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).

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Peptomyosin B

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RECEIVED NOVEMBER 10, 1954

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

below *p*H 4. When the *p*H was brought back to 6.5, recrystallization was immediate in the presence of pyrophosphate; in its absence, only an unmanageable gelatinous mass resulted.[3]

No evidence was found for the formation of compounds between the substances added and peptomyosin B, and recrystallization of washed crystals was never successful without the renewed addition of an active substance. This agrees with the tentative explanation that the substances added acted by immobilizing traces of an interfering agent—probably a heavy metal—coming predominantly from the NaCl used for solution, rather than by a direct effect on the protein.

A study of the effect of various ions on the solubility and stability of peptomyosin B yielded little significant information. At *p*H 6.5, the lowest salt molarity necessary to dissolve the protein crystals was about 0.05 with Na pyrophosphate or K ferro- and ferricyanide, 0.2 with most monodivalent salts, 0.5–0.8 with mono-monovalent salts. An unusual phenomenon was the higher solubility of the protein in the cold: at *p*H 6.5, a 1% protein solution was soluble in 0.6 *N* NaCl at 0°; upon warming to room temperature, an amorphous precipitate formed in a few minutes. This effect was reversible.

The *p*H of minimum solubility changed with the salt concentration, a fact also observed with myosin and actomyosin.[4] A rough test showed that, for NaCl molarity of 2.5, 1.0, 0.1 and 0, the *p*H range of minimum solubility was, respectively, 1.7–2, 2.0–4.5, 5.0–5.5 and 5–6. In the absence of salt, gelling occurred readily, even at room temperature, upon dialysis of an alkaline solution against water.

The protein yielded gelatinous precipitates with most protein precipitants. It was irreversibly precipitated by boiling in *N* NaCl at *p*H 6.5, but withstood boiling in 0.1 *N* HCl for several minutes without losing its ability to crystallize.

The crystals were strongly birefringent. Furthermore, double refraction of flow was easily shown when a 1% solution, squirted from a capillary pipet into a full beaker, was watched between crossed polaroid plates. The intensity of the phenomenon did not seem to be affected by the *p*H.

Viscosity.[5]—Viscosity measurements were made in 5-ml. Ostwald viscometers. The data were used as shown in Fig. 1 to estimate the intrinsic viscosity $[\eta]$. With the help of the expression $[\eta](100/\bar{v}) = \nu$, in which $\bar{v}$ is the partial specific volume (assumed to be 0.74) and ν Simha's[6] factor, one obtains $\nu = 169$. According to a table[7] this applies to elongated ellipsoids having an axial ratio of 48.7:1 and hence[8] a frictional coefficient $f_e/f_0 = 2.91$.

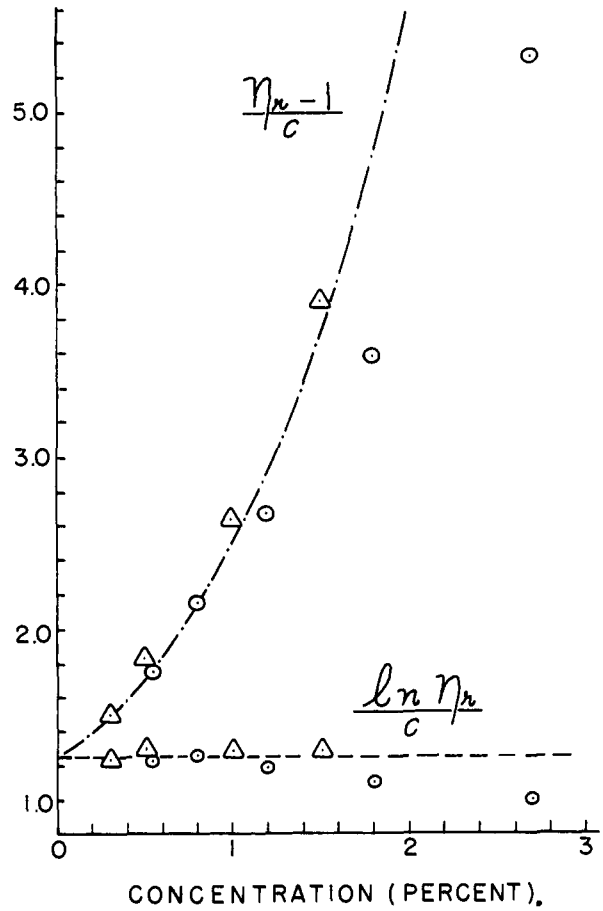

Fig. 1.—Viscosity of peptomyosin B: triangles, salt solution, 1 *M* in NaCl, 0.04 *M* in Na_2HPO_4, *p*H 7.3, temperature 30.1°; circles, 0.05 *N* HCl, *p*H 1.9, temperature 29.9°. The lower set of points represents experimental values of $(\ln \eta_r)/c$; the upper set represents the corresponding values of $(\eta_r - 1)/c$ (where η_r is the ratio of the viscosity of the solution to that of the solvent and c is the concentration of the protein in per cent.). The lower line shows the fit of a straight line to the former data, the upper line the corresponding fit of a curve to the latter data. The intrinsic viscosity $[\eta]$, defined as the limit of $(\ln \eta_r)/c$ as c approaches zero, is read from the fitted line where it intersects the ordinate axis; in this case $[\eta] = 1.25$ approximately.

Sedimentation.—The sedimentation rate was measured[9] in the Spinco centrifuge with the same solutions used for the first set of viscosity determinations. The boundaries were single and remarkably sharp, even at low concentration. The results, obtained at 25.5 to 28.4° and corrected to 20°, are shown in Fig. 2. From 0.1 to 1.0% concentration, the sedimentation rate was directly proportional to concentration, and yielded by extrapolation $s_{20} = 2.31 \times 10^{-13}$ at infinite dilution. Plotting $1/s$ *versus* concentration gave $s_{20} = 2.38 \times 10^{-13}$.

(3) Part of this work was done at the Biochemical Research Laboratory of the Massachusetts General Hospital, Boston. The author wishes to thank Professor F. Lipmann for his kind hospitality.

(4) A. Szent-Györgyi, "Chemistry of Muscular Contraction," 2nd ed., Academic Press, Inc., New York, N. Y., 1951, 162 pp.

(5) We are indebted to Dr. W. R. Thompson for mathematical assistance.

(6) R. Simha, *J. Phys. Chem.*, **44**, 25 (1940); J. W. Mehl, J. L. Oncley and R. Simha, *Science*, **92**, 132 (1940).

(7) J. T. Edsall, "Size, Shape and Hydration of Protein Molecules," in "The Proteins," H. Neurath and K. Bailey, ed., Academic Press, Inc., New York, N. Y., 1953, Vol. I, Part B, p. 689 (Table).

(8) T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Clarendon Press, Oxford, England, 1940, p. 41 (Table).

(9) We are indebted to Mr. W. H. Baker of this Laboratory for making the measurements of sedimentation rates.

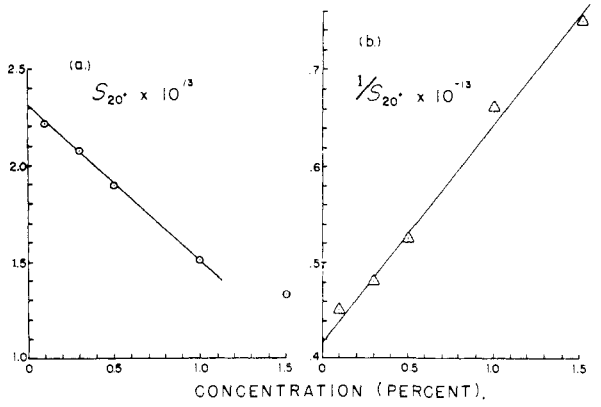

Fig. 2.—Sedimentation rate of peptomyosin B as a function of concentration.

Molecular Dimensions and Weight.—These were calculated with the help of the values obtained for the sedimentation rate, axial ratio and frictional coefficient of an assumed elongated ellipsoid. The solvent density, ρ, was 1.043; the solvent viscosity, η, 0.0108. The calculations gave: molecular weight 117,000; length 87 mμ; width 1.8 mμ.[10]

If a sphere of radius r and a prolate ellipsoid of polar axis $2a$ and equatorial radius b have the same volume, then $r^3 = ab^2$. Since, in general, $f_0 = 6\pi\eta rN$, and here, in particular, $f_e/f_0 = 2.91$ and $a/b = 48.7$ one has

$$f_e = 2.91(6)\pi\eta N(ab^2)^{1/3} = 2.91(6)\pi\eta N(3.65)b \quad (1)$$

For the sphere and ellipsoid, respectively, the sedimentation rates are

$$s_0 = \frac{M_0(1 - \bar{v}\rho)}{f_0} \text{ and } s_e = \frac{M_e(1 - \bar{v}\rho)}{f_e} \quad (2)$$

and the molecular weights

$$M_0 = M_e = \frac{4\pi Nab^2}{3v} = \frac{4\pi N(48.7)b^3}{3\bar{v}} \quad (3)$$

From (1), (2) and (3)

$$\frac{4\pi N(48.7)b^3}{3\bar{v}} = \frac{2.91(6)\pi\eta N(3.65)b \times s_e}{1 - \bar{v}\rho}$$

Substituting values of $\bar{v}$, η and ρ, and taking $s_e = 2.31 \times 10^{-13}$, one obtains $b = 0.891 \times 10^{-7}$, whence $a = 48.7b = 43.4 \times 10^{-7}$, $M_e = 117{,}000$.

Molecular Weight by Osmotic Pressure.—The author's method[11] was applied to the solution at *p*H 7.3 (Table I). Since the results varied little with concentration, the mean, 157,000, was a fairly close approximation to the weight at infinite dilution. Combining this with the sedimentation constant, one obtains: $f_e/f_0 = 3.35$; axial ratio 66:1; length 101 mμ; width 1.53 mμ.

Electron Microscopy.—Pictures of the protein in dilute HCl were kindly taken by Dr. H. Ruska. Tangled threads of extremely uneven width were the only structures observed. The narrowest formations had a diameter of the order of 10 mμ, much greater than those calculated above for the width of single particles, which would have been beyond the power of resolution of the microscope.

TABLE I

MOLECULAR WEIGHT OF PEPTOMYOSIN B FROM OSMOTIC PRESSURE DETERMINATIONS, *p*H 7.3

Concn. (%)	1.5	1.0	0.5
Calcd. mol. wt.	167,000	156,000	147,000
	159,000	163,000	147,000
Mean		157,000	

Physical State of Peptomyosin B.—The molecular weight of 117,000, calculated from sedimentation and viscosity measurements, comes fairly close to that of 157,000 obtained by osmometry. If, like tropomyosin,[12] the protein should have a partial specific volume of 0.71, the first figure would rise to 139,000. It is unnecessary to insist on the gratuitous assumptions made, such as rigidity, ellipsoidal shape and absence of hydration. The narrow solubility range of the protein and the tendency of its solutions to gel at low temperatures have thus far precluded diffusion and electrophoresis measurements. Although the physical state of the substance under various conditions cannot yet be fully described, the evidence so far suggests that it shifts from extremely thin needle- or thread-like single molecules (in high salt concentration at neutral *p*H, or low salt concentration at low *p*H) to stringy aggregates (in salt-free, moderately alkaline or acid solution) which easily form gels and yield the structures revealed by electron microscopy.

Peptomyosins A and B.—Since the effect of pepsin may be synthetic as well as lytic, it is conceivable that neither peptomyosin A,[1] nor B exist as such *in situ* and that both are artificial products. Their exact origin is not known, except to the extent that they seem to come from the less soluble part of muscle and that B has not been found in digests of rabbit myosin or actin (kindly supplied by Dr. T. Gergely).

They coexist in meat digests, and A can be isolated from the supernatant after removal of B, and thus prepared by a procedure much simpler than the original method.[1] The two proteins differ not only in their solubility, the appearance of their precipitates and the conditions necessary for crystallization, but also in their resistance to pepsin. At *p*H 1.5, A is very resistant to redigestion,[1] while B is fairly rapidly destroyed. If the initial *p*H of digestion of meat is 1.5, much less B is found than if it is 2.5. Both proteins have been isolated, in apparently undiminished amount, from a filtered digest (final *p*H 3.4) which had been kept 8 months at 4°.

The above information applies to beef skeletal muscle. Similar proteins have been isolated from several other species, and peptomyosin B also from beef heart.

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(10) The calculations were as shown (the symbols have their usual meaning). The same result is obtained with the help of the simplified form of Perrin's equation (as used by A. R. Peacocke and H. K. Schachman: *Biochim. et Biophys. Acta*, **15**, 198 (1954)), which gives $b = 0.884 \times 10^{-7}$ mμ. Since the sedimentation rate of very long particles depends almost entirely on width and very little on length, the sharp sedimenting boundary observed here could be offered by rods of uniform width but of uneven length. Osmotic pressure measurements (see below) showed that the average particle size did not differ much from that given by the above calculations.

(11) J. Bourdillon, *J. Biol. Chem.*, **127**, 617 (1939).

(12) K. Bailey, *Biochem. J.*, **43**, 271 (1948); K. Bailey, H. Gutfreund and A. G. Ogston, *ibid.*, **43**, 279 (1948); W. T. Ostbury, R. Reed and L. C. Spark, *ibid.*, **43**, 282 (1948).