of the metabolites preceeding X. The block

in the E.coli mutant JHM544 could be indicated as covering both steps 5 and 6, which is not unwarranted since they both represent the action of threonine deaminase. In this connection it would be of great interest to test this E. coli strain for the presence or absence of threonine deaminase in resting cells and in extracts. The lack of a methionine requirement in strain 44105, but the presence of it in 51504 is accounted for by the fact that the 51504 block also blocks the threonine to homoserine path, since step 4 is practically irreversible.

Teas (40) suggested that the in vivo conversion of alpha-ketobutyric into threonine occurs in Neurospora by a reversal of threonine deaminase. In scheme 5, this would imply that X is identical with alpha-aminocrotonic (since this is the probable path of deamination), that ketobutyric is converted directly into X as in step 12, and that steps 9 and 10 are identical. Ignoring for the moment the E coli mutant, and placing step 1 as a forerunner of aminobutyric, scheme 6 can be converted in a similar fashion. But we have found (table XXIII and p. 78) that extracts of strain 35423, blocked in step X to L-threonine and unable to use ketobutyric or aminobutyric in place of threonine, are able to deaminate threonine to yield alpha-ketobutyric and alpha aminobutyric acid. The following alternative interpretations of that observation may be suggested:

1) The Teas hypothesis is correct. The inability of 35423 to use alpha-aminobutyric and alpha-ketobutyric acid is due to "uptake" difficulties peculiar to that strain.

2) The Teas hypothesis is correct. The deaminase of 35423 is active *in vitro*, but the character of the 35423 mutation is such that the same process is inactivated *in vivo*. A similar hypothesis has been postulated by Wagner (109) to explain the ability of pantothenicless mutants of Neurospora to synthesize pantothenic acid *in vitro* from precursors the strains could not utilize *in vivo*.

3) A special case of the above hypothesis: the block in 35423 is concerned with the coupling of threonine deaminase with a reaction able to reverse it. What one may call a "one way block".

4) The synthesis of threonine from X is accomplished by an enzyme system different from threonine deaminase.

5) A special case of the above hypothesis: X is identical with alpha-aminocrotonic acid, which is an intermediate in threonine deamination. The deaminase catalyses only the irreversible conversion of threonine into ketobutyric, while the enzyme system in which 35423 is deficient is responsible for the conversion of aminocrotonic into threonine.

6) As a special case of hypothesis 5, we may conceive a situation similar to that encountered in the synthesis and breakdown of tryptophane, where there exists a different enzyme for each of the processes (although found

in different organisms) and each enzyme uses pyridoxal phosphate as coenzyme.

At present it is not possible to decide between the hypotheses presented. If the observations of Smythe and Halliday (indicating little if any reversibility of desulfhydration) can be extended to dehydration by threonine deaminase, then hypotheses postulating reversibility of threonine deaminase should be considered as improbable.

Hypotheses 2, 3, or 6 are particularly attractive because they assign a role to pyridoxal phosphate in the biosynthesis of threonine. In scheme 6 such a role is already implicated by the transamination of step 2. That is not the case of scheme 5 in which the named hypothesis comes closer than any other available explanation to suggest a relation between B_6 and threonine. The lines of evidence which favor the existence of such a relation are as follows:

a) Threonine, serine, cysteine, lysine, alanine, histidine, proline, phenylalanine, tyrosine, glycine and aspartic acid have been demonstrated in different experiments (111) (112) (113) (114) to be able to substitute for specific B_6 requirements of Lactobacilii or Sptreptococci.

b) Strain 44602 (B_6-less, pH sensitive) behaves as if threonine synthesis were limited when grown at high pH in the absence of B_6. Under those conditions it becomes

quite sensitive to methionine inhibition, and this inhibition is overcome by threonine (39) (51). Similar methionine-threonine relations are found in the sulfonamide requiring strain which can be better described as a special kind of threonineless strain (50).

c) Cercedo and DeRenzo (115) have found that B_6 deficient rats, kept on a low protein diet, are sensitive to homocysteine and methionine inhibition. It would be interesting to find out whether this inhibition is reversible by threonine and thus parallels the situation encountered in mutant 44602 of Neurospora.

Information that B_6 is concerned with the in vivo utilization or breakdown of serine and threonine comes from experiments in which B_6 antagonizes inhibitions by those compounds. Snell and Guirard (116) found that excess pyridoxine releases the inhibition of Lactobacilli by DL-serine, DL-threonine, beta-alanine, or glycine. Pyridoxine also reduces the toxicity of D-serine for rats (117) (118). It is possible that stimulation of serine and threonine deaminases are instrumental in such effects, provided that the deaminases attack the D amino acids too, or where inhibition may be due to the L form. Gale and Stephenson's serine deaminase of E. coli (21) was able to attack both D- and L-serine, though at somewhat different rates.

We have discussed earlier the low deaminase activity of extracts of 44602 grown on high pH "minimal" medium. This depression of deaminase activity by raising the pH of the medium is shared by strain E-18829, but is not found in the wild type strain used in our investigation. One may suspect that a causal correlation exists between: 1) this pH effect on deaminase activity, 2) the ability of both mutants to grow without supplement if the pH of the medium is raised, and 3) the symptoms of threonine deficiency found in both mutants (quite clear symptoms in strain E-18829, rather indirect ones in 44602). Perhaps raising the pH of the medium (which as found out by Strauss (39) amounts to enriching the medium with free ammonium ions) protects the mutants against a threonine deficiency by lowering the activity of threonine deaminase. However, on the basis of the nature of the data bearing on this point, we do not think the above hypothesis should be given very serious consideration. Likewise, we do not think that much can be made out of the differences in deaminase activity found among the threonineless mutants. H. K. Mitchell (personal communication) has found up to 4-fold differences in the activity of the "tryptophane enzyme" among a number of unrelated mutant strains picked up at random, and his observations resist any attempt to trace them back to known biochemical interactions.

Harold Garner (personal communication) has tested pyridoxine, alone and in conjunction with limiting amounts of threonine, for its ability to support growth of mutants 44104, 35423 and 44105, with negative results. Neither was it active as a sole growth substrate for 51504, nor could it increase the growth of 44105 when added in conjunction with limiting amounts of alpha-aminobutyric acid, alpha-ketobutyric acid, threonine, isoleucine or homoserine.

As we mentioned earlier, 44104 grows better on alpha-aminobutyric than on alpha-ketobutyric acid, while 44105 uses both equally well. Utilization of the latter compound is facilitated by the addition of pyridoxine to the growth medium of 44104 (pyridoxine and alpha-aminobutyric mixtures have not been tested to our knowledge). The <u>in vitro</u> behaviour of 44104 does not offer any explanation for these nutritional peculiarities, since the activities of glutamic-alphaketobutyric transaminase and ketobutyric decarboxylase of 44104 and wild type extracts were not appreciably different. In a scheme such as #5, where 44104 is pictured as an orthodox block of a biochemical step, it seems to us impossible to account for the different utilization of alpha-aminobutyric and alpha-ketobutyric without assuming either some sort of pleiotropic effect of the gene mutation or an effect of genetic "modifiers", on one of the following processes:

1) Uptake of alpha-ketobutyric acid.

2) Rate of alpha-ketobutyric destruction (not affecting, however, the in vitro decarboxylase activity).

3) The rate of the conversion of alpha-ketobutyric into alpha-aminobutyric, without affecting the in vitro catalysis of this step.

It is simpler to account for the properties of 44104 in a scheme like #6. There, 44104 can remedy its threonine deficiency if supplied with concentrations of the threonine and homoserine precursors able to overcome the drainage in the direction of homoserine. Alpha-aminobutyric appears to be able to do so, but ketobutyric probably dissipates before it can generate such a concentration of alpha-aminobutyric that the rate of step 3 will be greater than that of step 9. Similar considerations can explain why addition of pyridoxine enhances the sparing action of alpha-ketobutyric acid.

Whichever of the hypotheses offered may be closest to reality, the behaviour of 44104 seems to indicate that the relative positions of alpha-ketobutyric, alpha-aminobutyric, and compound X, are those indicated in schemes #5 and #6.

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VII) APPENDIX

A) COMPARISON OF THE ACTIVITY OF CALCIUM PYRIDOXAL PHOSPHATE AND OF PYRIDOXAL PHOSPHORIC ACID, AS COFACTORS IN THREONINE DEAMINATION

Table XXVII

Enzyme preparation: Lyophilizate from mycelium aged in a frozen condition, prepared as described in table XVII (p. 42) and kept in an evacuated desiccator in the icebox for 3 months, was used to make a Neurospora extract in the usual way (in M/4 phosphate buffer).
Incubation: 4 hours at 40°C
Reaction mixture: 0.6 ml enzyme
0.2 ml substrate or water
0.2 ml cofactor or water or oxalic acid solution

Substrate	Cofactor				
	No cofactor	Oxalic acid (M/290)	B_6-al-Ph, clear*	B_6-al-Ph, suspension**	B_6-al-Ph, calcium free***
	Micromoles of NH_3/ml				
No substrate	10.5			10.0	9.0
0.2 M L-threonine	11.1	10.9	18.2	20.8 21.2	20.3 19.3
Minus blank	0.6	0.4	8.2	11.0	10.8
R			14	18	27

Where two values appear, they represent determinations made on independent incubations.

* A solution made by suspending 200 micrograms of B_6-al-Ph in M/10, pH 7.8 phosphate; bringing most of the suspension into solution by vibration, and filtering off a small insoluble residue. The solution used here had been kept for 2 weeks in the ice box.

** A freshly made suspension of 1 mg. of B_6-al-Ph per ml. of water.

*** Prepared as "B_6-al-Ph suspension," but using oxalic acid (M/290: twice the equivalents necessary to bind all the calcium) instead of water. It was then centrifuged, and the white precipitate of calcium oxalate was discarded.

Table XXVII shows that no greater activation of threonine deaminase is obtained if pyridoxal phosphate is freed of calcium by addition of oxalic acid, or in other words, that the solubility of the calcium salt is high enough to permit maximum activation of the system. The slightly lower activity of the "clear" solution might be due to age or to lower cofactor concentration.

The stability of the calcium salt in phosphate buffer (pH 7.8) appears here greater than that stated in the circular which Merck & Co. sends out with the samples of pyridoxal phosphate. According to them, pyridoxal phosphate (as the Ca salt free acid?) is stable in solution (at pH 6 to 7) for only one week at 0°, and for 24 hours at 25°.

The values obtained for R* are remarkably high. However, due to the magnitude of the errors which may affect the ammonia values corrected for blank readings in the B_6-less mixtures, R values half as large as the given ones are also compatible with the experimental results. Still, the activation achieved in this experiment appears higher than that reported on table XVII for a similar enzyme preparation under slightly different conditions.

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* We have defined R as the ratio of deaminase activity with added pyridoxal phosphate to activity without added pyridoxal phosphate.

B) TEST OF ACTIVATORS OF THREONINE DEAMINASE REPORTED IN THE LITERATURE

The results of testing on Neurospora threonine deaminase the effect of substances reported to activate the deamination of serine and threonine by enzyme systems derived from other organisms, are shown on table XXVIII.

No significant activation appears by addition of any of the presumed cofactors, either alone or in conjunction with pyridoxal phosphate; while this latter substance caused a five-fold increase in deamination.

Addition of zinc sulfate and of yeast extract results in considerable inhibition. When a solution of calcium pyridoxal phosphate (buffered at pH 7.8) is mixed with zinc sulfate, a white precipitate separates. Comparable solutions of calcium pyridoxal phosphate and sodium sulfate do not precipitate upon mixing. These observations suggest that the inhibition caused by zinc may be due to the ability of such ions to bind pyridoxal phosphate.

C) IDENTIFICATION OF THE PRODUCTS OF THE DEAMINATION OF SERINE AND THREONINE.

The same enzyme preparation described on table XXVIII, supplemented with pyridoxal phosphate, was incubated with serine and threonine respectively for 7 hours at 37° C. The proteins were precipitated with trichloracetic acid. Upon addition to each supernatant of a solution of

TABLE XXVIII

Test of possible activators of threonine deaminase

Enzyme: Neurospora extract, precipitated with 55% saturated ammonium sulfate, dialyzed for 20 hours against M/10 pH 7.8 phosphate (using five changes of 3 liter batches of buffer)

Reaction mixture: 0.9 ml enzyme
0.2 ml 0.2 M L-threonine or water
0.2 ml B_6al-Ph (40 micrograms) or water
0.2 ml other additions (freshly made solutions titrated to pH 7.8) or water

Incubation: 5 hours at 40°C.

Presumed activators added	B_6al-Ph added	Substrate		
		No substrate	Threonine	Minus blank
		Micromoles of NH_3/ml		
None	none	0.5	1.4	0.9
	B_6al-Ph	0.5	5.8	5.3
500 micrograms of muscle adenylic ac.*	none		2.7	1.2
	B_6al-Ph	1.5	5.7	4.2
50 micrograms of muscle adenylic ac.*	none		1.8	1.0
	B_6al-Ph	0.8	6.3	5.5
Ditto, plus 14 micrograms pyridoxal HCl	none		1.6	1.1
	B_6al-Ph	0.5	6.1	5.6
14 micrograms pyridoxal HCl	none		1.6	1.1
	B_6al-Ph	0.5	6.3	5.8
50 micrograms of muscle adenylic ac. plus 1 mg glutathione	none		1.9	1.2
	B_6al-Ph	0.7	6.5	5.8
1 mg glutathione**	none		1.9	1.1
	B_6al-Ph	0.8	6.7	5.9
50 micrograms muscle adenylic acid plus 2.5 micrograms biotin	none		1.5	0.8
	B_6al-Ph	0.7	6.1	5.4
2.5 micrograms biotin***	none		1.4	0.6
	B_6al-Ph	0.8	5.9	5.1
0.1 micrograms biotin***	none		1.4	0.7
	B_6al-Ph	0.7	6.2	5.5
10 micromoles $MgCl_2$****	none		1.9	1.2
	B_6al-Ph	0.7	6.4	5.7
10 micromoles $ZnSO_4$*****	none		1.0	0.1
	B_6al-Ph	0.9	2.6	1.7
1 mg yeast extract ******	none		1.0	0.0
	B_6al-Ph	1.0	2.2	1.2

TABLE XXVII - continued

* Adenosine-5-phosphoric acid, purchased from Sigma Chemical Company. Wood and Gunsalus (24) obtained half maximum activation of their threonine deaminase preparations by addition of 245 micrograms adenylic acid per ml. Lichstein and Christman (23) encountered activation upon addition of 50 micrograms per ml. Gale and Stevenson's preparations responded to 0.01 microgram of muscle adenylic acid per ml (21). The latter two preparations consisted of resting cells.

** A product of H. M. Chemical Company. Wood and Gunsalus (24) added 4 mg of glutathione per ml of reaction mixture in order to demonstrate activation. Gale and Stevenson (21) added only 1 microgram.

*** Lichstein and Christman's cellular deaminase preparations (23) were activated by 0.1 or 0.5 micrograms of biotin per ml.

**** Chargaff and Sprinson (22) reactivated their dialyzed deaminase preparations by adding $MgCl_2$ at a concentration of 5 micromoles per ml.

***** Binkley (20) fails to report the concentration of zinc ions which activated serine deaminase.

****** Lichstein and Christman's aspartic acid deaminase (26) (27) preparation responded to yeast extract (desiccated, from Difco Labs., the same kind used in our experiments) at concentrations ranging from $\frac{1}{2}$ microgram to 10 mg per ml.

2,4-dinitrophenyl hydrazine in 2 N HCl, precipitates formed. They were recrystallized once from ethyl acetate, and their melting points compared with those of synthetic samples of the 2,4-dinitrophenyl hydrazones of pyruvic acid and alpha-ketobutyric acid, respectively (Table XXIX).

Melting points reported throughout this thesis have been determined in a Hershberg apparatus. The samples were introduced at room temperature, heated to about 20° below the expected melting point in the course of approximately 15 minutes, and heated for the last 10° at the rate of 1° per minute. All melting points are corrected.

D) IDENTIFICATION OF ALPHA-KETOBUTYRIC ACID AS A PRODUCT OF THE TRANSAMINATION BETWEEN ALPHA-AMINOBUTYRIC ACID AND ALPHA-KETOGLUTARIC ACID.

Seventy five ml. of Neurospora extract, dialyzed overnight and supplemented with 3 mg of calcium pyridoxal phosphate, were incubated anaerobically for 7 hours at about 35° C with 6 grams of DL-alpha-aminobutyric acid and 6 ml of an aqueous solution containing 3 grams of neutralized alpha-ketoglutaric acid. Control incubations were carried out with comparable concentrations in 1.1 ml volumes of reaction mixture with each of the following substrates: alpha-aminobutyric acid, alpha-ketoglutaric acid, and no substrate (negative control).

The test mixture was acidified with 0.1 volumes of

TABLE XXIX

Identification of deamination products

2,4-dinitrophenyl hydrazone of	M.p. (°C)	Yield (in %) of theoretical, calculated assuming complete deamination
a) Product of serine deamination	212	7%
b) Mixture of a) and c)	212-213	
c) Pyruvic acid	212	
Reported for pyruvic acid	215-216(22) 216 (119)	
d) Product of threonine deamination	194-194.5	14%
e) Mixture of d) and f)	194-194.5	
f) Alpha-ketobutyric acid	194-194.5	
Reported for alpha-ketobutyric acid	195 (22) 198-199 (9)	

TABLE XXX

Manometric assay of alpha-ketobutyric in the transamination reaction mixture and in the controls

Substrate incubated with the transaminase preparation	Micromoles of CO_2 per ml. of solution
Alpha-ketoglutaric (238 micromoles/ml.)	2.3
Alpha-aminobutyric (347 micromoles/ml.)	1.7
Alpha-ketoglutaric (238 micromoles/ml.) plus Alpha-aminobutyric (347 micromoles/ml.)	31
No substrate	0.4

TABLE XXXI

Identification of transamination product

2,4-dinitrophenyl hydrazone of	M.p.(°C)
a) Product isolated from reaction mixture	192-193
b) Mixture of a) and c)	192-193
c) Alpha-ketobutyric acid	194

5.6% sulfuric acid, boiled, and the proteins were centrifuged off. The occurrence of transamination in the test mixture was corroborated by paper chromatographic analysis of amino acids: the glutamic acid spot appeared far stronger in the test than in the control mixtures. Assay of alpha-ketobutyric acid gave the results shown on table XXX. Carbon dioxide evolution in the controls might be due to alpha-ketobutyric or to other compounds which could be decarboxylated (e.g. pyruvate)

Further work was done to find out whether or not the CO_2 yielding compound produced in the test mixture is alpha-ketobutyric acid. The values from the manometric assay correspond to 13% (or 11%, if the readings in the controls are subtracted) of the theoretical yield, calculated assuming that each mole of alpha-ketoglutaric acid present gives rise to one mole of alpha-ketobutyric.

Extraction of the acidified test mixture with ether and further manipulation of this extract failed to yield any alpha-ketobutyric acid. Manometric assay of the extracted reaction mixture showed that the CO_2-yielding compound was still in it. The mixture was stored in the deep freezer for two weeks. It was then further acidified until a 10% content of sulfuric acid was reached and extracted overnight with ether by means of a continuous-extraction apparatus. The ether phase was washed with water, dried over sodium sulfate, and the ether was driven off. The resulting mixture was distilled under reduced pressure

in a small still designed by Dr. Norman Good. Fraction 1 was collected by heating the water bath to about 80°C. Fraction 2 was collected while the water bath was boiling. Fraction 2, cooled in ice water, solidified for the most part giving rise to flake-like crystals like those of alpha-ketobutyric acid. Each fraction added to 60 ml of ca. 0.6 % 2,4-dinitrophenyl hydrazine solution in 2N HCl, gave rise to a yellow precipitate. Chromatographic analysis revealed that in all probability the precipitate from fraction 1 consisted mainly of the dinitrophenyl hydrazone of propionaldehyde (propionaldehyde could be smelled in the reaction mixture), while the precipitate from fraction 2 gave rise to two spots which corresponded to those given by the dinitrophenyl hydrazones of propionaldehyde and alpha-ketobutyric acid.

The precipitate and mother liquor from fraction 2 were extracted with ether, and the ether extracted with 10% sodium carbonate. Upon acidification, a yellow precipitate was obtained from this solution. Filtered, washed and dried, it weighed 127 mg. This corresponds to ca. 1/5 of the theoretical yield, as calculated from the manometric alpha-ketobutyric assay. It behaved chromatographically as pure alpha-ketobutyric, and gave a melting point of 180-185° C. After recrystallization from ethyl acetate, and washing with water, its melting point was compared with that of a synthetic sample of alpha-ketobutyric dinitrophenyl hydrazone (Table XXXI).

E) ADDITIONAL LITERATURE REVIEW

After the completion of this thesis our attention was drawn to a paper in which Braunstein and Azarkh (120) suggest that serine dehydrase (deaminase) might be activated by pyridoxal phosphate, since: 1) vitamin B_6 antagonizes the inhibitory properties of DL- and D-serine towards rats, and 2) vitamin B_6 is implicated in the enzymatic breakdown of cysteine and tryptophane, reactions which are thought to take place along a path similar to that of serine deamination. A relation between cysteine deaminase activity and level of vitamin B_6 in the diet of rats is established in that paper. Such a relation is further elucidated by Kallio (121), who was able to demonstrate activation of cysteine desulfhydrase and homocysteine desulfhydrase from bacteria by in vitro addition of pyridoxal phosphate.

The participation of pyridoxal phosphate in the enzymatic transfer of sulfur from homocysteine to serine (presumably via cystathionine) to yield cysteine, has been demonstrated by Braunstein and Goriachenkova (122). One cannot decide from their experiments whether pyridoxal phosphate activates the synthesis, or the breakdown of cystathionine, or both.

REFERENCES IN THE APPENDIX

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