[CONTRIBUTION FROM THE LABORATORY OF BIOCHEMISTRY, NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH]

Studies on the Relationship between the Enzymatic Synthesis of Glutamine and the Glutamyl Transfer Reaction[1]

BY LEON LEVINTOW, ALTON MEISTER, GEORGE H. HOGEBOOM AND EDWARD L. KUFF

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A purified preparation from peas that catalyzes glutamine synthesis and the glutamyl transfer reaction has been studied in the ultracentrifuge. Both enzymatic activities have been shown to be associated with the same, monodisperse component ($s_{20,w}$ = 13.6–14.0 × 10^{-13}). This observation supports the concept that a single enzyme catalyzes both reactions. When the glutamyl transfer reaction was carried out in the presence of labelled L-glutamate, only a small fraction of the radioactivity appeared in the γ-glutamylhydroxamate produced in the reaction. This finding has been interpreted as rendering unlikely the concept that the transfer reaction proceeds *via* complete reversal of glutamine synthesis, but consistent with the concept that the same or a similar intermediate is formed in both reactions. The relationship between the two reactions is considered in the light of these findings.

Introduction

Enzyme preparations that catalyze the synthesis of glutamine

$$\text{glutamate} + \text{ATP}^{2} + \text{ammonia} \rightleftharpoons \text{glutamine} + \text{ADP} + \text{orthophosphate} \quad (1)$$

also catalyze a glutamyl transfer reaction which requires catalytic amounts of ADP and phosphate or arsenate

$$\text{glutamine} + \text{hydroxylamine} \longrightarrow \gamma\text{-glutamylhydroxamate} + \text{ammonia} \quad (2)^{3,4}$$

Inasmuch as (a) reaction (1) is reversible and (b) hydroxylamine can replace ammonia in reaction (1), it is possible to visualize reaction (2) as the reversal of reaction (1) followed by the synthesis of γ-glutamylhydroxamate from hydroxylamine and the ATP and glutamate thus formed, and regeneration of ADP and orthophosphate for another cycle. This hypothesis has now been tested by carrying out reaction (2) in the presence of L-glutamate 2-C^{14}. The results indicate that only a small fraction of the γ-glutamylhydroxamate formed in reaction (2) arises *via* a complete reversal of reaction (1), and that glutamate therefore is not an obligatory intermediate in reaction (2).

The concept that reactions (1) and (2) are catalyzed by the same enzyme has rested chiefly on the parallel concentration of the two activities during the course of enzyme fractionation.[3,4] Further evidence on this point has now been obtained by study of a purified pea enzyme in the ultracentrifuge. The data from enzymatic assay of fractions obtained by tube section and direct sampling techniques indicate that both enzymatic activities are associated with the same monodisperse component.

All known enzyme preparations that catalyze glutamine synthesis exhibit glutamyl transfer activity as well. The present experiments indicate that the converse is also true; preparations which catalyze the transfer reaction in the presence of ADP and orthophosphate also catalyze glutamine synthesis. The relative rates of the two reactions under standard test conditions vary from about 1:1 to 1:4.5 with enzymes from different sources. The glutamyl transfer reaction considered in this paper differs from another type of glutamyl transfer reaction, which requires neither ADP nor phosphate.[4]

Experimental

Material and Methods.—L-Glutamate and L-glutamine were prepared as previously described.[5] We are indebted to Dr. Carl G. Baker for L-glutamate-2-C^{14}, prepared by enzymatic resolution[6] of the racemate. L-Pyrrolidone carboxylate was obtained from L-glutamic acid-γ-ethyl ester according to Bergmann and Zervas.[7] Disodium ATP and sodium ADP were obtained from Pabst Laboratories.

dl-β-Methyl-DL-glutamic acid (2-amino-3-methylglutaric acid),[8] *dl*-γ-methyl-DL-glutamic acid (2-amino-4-methylglutaric acid),[9] *dl*-β-methyl-DL-glutamine (2-amino-3-methylglutaramic acid),[8] *dl*-γ-methyl-L-glutamine (2-amino-4-methylglutaramic acid)[9] and α-methyl-DL-glutamine (2-amino-2-methylglutaramic acid)[9] were prepared as described. The authors thank Dr. Karl Pfister of Merck and Company for α-methyl-DL-glutamic acid (2-amino-2-methylglutaric acid), β-hydroxy-DL-glutamic acid, and *allo*-β-hydroxy-DL-glutamic acid. γ-Methyleneglutamic acid and γ-methyleneglutamine were samples isolated from the peanut plant[10] and the tulip bulb,[11] and generously provided by Dr. L. Fowden and Dr. F. C. Steward. These two compounds are tentatively considered to be of the DL and L configurations, respectively.

The purified pea enzyme was prepared by Elliott's procedure,[3] modified as previously described.[5] A preparation at Elliott's stage 6, which was entirely stable under experimental conditions, was employed for the ultracentrifugal studies. Dr. P. K. Stumpf kindly supplied an acetone powder of pumpkin seedlings, from which purified enzyme was prepared by the procedure of Stumpf, *et al.*[12] The sheep brain enzyme was prepared by the method of Elliott.[13] Acetone powders of pigeon liver, rat liver and human liver were extracted with 10 volumes of cold 0.05 *M* potassium bicarbonate, clarified by centrifugation, and dialyzed for 6 hours against 0.05 *M* potassium bicarbonate.

Initial attempts to resolve mixtures of glutamate, glutamine and γ-glutamylhydroxamate for determination of the radioactivity of each compound were unsuccessful because of the instability of γ-glutamylhydroxamate. Considerable cyclization of γ-glutamylhydroxamate to pyrrolidone carboxylate occurred during paper chromatography of the reac-

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(2) Abbreviations employed: ATP, adenosine triphosphate; ADP, adenosine diphosphate; ATPase, adenosine triphosphatase.

(3) W. H. Elliott, *J. Biol. Chem.*, **201**, 661 (1953).

(4) H. Waelsch, in W. D. McElroy and B. D. Glass, "Phosphorus Metabolism," Vol. 2, Williams and Wilkins, Baltimore, Md., 1952, p. 109.

(5) L. Levintow and A. Meister, *J. Biol. Chem.*, **209**, 265 (1954).

(6) L. Levintow and J. P. Greenstein, *Arch. Biochem. Biophys.*, **31**, 77 (1951).

(7) M. Bergmann and L. Zervas, *Z. physiol. Chem.*, **221**, 51 (1933).

(8) A. Meister, L. Levintow, R. E. Greenfield and P. A. Abendschein, *J. Biol. Chem.*, **215**, 441 (1955).

(9) A. Meister, *ibid.*, **210**, 17 (1954).

(10) J. Done and L. Fowden, *Biochem. J.*, **51**, 451 (1952).

(11) R. M. Zacharias, J. K. Pollard and F. C. Steward, THIS JOURNAL, **76**, 1961 (1954).

(12) P. K. Stumpf, W. D. Loomis and C. Michelson, *Arch. Biochem.*, **30**, 126 (1951).

(13) W. H. Elliott, *Biochem. J.* **49**, 106 (1951).

tion mixtures. Accordingly, the instability of γ-glutamylhydroxamate was utilized to separate it from glutamate and glutamine. Reaction mixtures were heated in a water-bath at *p*H 7.0 and 100° for 7.5 minutes. Under these conditions, more than 95% of the γ-glutamylhydroxamate was converted to pyrrolidone carboxylate. Less than 10% of the glutamine was cyclized under these conditions, and the glutamate was completely stable. Paper chromatograms of reaction mixtures thus treated were developed in formic acid–water–*t*-butyl alcohol as previously described.[5] This procedure resolved glutamate, glutamine and the pyrrolidone carboxylate derived from γ-glutamylhydroxamate. The compounds were located by radioautography, the ninhydrin spot test and by a modification of the procedure of Rydon and Smith[8,14]; they were eluted from the paper with water and directly plated. Radioactivity was measured with a gas-flow Geiger counter. In some instances paper discs were counted directly; in these cases appropriate corrections for self-absorption were applied.

The validity of the separation technique was tested with a reaction mixture in which γ-glutamylhydroxamate was synthesized from ATP, hydroxylamine and L-glutamate-2-C^{14} (Table I). The specific activity of the hydroxamate, measured as pyrrolidone carboxylate, was the same, within experimental error, as that of the glutamate.

Details of the techniques of ultracentrifugation in a horizontal preparative rotor and of the sampling of the sedimenting materials have been described.[15] The concentration of protein was estimated by absorption measurements at 280 mμ. γ-Glutamylhydroxamate was determined by the method of Lipmann and Tuttle,[16] using a standard solution of the authentic compound.[5]

TABLE I

ENZYMATIC SYNTHESIS OF γ-GLUTAMYLHYDROXAMATE FROM L-GLUTAMATE-2-C^{14}

The reaction mixture contained 10 μmoles of magnesium chloride, 4.0 μmoles of L-glutamate-2-C^{14}, 4 μmoles of ATP, 20 μmoles of neutralized hydroxylamine hydrochloride, 5 μmoles of β-mercaptoethanol, 10 μmoles of imidazole buffer *p*H 7.0, and purified pea enzyme (25 γ N) in a volume of 0.14 ml. The mixture was incubated at 37° for 30 minutes.

	Enzyme omitted			Complete system		
	μmoles	C.p.m.	C.p.m./μmole	μmoles	C.p.m.	C.p.m./μmole
L-Glutamate	4.00	38,500	9620	1.50	15,200	10133
L-γ-Glutamylhydroxamate	0	0	0	2.50	24,100	9640

Assays of enzymatic activity were carried out by measuring the initial rates of γ-glutamylhydroxamate formation at 37° in the following incubation mixtures. The conditions used for reaction (1) were as follows: 50 μmoles of magnesium chloride, 50 μmoles of monosodium glutamate, 10 μmoles of ATP, 100 μmoles of neutralized hydroxylamine hydrochloride, 25 μmoles of β-mercaptoethanol, 50 μmoles of imidazole buffer *p*H 7.0, and enzyme in a final volume of 1.0 ml. Blanks consisting of the complete mixture except for ATP were subtracted. The conditions used for reaction (2) were as follows: 5 μmoles of manganous chloride, 50 μmoles of L-glutamine, 0.1 μmoles of ADP, 20 μmoles of potassium phosphate buffer *p*H 7.0, 100 μmoles of neutralized hydroxylamine hydrochloride, and enzyme in a final volume of 1.0 ml. Blanks consisting of the complete system except for glutamine were subtracted. ATPase[17] and glutamine arsenolysis activity[5] were determined as described.

Results

Experiments with Labeled Glutamate.—When the glutamyl transfer reaction (2) was carried out in the presence of labeled glutamate, only a small fraction of the radioactivity was incorporated into the γ-glutamylhydroxamate (Table II). This finding is not consistent with the concept that the transfer reaction proceeds primarily *via* reversal of glutamine synthesis with glutamate as an intermediate. The data indicate that free L-glutamate as such participates in the formation of at most only a small part of the γ-glutamylhydroxamate formed.

TABLE II

GLUTAMYL TRANSFER REACTION IN THE PRESENCE OF L-GLUTAMATE-2-C^{14}

The reaction mixture contained 2 μmoles of manganous chloride, 3.25 μmoles of L-glutamate-2-C^{14}, 10 μmoles of L-glutamine, 0.2 μmoles of ADP, 10 μmoles of potassium phosphate buffer *p*H 7.0, 20 μmoles of neutralized hydroxylamine hydrochloride, and purified pea enzyme (50 γ N) in a volume of 0.18 ml. The mixture was incubated 30 minutes at 37°.

	Enzyme omitted			Complete system		
	μmoles	C.p.m.	C.p.m./μmole	μmoles	C.p.m.	C.p.m./μmole
L-Glutamate	3.25	77,100	23,700	3.25	76,000	23,400
L-Glutamine	10.0	0	0	4.60	330	72
L-γ-Glutamylhydroxamate	0	0	0	5.40	830	154

Ultracentrifugal Studies.—In view of the evidence suggesting that the purified enzyme catalyzes two distinct reactions, ultracentrifugal studies were carried out, using the optical method and enzymatic assays as analytical tools. Examination of an 0.8% solution of a preparation (Elliott's stage 6[3]) in the optical ultracentrifuge (Fig. 1) demonstrated the presence of several components. The most rapidly sedimenting component ($s_{20,w} = 13.9 \times 10^{-13}$) appeared as a small, symmetrical refractive index peak and was found, by planimetry and correction to zero time,[18] to comprise approximately 20% of the total refractive index gradient. The remainder of the material in the preparation appeared as a broad, slowly sedimenting peak (average $s_{20,w} = 4.1 \times 10^{-13}$), the shape and width of which indicated polydispersity.

Two types of experiments were carried out in order to determine the relationship of the refractive index peaks in Fig. 1 to the enzymatic activities of the preparation. First, the material studied in the optical ultracentrifuge was diluted 10-fold, and a 4.53-ml. aliquot was centrifuged at 24° for 97 minutes at 39,460 r.p.m. in the Spinco swinging bucket rotor (SW-39) as described previously.[15] Stabilization of the fluid column against convective disturbance was effected through the use of a linear concentration gradient of sucrose ranging from zero at the top of the tube to 1.68% at the bottom.[15,19] The solvent was also 0.15 *M* with respect to sodium chloride. After centrifugation, the Lusteroid tube was sectioned near its midpoint. Protein concentration and enzymatic activity were determined on the material remaining above the plane of section, and the values compared with those of the original solution. The upper half of the fluid column contained 37.2% of the total glutamine synthesis activity, 39.0% of the total glutamyltransferase activity, and 70.7% of the total protein. Knowing the radial distance from the axis of rotation to the meniscus (6.08 cm.) and

(14) H. N. Rydon and P. W. G. Smith, *Nature*, **169**, 922 (1952).

(15) G. H. Hogeboom and E. L. Kuff, *J. Biol. Chem.*, **210**, 733 (1954).

(16) F. Lipmann and L. C. Tuttle, *ibid.*, **159**, 21 (1945).

(17) L. Levintow, *J. Natl. Cancer Inst.*, **15**, 347 (1954).

(18) T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Oxford University Press, Oxford, 1940.

(19) A method for the reproducible production of a linear sucrose concentration gradient from the top to the bottom of the fluid column will be described (G. H. Hogeboom and E. L. Kuff, to be published).

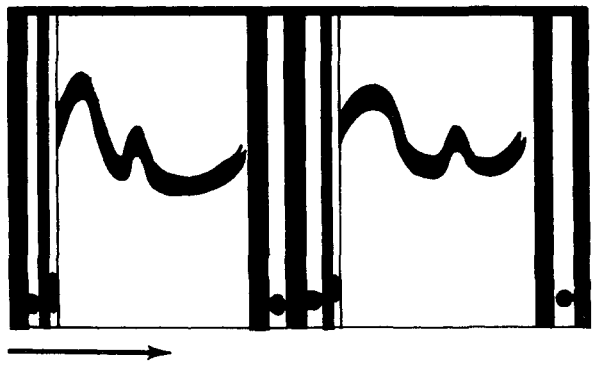

Fig. 1.—Sedimentation pattern of a purified enzyme preparation from peas which catalyzes the synthesis of glutamine. Total protein concentration, 0.8%; solvent, 0.15 *M* NaCl; speed, 50,740 r.p.m. (Spinco Model E ultracentrifuge, Type A analytical rotor); photographs of refractive index patterns taken at 39 and 55 minutes after attainment of speed; rotor temperature, 24°; bar angles, 30 and 28°; magnification, 2 diameters. Sedimentation constants, after correction to water at 20° (1), were 13.9×10^{-13} for the symmetrical, rapidly-sedimenting peak and 4.1×10^{-13} for the broad, slowly-sedimenting peak.

to the plane of section (7.82 cm.), it was possible to calculate sedimentation constants according to an equation derived previously.[15] After viscosity and density corrections to water at 20°, the sedimentation constants obtained for the two enzyme activities (Table III) were in agreement within the experimental error of the assay procedures and corresponded closely to that of the rapidly sedimenting component of Fig. 1. The average sedimentation constant for the total protein was much lower than the constants for the enzyme activities. These findings are consistent with the view that no enzyme activity was associated with the slowly-sedimenting material in the preparation.

TABLE III

SEDIMENTATION CONSTANTS OF ENZYME ACTIVITIES DETERMINED BY TUBE-SECTIONING AND SAMPLING TECHNIQUES

Expt.		Total protein concn., %	Sedimentation constant ($s_{20,w} \times 10^{13}$): γ-Glutamylhydroxamate synthesis	Glutamyltransferase	Total protein
Tube-sectioning		0.08	14.0	13.6	6.3
Sampling	a	.08	14.0	14.0	
	b	.08	13.6[a]	13.6[a]	

[a] Experiment shown in Fig. 2.

As shown previously,[15] the tube-sectioning method necessarily gives an *average* sedimentation constant and is thus suitable only for the study of monodisperse compounds. Since in at least one instance a soluble enzyme was found to be polydisperse,[20] a second type of experiment, utilizing a direct sampling technique, was carried out.

In two experiments, 4.53-ml. aliquots of the diluted enzyme preparation were centrifuged for 126 minutes under exactly the same conditions as in the tube-sectioning experiment. The fluid column was then sampled[15] at successive levels, and enzyme assays were carried out on the fractions obtained. The activity of each sample, expressed as a fraction of the original activity (A_F of Fig. 2) was plotted against the radial distance from the meniscus to the midpoint of the sample, and a smooth curve drawn between the points. It can be seen from the data obtained in one of the experiments (Fig. 2) that the position of the sedimentation boundary for the two enzyme activities was identical within the limits of error of the enzyme assays. The exact position of the centrifugal limb of the sedimentation boundary was somewhat uncertain, however, because of imperfect agreement between the determinations of enzyme activity. Since the assay of glutamyltransferase activity is probably more precise, the curve in this region was weighted slightly in favor of the result of these determinations. Translation of the sedimentation boundary into a concentration increment *vs.* distance curve[15] (Fig. 2) yielded a sharp peak, typical of a monodisperse substance. The slight skewing of the peak was probably the result of experimental error in the enzyme assays.

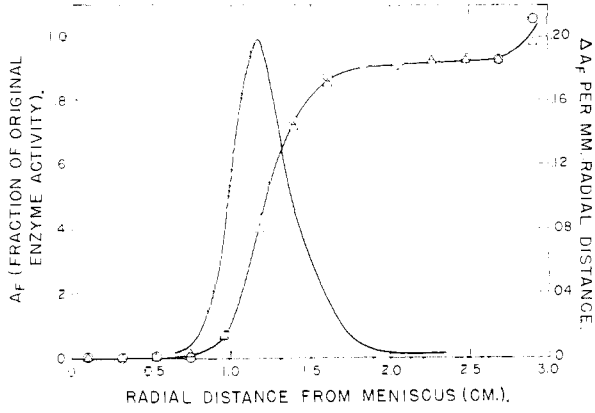

Fig. 2.—Sedimentation diagram and increment curve obtained in a sampling experiment with a purified enzyme preparation from peas. The sedimentation diagram, showing the points determined experimentally, is plotted against the left-hand ordinate and the increment curve (no points) against the right-hand ordinate: Δ, glutamine synthesis activity; O, glutamyltransferase activity. See text for experimental details.

Sedimentation constants were obtained from the two sampling experiments (Table III) after measuring graphically the distance from the meniscus to the point of maximum concentration increment (*i.e.*, the apex of the concentration increment *vs.* distance curve).[15] These values, which are included in Table III, are in agreement with those obtained in the tube-sectioning experiment and again correspond to that of the rapidly-sedimenting component observed in the optical ultracentrifuge (Fig. 1).

Studies with Various Enzymes.—The foregoing data provide strong evidence that, in pea preparations at least, the same enzyme catalyzes reactions (1) and (2). Aspects of the relationship in other tissues were studied by comparison of rates of the two reactions under standard test conditions (Table IV). The relative ratios of the activities

(20) E. L. Kuff, G. H. Hogeboom and M. J. Striebich, *J. Biol. Chem.*, **212**, 439 (1955).

exhibited considerable variation, which cannot be ascribed entirely to the presence of ATPase in some of the preparations. The ratios rather appeared to be characteristic of the particular tissue. Of all the tissues studied, the relative glutamyltransferase activity of pumpkin seedlings was highest. This is the tissue from which glutamyltransferase was originally purified[12]; the relatively low activity of the synthesis system in both the crude and purified preparations probably accounts for the fact that this activity has not previously been noted. It is of interest that there was a similar ratio between the rate of arsenolysis of glutamine and the rate of glutamine synthesis in both the purified pea and pumpkin preparations.

TABLE IV

RELATIVE RATES OF ENZYMATIC REACTIONS IN VARIOUS PREPARATIONS

Source of prepn.	γ-Glutamylhydroxamate synthesis	Glutamyltransferase	ATPase	Glutamine arsenolysis
Pea[a]	100	92	0	68
Pigeon liver[b]	100	110	42	
Sheep brain[a]	100	200	6	
Rat liver[b]	100	230	140	
Human liver[b,c]	100	230	1400	
Pumpkin[b]	100	450	360	
Pumpkin[a]	100	450	32	69

[a] Purified. [b] Crude. [c] Prepared from a specimen obtained 6 hours after death of a patient with carcinoma of the breast. The rate of γ-glutamylhydroxamate synthesis *of each preparation* is arbitrarily given a value of 100.

Studies with Substituted Glutamate and Glutamine Derivatives.—A summary of the relative rates of synthesis and transfer with several substituted glutamate and glutamine derivatives is given in Table V. All of the analogs of glutamate which were tested as substrates for the purified pea enzyme were active in synthesis with ammonia and hydroxylamine. The corresponding glutamine derivatives formed were identified by paper chromatographic procedures. Amide synthesis proceeded at about the same rate or more slowly than did synthesis of the corresponding hydroxamate. This observation lends support to the suggestion[21] that activation of the amino acid substrate may precede reaction with ammonia or hydroxylamine, and that reaction of the activated complex with ammonia may become the rate-limiting step. Alternatively, the higher rates of synthesis with hydroxylamine may reflect a greater reactivity of this base as compared to ammonia.[22]

The activity of α-methylglutamate has previously been reported with enzyme preparations from pigeon liver[23] and sheep brain.[24] Although γ-methyleneglutamate and its amide occur in plants,[10,11] synthesis of the latter from the former compound was one of the slowest reactions in this series. It is of interest that *allo*-β-hydroxy-DL-glutamate was considerably more active than was β-hydroxy-DL-glutamate. *allo*-β-Hydroxyglutamate is likewise decarboxylated 3 to 10 times faster than β-hydroxyglutamate by preparations of *E. coli*.[25]

TABLE V

RELATIVE RATES OF TRANSFER AND SYNTHESIS REACTIONS WITH SUBSTITUTED GLUTAMINE AHD GLUTAMATE DERIVATIVES AND THE PURIFIED PEA ENZYME[a]

Substituent	Synthesis: Amide	Synthesis: Hydroxamate	Transfer reaction
None	100	100	100
α-Methyl	85	84	50
β-Methyl	1	28	0
γ-Methyl	22	33	0
γ-Methylene	3	5	2
β-Hydroxy	6	8	
allo-β-Hydroxy	16	80	

[a] The rates with glutamate and glutamine are arbitrarily given a value of 100.

The only glutamine derivative which was appreciably active in the glutamyl transfer reaction was α-methylglutamine. The relatively narrow specificity of this reaction is in keeping with previously reported findings that D-glutamine and L-homoglutamine were not significantly active.[21]

Discussion

The fact that both glutamine synthesis and glutamyltransferase activities sediment together in the ultracentrifuge in association with a single, monodisperse component is consistent with the previous suggestion that these reactions are catalyzed by the same enzyme.[3,4] Moreover, no convincing evidence exists to the contrary. No enzyme preparation from any source is known which catalyzes only one of these reactions. It is a matter of some interest, however, that the relative rates of the two reactions vary considerably with enzymes from different tissues. This circumstance may be a reflection of different affinities, with enzymes from different sources, for the respective substrates of the synthesis and transfer reactions. The ratios of the hydrolysis and transfer reactions catalyzed by papain and trypsin with the same substrates have likewise been shown to be different.[26] The different ratios between amide synthesis and transfer given in Table V may also be explained by postulating different enzyme-substrate affinities.

The results of the present experiments with

(21) L. Levintow and A. Meister, THIS JOURNAL, **75**, 3039 (1953).

(22) Both isomers of glutamate were active substrates for this enzyme when Mg^{++}, Mn^{++} or Co^{++} was employed to fulfill the cation requirement. $CoCl_2$ at its optimal concentration range (0.010 to 0.025 *M*) was only one-eighth as effective an activator as was $MgCl_2$ (0.050 *M*). The concentration of cation had some effect on the relative activity of the isomers; thus with 0.010 *M* Co^{++}, the rate of synthesis of L-γ-glutamylhydroxamate was 3 times that of the D-isomer, but with 0.025 *M* Co^{++}, the rates of synthesis of the two isomers were identical. An enzyme from lupine seeds has been reported to be specific for L-glutamate when the system is activated with Co^{++} (G. Denes, *Biochim. et Biophys. Acta*, **15**, 296 (1954)). Co^{++} can also activate the transfer reaction; in this reaction it is likewise less effective than either Mn^{++} or Mg^{++}. We recently also have had an opportunity to study the specificity of the nucleotide requirement of the synthesis system using the enzyme from peas. Inosine triphosphate, cytidine triphosphate and uridine triphosphate were active in replacing ATP, although the rates of reaction were much slower. We thank Dr. Manuel F. Morales and Dr. W. Wayne Kielley for generously providing pure samples of these nucleotides.

(23) B. M. Braganca, J. H. Quastel and R. Schucher, *Arch. Biochem. Biophys.*, **41**, 478 (1952).

(24) N. Lichtenstein, H. E. Ross and P. P. Cohen, *J. Biol. Chem.*, **201**, 117 (1953).

(25) W. W. Umbreit and P. Heneage, *ibid.*, **201**, 15 (1953).

(26) J. Durell and J. S. Fruton, *ibid.*, **207**, 487 (1954).

labeled L-glutamate render improbable the concept that the transfer reaction is associated with complete reversal of glutamine synthesis. Rather it appears more reasonable to assume the formation of an intermediate compound common to both reactions, although at this time the nature of such an intermediate is not clear.

That such an intermediate is bound to enzyme may be suspected on the basis of failure to detect a free intermediate or demonstrate a half-reaction, but this is at best only negative evidence. Likewise, caution should be exercised in drawing conclusions as to the nature of postulated intermediates on the basis of radioactivity exchange experiments, particularly since such experiments have not been carried out with homogeneous enzyme preparations.[5,27,28] The possibility that such exchange reactions are due to the presence of contaminating enzymes or substrates must be considered in the interpretation of these data. Moreover, a tenable scheme for the mechanism of glutamine synthesis must be consistent with the experimental finding that purified enzyme preparations require both phosphate and ADP for the glutamyl transfer reaction. It may be concluded that the evidence presently at hand does not permit final conclusions as to the sequence of reactions and the nature of the intermediates involved.

(27) G. C. Webster and J. E. Varner, THIS JOURNAL, **76**, 633 (1954).
(28) M. Staehelin and F. Leuthardt, *Helv. Chim. Acta*, **38**, 184 (1955).

BETHESDA, MD.

[CONTRIBUTION FROM THE DIVISION OF LABORATORIES AND RESEARCH, NEW YORK STATE DEPARTMENT OF HEALTH]

Peptomyosin B

BY JAQUES BOURDILLON

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A second protein called peptomyosin B has been isolated from beef skeletal muscle digested with pepsin. It crystallizes at *p*H 6.5 in the presence of metal-binding substances such as pyrophosphate. It is very stable, particularly in acid medium. It is homogeneous in the ultracentrifuge and has a sedimentation constant of $s_{20} = 2.31 \times 10^{-13}$ at infinite dilution. The viscosity increment is $\nu = 169$, hence the axial ratio (assuming an elongated ellipsoid) 49:1, and the frictional ratio $f_e/f_0 = 2.91$. Calculated from these data, the molecular weight (assuming a partial specific volume of 0.74 and no hydration) is 117,000, the length of the molecule, 87 mμ, the width, 1.8 mμ. From osmotic pressure measurements, the molecular weight is 157,000 which, combined with the sedimentation constant, gives $f_e/f_0 = 3.35$, axial ratio 66:1, length of the molecule 101 mμ, width 1.5 mμ.

Several years ago, the author[1] isolated from peptic digests of beef skeletal muscle a crystalline protein which was called peptomyosin. The present paper describes the properties of another protein, called peptomyosin B, obtained from the same digests.

Isolation.—Five hundred grams of chopped beef muscle was stirred with 1 liter of water, approximately 15 ml. of concentrated HCl (to lower the *p*H to 2.5), and 1 g. of commercial pepsin. The suspension was placed overnight at 37°, then filtered at room temperature through fluted paper. The filtration took 24 to 48 hours, at the end of which about 600 ml. of filtrate was obtained. An alternate method consisted of straining the digest through cheese cloth, shaking it for one minute with 1/4 volume ether, centrifuging and collecting the clear under layer. The fluid was neutralized to *p*H 6.5 with *N* NaOH, at which point a voluminous white precipitate formed which was entirely composed of very thin, hair-like needles. It was collected and dissolved in 100 ml. of *N* NaCl, followed by 25 ml. of an 0.1 *M* solution of Na pyrophosphate adjusted to *p*H 6.5 with HCl. Upon slow addition of 1 liter of water, the protein precipitated in crystalline form.[2] Such crystal suspensions have been kept for months in the refrigerator in the presence of thymol and "Merthiolate" without spoilage. They could also be washed with water and dried with acetone without denaturation. The yield was about 1% of the wet weight of muscle.

General Properties.—The elementary analysis gave the following percentages: C, 46.1; N, 15.9; P, 0.05; carbohydrate (carbazole), less than 0.5; ash, 0.24.

(1) J. Bourdillon, *Arch. Biochem.*, **16**, 61 (1948).
(2) This material may be more accurately referred to as regularly needle-shaped and strongly birefringent since no crystal faces were seen. The term "crystalline" is used for convenience only. Precipitation in this form occurred so suddenly (when the salt molarity was about 0.2) that it did not lend itself to further purification of the protein. This very fact might suggest significant homogeneity.

An outstanding feature of the protein was its readiness to crystallize from a neutral solution upon removal of salt, even in the presence of considerable amounts of extraneous material, provided there was added any one of a large number of substances (cyanide, oxalate, citrate, Versene, ATP, some amino acids, some proteins) whose common property appeared to be that of forming with metals insoluble or poorly ionized complexes. The most effective agent was pyrophosphate. The phenomenon was illustrated as follows. In a series of small tubes was placed 0.3 ml. of an 0.5% solution of washed crystals dissolved in *N* NaCl. To this was added slowly, at the rate of one drop every 2 to 3 seconds, 2.7 ml. of Na pyrophosphate (adjusted to *p*H 6.5) in decreasing 3-fold serial dilutions. In the first tube, the pyrophosphate concentration, 0.025 *M*, was so high that it prevented precipitation. Optimal conditions for crystallization were found in the next four tubes (pyrophosphate molarity of 10^{-2} to 10^{-3}). The seventh tube (pyrophosphate molarity of 0.3×10^{-4}) still showed a slight sheen, indicative of partial crystallization. In the ninth tube (no pyrophosphate), the precipitate was entirely amorphous. The contrast between the milky white, shiny crystalline precipitate obtained with pyrophosphate, and the gelatinous, translucent masses obtained without, was quite striking. This was due in part to the much larger volume of the crystalline material, which, after thoroughly pressing between sheets of filter paper, still contained 49% water. Insoluble at *p*H 6.5 in the absence of salt, the crystals dissolved above *p*H 7.5 or