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The Molecular Weights of Two Forms of Carbamoyl Phosphate Synthase from Rat Liver

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1. *N*-Acetylglutamate-dependent carbamoyl phosphate synthase from rat liver was centrifuged in sucrose density gradients. The concentration-dependence of *s* was consistent with a chemical equilibrium existing between the 11S and 7.5S forms of the enzyme. 2. Under conditions favouring the 11S form, the properties of the enzyme in ultra-short-column equilibrium experiments suggest a molecular weight of 316000±42000 for the 11S form. 3. Under conditions favouring the 7.5S form, high-speed equilibrium-sedimentation measurements gave a value of 160000±10000 as the molecular weight of the 7.5S form of the enzyme.

Of the two types of carbamoyl phosphate synthase activity (EC 2.7.2.5) found in rat liver (Nakanishi *et al.*, 1968) the type thought to be involved in urea biosynthesis, carbamoyl phosphate synthase I (Cohen, 1970), is characterized by its mitochondrial localization, its requirement for ammonia rather than glutamine as a substrate, and its requirement for *N*-acetyl-L-glutamate as an activator. Until recently (Guthöhrlein & Knappe, 1968) the enzyme had not been purified from mammalian liver, although it had been obtained from frog liver (Marshall *et al.*, 1958). The purified mammalian enzyme was found by Guthöhrlein & Knappe (1968) to be capable of existing in three states, one sedimenting at 7.5S and slowly activated by *N*-acetyl-L-glutamate, the other two sedimenting at 11S, one being converted rapidly into the other (activated) state on addition of *N*-acetyl-L-glutamate. The 11S forms of the enzyme were reported to have a molecular weight of 250000 on the basis of sedimentation and diffusion measurements. It was suggested that the 7.5S form of the enzyme may arise by dissociation of an 11S form into two subunits of equal molecular weight. The experiments described in the present paper were aimed at establishing the molecular weights of the 11S and 7.5S forms of the enzyme.

Experimental

Materials

Water was deionized and distilled from an all-glass still. ATP, NADH, phosphoenolpyruvate, β-mercaptoethanol, bovine serum albumin fraction V, lactate dehydrogenase type III, pyruvate kinase type II and catalase (stock C-10) were from Sigma (London) Chemical Co. Ltd., London, S.W.6, U.K. *N*-Acetyl-L-glutamic acid was from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Electrophoretically homogeneous rabbit immunoglobulin G (IgG) was a gift from Dr. R. H. Pain. Chromedia DEAE-cellulose (DE-32) was from BDH Chemicals Ltd., Poole, Dorset, U.K. Other chemicals were AnalaR grade where obtainable.

Preparation of carbamoyl phosphate synthase

In this laboratory some difficulty was experienced in obtaining preparations of reproducible purity and stability. Thus very low activity was sometimes recovered from the first column step (see below); such preparations were discarded. The final preparation was usually stable as a suspension in ammonium sulphate, but with some preparations the specific activity fell by as much as 50% after storage for a week at −20°C. The livers from four rats were homogenized to yield a washed crude mitochondrial pellet as described by Guthöhrlein & Knappe (1968), but with a solution that contained 20mM- instead of 5mM-β-mercaptoethanol. The pellet, suspended in a volume of homogenizing solution equal to half the original weight of liver, was passed through a French pressure cell at 10^8 N/m^2 (15000lb/in^2). Particulate material was removed by centrifuging for 1h at 120000*g*. The supernatant was applied to a column (2.3cm×50cm) of DEAE-cellulose that had been equilibrated with buffer containing 20mM-tris–HCl, pH7.3, 7mM-KCN, 50mM-KCl, 20mM-β-mercaptoethanol and 20% (v/v) glycerol. On elution with the same buffer, the enzyme activity emerged with the second peak of protein. The pooled fractions containing activity were concentrated by vacuum dialysis against a buffer with the same composition except that it was adjusted to pH7.6 and contained 10mM-KCl. The concentrated protein solution (10ml) was

applied to a DEAE-cellulose column (1.2cm×25cm) equilibrated with the pH7.6 buffer and eluted with a linear gradient (total vol. 1 litre) containing 10–500mM-KCl in the same buffer. The enzyme emerged at constant specific activity as the first major peak of protein; fractions containing 50–100mg of protein (specific enzyme activity between 0.7 and 1 unit/mg) were precipitated and stored as a suspension in ammonium sulphate as described by Guthöhrlein & Knappe (1968). These workers used a method of measuring protein concentration that gives values 16% lower than the method used here. When allowance is made for this, the enzyme they prepared appears to have had, by colorimetric assay of citrulline formation, a specific activity 25% higher than my preparations. The overall yield of activity from the disrupted mitochondrial pellet was 20%. The preparation ran as a single broad band in polyacrylamide-gel electrophoresis after loading 100μg of protein directly on a small-pore gel of composition and size similar to that described by Davis (1964). The protein migrated in a similar way in gels containing 5, 4 or 3% (w/v) acrylamide.

For each experiment, enzyme was dissolved in buffer containing 20mM-tris–HCl, pH7.5, 7mM-KCN, 100mM-KCl and 5mM-β-mercaptoethanol. A sample of suspension was centrifuged, the pellet dissolved in buffer and the solution dialysed against buffer plus 10mM-*N*-acetyl-L-glutamate in some experiments.

Measurement of protein concentration

The method of Lowry *et al.* (1951) was used with unpurified extracts, after the protein had been precipitated with a final concentration of 500mM-$HClO_4$ and the precipitate dissolved in 1M-NaOH. For the purified enzyme measurements of E_{280} were made, the value 8.4 being used for $E^{1\%}_{1cm}$. This was obtained by assuming the refractive increment of the protein to be 0.00178dl·g^{-1} and measuring the difference in refractive index between dialysed enzyme solution and solvent during centrifugation at 2600rev./min in a synthetic-boundary cell (Richards *et al.*, 1968).

Assay of enzyme activity

The method of Fahien & Cohen (1964) was adapted for use in a Hilger–Gilford spectrophotometer. The reaction mixture, at 37°C, contained 5mM-ATP, 10mM-$MgCl_2$, 100mM-$KHCO_3$, 65mequiv. of NH_4^+ ions/litre (from 10mM-NH_4Cl and $(NH_4)_2SO_4$ in the linking enzymes), 2.5mM-phosphoenolpyruvate, 160μM-NADH, 20μg of lactate dehydrogenase and 4μg of pyruvate kinase. The change in E_{340} was followed after addition of enzyme to two cells, one containing 12mM-*N*-acetyl-L-glutamate in addition to the other components, so as to give a final volume of 250μl. A unit of enzyme activity is the activity that formed ADP at a rate of 2μmol/min.

Ultracentrifuge experiments with sucrose density gradients

Linear 5–20% (w/v) sucrose density gradients (4.7ml) were prepared and centrifuged (MSE Superspeed 50) as described by Martin & Ames (1961). Gradients contained the buffer described above in addition to sucrose. Some gradients contained also 5mM-ATP, 10mM-$MgCl_2$, 65mM-NH_4Cl, 100mM-$KHCO_3$ and 12mM-*N*-acetyl-L-glutamate. Enzyme (0.1ml) dissolved in buffer was loaded on each test gradient. Gradients were centrifuged for 8h (20°C) or 15h (4°C) at 38000rev./min. Sedimentation coefficients were estimated by linear interpolation by using catalase ($s_{20,w}$ = 11.35S) and rabbit immunoglobulin ($s_{20,w}$ = 6.45S) as standards in one gradient (which contained no mercaptoethanol or substrates) centrifuged together with two test gradients.

Analytical sedimentation experiments

The solvent for these experiments was the tris–HCl–KCN–KCl–mercaptoethanol buffer. In some experiments, 10mM-*N*-acetyl-L-glutamate was added. A Beckman model E analytical ultracentrifuge was used. In sedimentation-velocity experiments solutions were centrifuged respectively at 60000rev./min (3 and 12mm centre pieces) and 52000rev./min (30mm centre pieces), with schlieren optics. Unless otherwise stated, 12mm light-path single-sector cells were used, except when the protein concentration exceeded 10mg/ml (3mm cells) or was less than 2mg/ml (30mm cells), and the phase-plate angle was 70°. Proportions of various components were estimated by integrating schlieren peak areas and from u.v.-scanner traces. For equilibrium experiments, 12mm cells were used. In ultra-short-column equilibrium experiments (Yphantis, 1960) column heights were 0.8mm, rotor speed was 6056rev./min and exposures made after 4.5h were analysed. A sample of the same solution used for the equilibrium experiment was centrifuged at 6000rev./min in a synthetic-boundary cell; the area of the schlieren pattern was used as a measure of concentration. In high-speed equilibrium experiments (Yphantis, 1964) column heights were 4mm, the rotor speed was 13000rev./min and exposures made after 64h were analysed. In computing results, the density and viscosity of both solvents was assumed to be equal to that of 100mM-KCl and the partial specific volume of the enzyme was assumed to be 0.73ml·g^{-1}.

Table 1. *Sedimentation of carbamoyl phosphate synthase in sucrose density gradients*

Sedimentation coefficients were determined by measuring the position of either protein or enzyme activity after centrifugation as described in the text. Substrates, where added, were ATP, $MgCl_2$, NH_4Cl, $KHCO_3$, together with *N*-acetyl-L-glutamate. The final concentration of protein in the gradient was approx. 1 mg/ml in the peak fraction for a loading of 1 mg of protein.

Temp. (°C)	Additions	Protein loaded (mg)	$s_{20,w}$ (S)	
			Protein	Enzyme activity
20	None	0.1	8.9	—
20	None	0.15	9.1	9.1
20	None	0.5	10.1	9.7
20	Substrates	0.067	7.3	6.9
20	Substrates	0.15	8.0	8.0
20	Substrates	1.3	9.2	9.6
4	None	0.15	8.1	7.9
4	Substrates	0.15	7.3	7.0

Results

Zone and boundary sedimentation of carbamoyl phosphate synthase

The enzyme was centrifuged in sucrose density gradients in the absence or in the presence of *N*-acetyl-L-glutamate plus substrates. In each run a single peak of protein was observed. The positions of protein and enzyme activity respectively were coincident within 0.5 S (Table 1). Compared with values of s found at 20°C in the absence of *N*-acetyl-L-glutamate and substrates, lower values of s were found at equivalent protein loadings both at 4°C and in the presence of *N*-acetyl-L-glutamate and substrates at 20°C. Increased protein loading, tested at 20°C, was associated with increased values of s.

For analytical sedimentation-velocity and equilibrium measurements two conditions were chosen so as to observe the 11 S to 7.5 S forms of the enzyme respectively (Guthöhrlein & Knappe, 1968). Under both conditions in sedimentation-velocity experiments the protein was composed of major and minor components, which, from estimates of optical recovery when sedimentation was almost complete, together accounted for 90–100% of the total sedimenting material present shortly after boundary formation. The proportion of protein present as a minor component was not altered by varying the protein concentration.

In buffer at 20°C, $s^0_{20,w}$ for the major component was 10.8 S. The concentration-dependence of s was linear, obeying the equation $s = s^0(1-k_s c)$ (where c is protein concentration, and k_s is a constant: 6.2 ml·g^{-1}) (Fig. 1). A slower-sedimenting component, for which $s^0_{20,w}$ was estimated to be 7.5–8.5 S, was incompletely resolved. It comprised 20–30% of the total protein. Material sedimenting more rapidly than the main component accounted for up to 10% of the total protein.

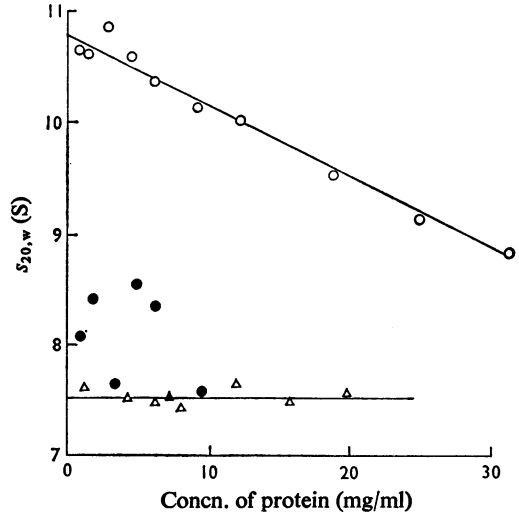

Fig. 1. *Concentration-dependence of sedimentation coefficients of carbamoyl phosphate synthase*

Sedimentation velocity was measured as described in the text, with single-sector cells. Enzyme dialysed against buffer, centrifuged at 20°C: o, main component; ●, minor component. △, Enzyme dialysed against buffer containing 10 mM-*N*-acetyl-L-glutamate, centrifuged at 5°C; ▲, the same after storage at 4°C for 9 days.

In buffer plus 10 mM-*N*-acetyl-L-glutamate at 5°C, $s^0_{20,w}$ for the major component was 7.5 S. The concentration-dependence of s was linear, k_s being zero

(Fig. 1). Under these conditions material sedimenting in a position consistent with a value of 11S accounted for 10–15% of the total material, but it was not sufficiently well resolved for accurate measurement of s. Material sedimenting more rapidly than this accounted for another 10–15% of the total material. The proportion of minor components did not vary significantly as a function of protein concentration. There was no evidence for the presence of material sedimenting more slowly than the main component. After storage at 4°C for 9 days in buffer plus *N*-acetyl-L-glutamate the protein sedimented with an unchanged value of $s_{20,w}$ for the main component (Fig. 1) and with no change in the proportions of components except for a slight increase in the proportion of the most rapidly sedimenting material.

Equilibrium sedimentation of carbamoyl phosphate synthase

An attempt was made to measure the molecular weight of the 11S form of the enzyme by the ultra-short-column method with the enzyme in buffer at 20°C. This method, with its sacrifice of accuracy for speed, was used in preference to a longer-column method because of the enzyme's tendency to lose activity and to produce aggregate during equilibration. Fig. 2 shows the effect of protein concentration on the apparent values of weight-average molecular weight ($\bar{M}_w$) and z-average molecular weight ($\bar{M}_z$) giving values of 235000±7000 and 290000±29000 for $\bar{M}_w^0$ and $\bar{M}_z^0$ respectively. The plots used to determine $\bar{M}_z$ were linear over 70–80% of each column.

In solutions containing *N*-acetyl-L-glutamate at 5°C the properties of the enzyme were appropriate for experiments using the high-speed equilibrium method, since the main 7.5S component was the slowest sedimenting, and therefore probably the low-molecular-weight component in solution. Enzyme (10mg) dissolved in 0.2ml of buffer was dialysed at 4°C against buffer plus 10mM-*N*-acetyl-L-glutamate. The protein solution was then passed through a column (1.2cm×20cm) of Sephadex G-150 (medium grade) equilibrated with the same buffer. The protein emerged as a single peak with a slight shoulder on the leading edge. The more nearly excluded material was pooled as fraction A; protein from the second part of the main peak was collected as solution B. A portion of solution B was kept for the equilibrium experiment. The rest of solution B and solution A were concentrated by vacuum dialysis, after which sedimentation coefficients were measured (at 5°C, in the 12mm double-sector cell). Values of $s_{20,w}$ for solutions A (4.8mg/ml) and B (1.5mg/ml) were 7.91 and 7.75S respectively. Solution A was estimated to contain 10–15% of material sedimenting more rapidly, and solution B to have 10% or less. Results

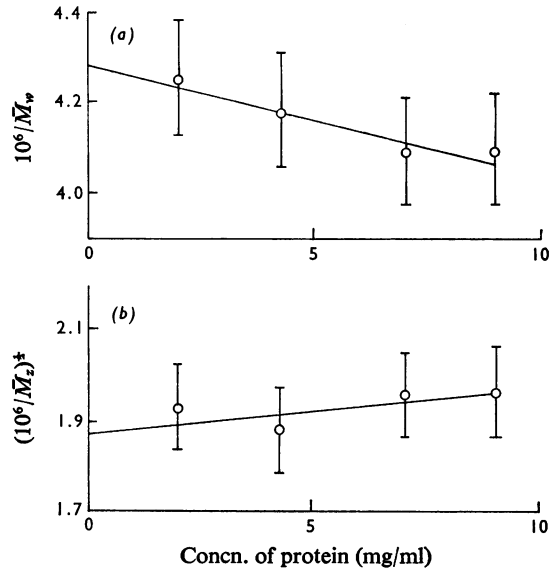

Fig. 2. *Sedimentation-equilibrium measurements on the 11S form of carbamoyl phosphate synthase*

The ultra-short-column method as described in the text was used. Enzyme was equilibrated with buffer containing no *N*-acetyl-L-glutamate. The temperature was 20°C and the phase-plate angle 70°. Effect of initial protein concentration on $\bar{M}_w$ is shown; the limits of the vertical lines correspond to a 3% error (*a*) or to a 10% error (*b*).

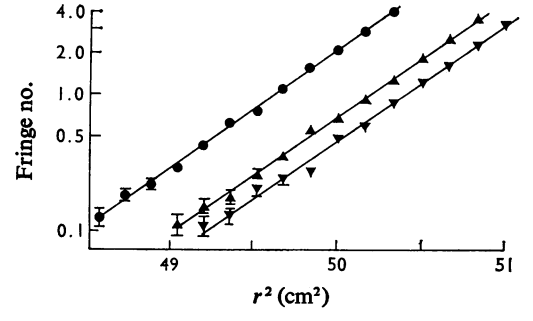

Fig. 3. *Sedimentation-equilibrium measurements on the 7.5S form of carbamoyl phosphate synthase*

The high-speed method was used as described in the text, with enzyme solution B. Vertical lines are shown where the assessed 5μm error of measurement exceeds the symbol size. The mean position of the bottom meniscus corresponded to $r^2 = 51.7$. The temperature was 5°C. Initial protein concentrations (mg/ml): ●, 0.498; ▲, 0.249; ▼, 0.125.

Table 2. *Weight-average molecular weights of the 7.5S form of carbamoyl phosphate synthase*

Values of $\bar{M}_w$ were calculated from the slopes of the plots in Fig. 3.

Initial concn. of protein (mg/ml)	$\bar{M}_w$
0.498	172000
0.249	172000
0.125	168500

of equilibrium measurements are shown in Fig. 3. The slope of each line was constant over the observed values of r^2. Table 2 shows the values of $\bar{M}_w$ calculated from these results.

Discussion

The coincidence of protein and enzyme-activity peaks in sucrose density gradients provides a check, albeit imprecise, on the purity of the preparation. The observation of single peaks, with values of s intermediate between 11S and 7.5S and with increased values at higher protein concentrations (Table 1), suggests that the enzyme sediments under these conditions as an associating system, approaching chemical equilibrium.

It is reasonable to conclude that the material sedimenting more slowly than the 11S peak in analytical sedimentation-velocity experiments was enzyme in the 7.5S state. Similarly the faster-sedimenting material observed at 5°C in the presence of *N*-acetyl-L-glutamate was assumed to be enzyme in the 11S state, together with some aggregate. A possible alternative explanation would be that the preparation was contaminated with two non-enzyme proteins with sedimentation coefficients of approx. 7.5S and 11S respectively. However, if this were the case, some evidence for heterogeneity might have been expected in polyacrylamide-gel electrophoresis. As noted in the Experimental section, there was no evidence for more than one band in electrophoresis, although the single band was rather broad, as was observed by Guthöhrlein & Knappe (1968) for their preparation.

At 20°C the proportion of 7.5S and 11S material did not alter detectably as a function of protein concentration, and a plot of $s_{20,w}$ against concentration was linear (Fig. 1). On the basis of the theory of Gilbert (1959) for rapidly equilibrating systems, these observations imply that there was no significant interconversion between 11S and 7.5S forms during the course of analytical sedimentation-velocity experiments at 20°C, without added *N*-acetyl-L-glutamate. This is supported by the observation of a typical value of k_s compared with values for a range of globular proteins (Creeth & Knight, 1965). Under the corresponding conditions the results of sedimentation in sucrose density gradients (Table 1) suggest a partial interconversion, which was presumably possible because of the longer time-scale of these experiments.

The results of sedimentation-velocity experiments at 5°C in the presence of *N*-acetyl-L-glutamate (Fig. 1) show values of $s_{20,w}$ that are independent of protein concentration. This is consistent with the existence of a chemical equilibrium between the main 7.5S component and a dimer, with the usual concentration-dependence of s being offset by concentration-dependent association.

The values of molecular weight measured by the ultra-short-column equilibrium method are interpreted on the assumption that, apart from any more rapidly sedimenting material, 70–80% (w/w) of the protein was a dimer and the rest monomer. The value of $\bar{M}_w^0$ (Fig. 2*a*) would correspond to a dimer molecular weight of 270000±18000. However, this value of $\bar{M}_w^0$ may be an underestimate because the method used depends on conservation of mass. This condition will not hold if heavy components are distributed wholly in the bottom part of the column at equilibrium. From the plots used to determine $\bar{M}_z$ it was evident that the protein contained material with $\bar{M}_z$ greater than that of most of the protein, presumably corresponding to material sedimenting more rapidly than 11S. Fig. 2(*a*) shows that the apparent value of $\bar{M}_w$ decreased slightly at low protein concentration. This might reflect a tendency towards greater dissociation at low concentration, or concentration-dependent adsorption of protein to the cell walls. The latter explanation was suggested by Yphantis (1960) to explain similar results with ribonuclease and β-lactoglobulin.

Estimates of $\bar{M}_z$ do not depend on knowledge of protein concentration. Thus loss of protein would not affect individual values of $\bar{M}_z$, although it would (if the loss was concentration-dependent) introduce an error into $\bar{M}_z^0$, which would be small for typical globular proteins. If most of the protein (70–80%, w/w) at equilibrium was dimer, the rest being monomer, the observed value of $\bar{M}_z^0$ corresponds to a dimer molecular weight of 316000±42000.

The molecular weight of the 7.5S form of the enzyme was estimated at protein concentrations where very little dimer should have been present (Fig. 1). Achievement of chemical equilibrium between dimer and monomer favouring dimer more strongly than suggested by sedimentation-velocity measurements, or heterogeneity from other causes, should have resulted in one or more curved plots in Fig. 3. If the material sedimenting more rapidly than 7.5S was a dimer accounting for 10% of the total protein, then the monomer molecular weight would be lower than that observed, with a lower limit of 150000.

It is concluded that the molecular weight of the 7.5S forms of the enzyme is 160000±10000 and that the molecular weight of the 11S form of the enzyme is consistent with its being a dimer composed of two 7.5S monomers. It is not clear why these results do not agree with those of Guthöhrlein & Knappe (1968), who combined the sedimentation coefficient with a height–area diffusion coefficient for a single concentration of the enzyme, to obtain a molecular weight of 250000. With the present preparation, presence of slower-sedimenting material would have led to an underestimate of the molecular weight of the 11S form through its effect on the diffusion coefficient, but although no detailed analysis of the extent of heterogeneity during sedimentation was reported by Guthöhrlein & Knappe (1968), it seems unlikely that this could wholly explain the discrepancy.

These results can be compared with measurements of molecular weight on the enzyme from frog liver. The value of 315000 for the native enzyme (Marshall *et al.*, 1961) and recent estimates of subunit molecular weight (J. R. Strahler & P. P. Cohen, unpublished work; cited by Cohen, 1970) are in close agreement with the present results for the rat liver enzyme.

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References

Cohen, P. P. (1970) *Science* **168**, 533
Creeth, J. M. & Knight, C. G. (1965) *Biochim. Biophys. Acta* **102**, 549
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 402
Fahien, L. A. & Cohen, P. P. (1964) *J. Biol. Chem.* **239**, 1925
Gilbert, G. A. (1959) *Proc. Roy. Soc. Ser. A* **250**, 377
Guthöhrlein, G. & Knappe, J. (1968) *Eur. J. Biochem.* **7**, 119
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265
Marshall, M., Metzenberg, R. L. & Cohen, P. P. (1958) *J. Biol. Chem.* **233**, 102
Marshall, M., Metzenberg, R. L. & Cohen, P. P. (1961) *J. Biol. Chem.* **236**, 2229
Martin, R. G. & Ames, B. N. (1961) *J. Biol. Chem.* **236**, 1372
Nakanishi, S., Kazuhiko, I. & Tatibana, M. (1968) *Biochem. Biophys. Res. Commun.* **33**, 774
Richards, E. G., Teller, D. C. & Schachman, H. K. (1968) *Biochemistry* **7**, 1054
Yphantis, D. A. (1960) *Ann. N.Y. Acad. Sci.* **88**, 586
Yphantis, D. A. (1964) *Biochemistry* **3**, 297