Studies on the Enzymatic Binding of Aminoacyl Transfer Ribonucleic Acid to Ribosomes in a *Drosophila in Vitro* System*

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ABSTRACT: An enzyme which catalyzes the *in vitro* codon-specific binding of AA-tRNA to ribosomes has been obtained partially purified from *Drosophila melanogaster*. The purified binding enzyme fraction supported *in vitro* amino acid polymerization in a system which required ribosomes, polyuridylic acid, AA([^{14}C]Phe)-tRNA, guanosine triphosphate (GTP), magnesium ions, potassium ions, and a complementary supernatant factor. The complementary factor was shown to be involved in a reaction separate from the binding reaction.

Enzymatic binding was uncoupled from polymerization by assaying in the presence of *N*-ethylmaleimide, an alkylating reagent which completely inhibits the polymerization reaction. The binding reaction rate was proportional to limiting amounts of ribosomes, binding enzyme fraction, polyuridylic acid, AA-tRNA, and GTP added. The GTP analog, 5′-guanylylmethylenediphosphonate, also supported binding in the absence of added GTP. Magnesium ions were required for enzymatic binding, while potassium ions progressively inhibited the reaction.

It is now generally established that the process of chain elongation during protein biosynthesis is a composite of three major enzyme-catalyzed steps: (1) binding of AA-tRNA to the ribosome–mRNA complex, (2) formation of the peptide bond, and (3) translocation of the mRNA and peptidyl-tRNA. The enzyme-catalyzed AA-tRNA binding step has been studied in bacteria (Lucas-Lenard and Haenni, 1968; Ono *et al.*, 1969; Ravel, 1967; Shorey *et al.*, 1969), yeast (Ayuso and Heredia, 1968; Heredia and Halvorson, 1966), and mammals (Arlinghaus *et al.*, 1964; Ibuki and Moldave, 1968; Lin *et al.*, 1969).

The present sampling of species provides only limited opportunity for comparative studies of the mechanism of protein biosynthesis. Such information would provide valuable insight into the metabolic control mechanisms and the detailed enzymology of protein biosynthesis. *Drosophila* was chosen for the present investigation because it is easy to culture, because of the ample and continuously increasing background on its biochemical and developmental genetics, and because it is a representative of the protostome branch of animal evolution (Barnes, 1963).

Although *in vitro* protein biosynthesis has been demonstrated previously in *Drosophila* and other insects, the *in vitro* systems employed were undefined with respect to purified supernatant polymerization factors (Fox *et al.*, 1965; Ilan, 1968; Jenny *et al.*, 1962; Litvak *et al.*, 1967; Moon, 1967; Rose and Hillman, 1969; Suzuka and Shimura, 1960; Takeyama *et al.*, 1958). This investigation was initiated in the interest of studying the reactions involved in the process of protein biosynthesis in a defined *in vitro* system using *Drosophila* ribosomes and transfer enzymes.

A previous report from this laboratory concerned the partial purification of a soluble factor from *Drosophila* which catalyzes the binding of AA-tRNA to *Drosophila* ribosomes (Pelley and Stafford, 1970). We now submit a more detailed report on the binding enzyme fraction and some of the characteristics of the reaction which it catalyzes.

Materials

Uniformly labeled L-[^{14}C]phenylalanine was purchased from New England Nuclear. Chromatographically pure [^{12}C]amino acids were obtained from Mann Research. Poly U, ATP, and GTP were purchased from P-L Biochemicals. TES[1] buffer was obtained from Sigma Chemical Co. Poly A, *N*-ethylmaleimide, dithiothreitol, and phosphoenolpyruvate were purchased from Calbiochem. *Escherichia coli* B tRNA was purchased from Schwarz. GMD was obtained from Miles Laboratories. Aged calcium phosphate gel (Sigma) was equilibrated with Tris–EDTA buffer (see below) and stored at 0–3° as a suspension of 20 mg/ml (dry weight/buffer).

Methods

Preparation of AA-tRNA Binding Enzyme. Drosophila melanogaster stock cultures were maintained at room temperature on standard cornmeal, agar, molasses, and brewer's yeast medium. One gram of larvae was homogenized per 1 ml of buffer containing 50 mM Tris-HCl, pH 7.2 at 25°, 100 mM KCl, 6 mM $Mg(CH_3CO_2)_2$, 1 mM EDTA, 250 mM sucrose, 7 mM β-mercaptoethanol, and 1 mM phenylthiourea in a Waring Blendor for 1 min at full speed. All enzyme purification steps were performed at 0–5°. After filtering the homogenate through a layer of cheesecloth and a layer of Miracloth

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[1] Abbreviations used are: TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; GMD, 5′-guanylylmethylenediphosphonate.

(Calbiochem) it was further treated in a glass Dounce homogenizer (Kontes Glass Co.) using four strokes with the tight pestle. The homogenate was then centrifuged for 30 min at 25,000*g*, the pellet was discarded, and the supernatant was centrifuged again for 20 min at 230,000*g* in a Spinco L2-65 ultracentrifuge using the Model 65 rotor (Beckman Inst., Inc.). The bulk of the enzymatic activity was precipitated from the 230,000*g* supernatant between 45 and 60% saturation with ammonium sulfate. The pH was maintained between 7.0 and 7.5 by diluting the 230,000*g* supernatant with an equal volume of Tris–EDTA buffer (50 mM Tris-HCl, pH 7.4 at 25°, 0.1 mM EDTA) and by adding ammonium carbonate in a weight ratio of 1:100 to ammonium sulfate. The precipitate was sedimented by centrifugation, dissolved in a minimal volume of Tris–EDTA buffer, and filtered through a Sephadex G-25 (medium) column equilibrated with Tris–EDTA buffer. The sample volume was never greater than one-half the void volume of the column. Aged calcium phosphate gel was equilibrated with Tris–EDTA buffer and used as a suspension of 20 mg/ml. The 45–60 ammonium sulfate fraction was adsorbed to the gel in Tris–EDTA buffer at a concentration of 5 mg of protein/ml and a gel:protein ratio of 0.45. The enzyme fraction was eluted from the gel between 0.1 M and 0.25 M potassium phosphate buffer, pH 7.5, and concentrated by ultrafiltration in an Amicon Model 50 Diaflo chamber after first being made 10% (v/v) glycerol. Ultrafiltration was performed with PM-10 membrane (Amicon) at 50 psi of N_2. The concentrated solution was chromatographed on a 2.5 × 70 cm column of Sephadex G-200 which had been equilibrated with 50 mM Tris-HCl, pH 7.4 at 25°, 150 mM KCl, 7 mM β-mercaptoethanol, 0.1 mM EDTA, and 20% (v/v) glycerol. The binding factor always eluted in the first major peak of material absorbing at 280 mμ. The active fractions were pooled and concentrated by ultrafiltration using a UM-2 membrane (Amicon). The concentrated sample was then dialyzed for 4–5 hr against 20% (v/v) glycerol in Tris–EDTA buffer and stored until use in liquid nitrogen. The material prepared as described above is referred to in the text as the binding enzyme fraction.

Preparation of Ribosomes. The ribosomes were prepared at 0–5° from the microsomal pellets resulting from the 230,000*g* centrifugation during the purification of the binding enzyme. The pellets were dissolved in a buffer solution composed of 50 mM Tris-HCl, pH 7.2 at 25°, 100 mM KCl, 5 mM $Mg(CH_3CO_2)_2$, 7 mM β-mercaptoethanol, 1 mM phenylthiourea, and 1% (v/v) Triton X-100 by gentle homogenization. This solution was layered over 2 ml of a buffer solution containing 50 mM Tris-HCl, pH 7.6 at 25°, 50 mM KCl, 5 mM $Mg(CH_3CO_2)_2$, 40% sucrose, and 7 mM β-mercaptoethanol in Oak Ridge type tubes (International Equipment Co.) and was centrifuged for 90 min at 230,000*g*. The ribosomal pellets were dissolved in a buffer solution containing 50 mM Tris-HCl, pH 7.4 at 25°, 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothrietol, and 20% (v/v) glycerol, centrifuged for 10 min at 10,000*g*, and stored in liquid nitrogen until use as sucrose-washed ribosomes.

Acylation of Escherichia coli tRNA. E. coli B tRNA was acylated in a total volume of 50 ml in a mixture containing 100 mM Tris HCl, pH 7.5 at 25°, 30 mM $MgCl_2$, 2.5 mM ATP, 10 mM dithiothreitol, 500 mg of tRNA, 0.03 mM L-[^{14}C]-phenylalanine, 0.02 mM of 19 remaining [^{12}C]amino acids, and 75 mg of an aminoacyl synthetase preparation (Hoskinson and Khorana, 1965) which had been freshly filtered through Sephadex G-25 (medium). Incubation was for 15 min at 37° and the reaction was terminated by adding an equal volume of freshly distilled, water-saturated phenol. The aqueous phase was removed and the AA-tRNA was precipitated from it by the addition of two volumes of cold ethanol and collected by centrifugation. The precipitate was then dissolved in a minimal volume of 10 mM potassium acetate buffer, pH 5.0, and chromatographed on Sephadex G-25 (medium). The radioactive fractions eluting in back of the void volume were pooled, adjusted to a concentration of 10 mg/ml, and stored frozen at −20°.

In Vitro Polymerization Reaction Conditions. The basic reaction mixture for determination of *in vitro* amino acid polymerization contained, except where noted, 0.05 M TES buffer, pH 7.6, 7 mM $MgCl_2$, 0.06 M KCl, 2 mM dithiothreitol, 0.5 mM GTP, 10 mM phosphoenolpyruvate, 50 μg of polyuridylic acid, 250 μg of AA([^{14}C]Phe)-tRNA, 200 μg of sucrose-washed ribosomes, and variable amounts of the binding enzyme fraction, as indicated, in a total volume of 0.25 ml. The reaction was incubated for 10 min at 37° and was terminated by adding 50 μl of 6 N NaOH to give a final concentration of 1 N. The tubes were then incubated for 10 min at 37°. After replacing the tubes in ice–water, 0.5 ml of 20% trichloroacetic acid was added. The precipitates were then collected on glass fiber filters and washed with 25–30 ml of 5% trichloroacetic acid. After a final wash with 10 ml of acetone the filter was glued to a planchet and the radioactivity was counted on a low-background gas-flow Geiger counter.

AA-tRNA Binding Reaction Conditions. The basic reaction mixture for determination of *in vitro* binding of AA-tRNA to ribosomes contained 0.05 M TES buffer, pH 7.6, 6 mM $MgCl_2$, 0.4 mM *N*-ethylmaleimide, 0.5 mM GTP, 100 μg of poly U, 200 μg of AA([^{14}C]Phe)-tRNA, 400 μg of sucrose-washed ribosomes, and 100–150 μg of the binding enzyme in a total volume of 0.5 ml. The use of *N*-ethylmaleimide permitted the enzymatic binding of AA-tRNA while it completely inhibited the overall polymerization reaction. The reaction was incubated for 2.5 min at 37° and then was terminated by dilution with 3 ml of cold buffer solution containing 50 mM Tris-HCl, pH 7.5 at 25°, 50 mM KCl, and 5 mM $MgCl_2$. The radioactivity bound to ribosomes was then determined by the method of Nirenberg and Leder (1964), except that the wash buffer contained $MgCl_2$ at 5 mM instead of 20 mM and two stacked nitrocellulose filters were used per tube in order to obtain acceptable standard deviations.

Preparation of rRNA. rRNA was prepared by the phenol extraction of a preparation of sucrose-washed ribosomes. The aqueous phase was reextracted with phenol and precipitated with two volumes of cold 95% ethanol. The precipitate was collected by centrifugation and dissolved in 0.01 M potassium acetate buffer at pH 5.0. After dialyzing for 16 hr against the same buffer the sample was frozen and stored at −20°.

Quantitative Analysis. Protein concentration was measured by the method of Lowry *et al.* (1951). Ribosome concentration was based on the assumption that $12.5A_{260}$ is equivalent to a concentration of 1 mg/ml.

Results

Differential Requirement of Sucrose-Washed and Salt-Washed Ribosomes for Supernatant Fractions. The early

TABLE I: Supernatant Enzyme Requirements of Sucrose-Washed and Salt-Washed Ribosomes for Polymerization Reaction.[a]

Supernatant Fraction Added	Sucrose-Washed Ribosomes (500 μg)	Salt-Washed Ribosomes[c] (540 μg)
None	5	6
230,000*g* supernatant (500 μg)	876	360
Stored 45–65 $(NH_4)_2SO_4$ fraction[b] (520 μg)	810	42

[a] Results are expressed as counts per minute incorporated into protein. Polymerization was carried out as described in Methods. [b] Prepared identically with the 45–60 $(NH_4)_2SO_4$ fraction except second precipitation was at 65% ammonium sulfate. Storage was for 3 days at 0° in Tris-EDTA buffer. [c] Salt-washed ribosomes were prepared by resuspending sucrose-washed ribosomes in 50 mM Tris-HCl, pH 7.4 at 25°, 500 mM KCl, and 5 mM $Mg(CH_3CO_2)_2$ and centrifuging the suspension for 45 min at 230,000*g*. The pellets were then treated as described in Methods for sucrose-washed ribosomes.

experiments in this study involved two experimental approaches: (1) the purification of ribosomes by washing with sucrose and with high levels of salt, and (2) a primary purification of the 230,000*g* supernatant factors required for *in vitro* amino acid polymerization. It was found that a freshly prepared fraction of the 230,000*g* supernatant which precipitated between 45 and 65% saturation with ammonium sulfate supported polymerization on both sucrose-washed and salt-washed ribosomes. In contrast, as shown in Table I, these ribosomes showed different responses to a sample of the 45–65 ammonium sulfate fraction which had been stored in Tris–EDTA buffer at 0° for 3 days. In this experiment the 230,000*g* supernatant was freshly prepared. Both types of ribosomes required the 230,000*g* supernatant; however, the stored 45–65 ammonium sulfate fraction lost its ability to support polymerization with salt-washed ribosomes. Since the sucrose-washed ribosomes retained polymerization activity with the stored 45–65 ammonium sulfate fraction, they must also have retained the unstable supernatant factor in an active form during their preparation. A similar result also has been obtained in the rat liver system and in the reticulocyte system. In both of these systems the transfer enzymes, termed aminoacyl transferase II and TF-II, respectively, were found to contaminate crude preparations of ribosomes (Gasior and Moldave, 1965; Arlinghaus *et al.*, 1968). These enzymes were also removed from the ribosomes by salt washing with a subsequent decrease in their stability to storage. We have not tested the supernatant from the salt wash for the presence of the unstable factor. The *Drosophila* sucrose-washed ribosomes and the ribosome bound enzyme remained active for several months when stored at −30° and apparently indefinitely under liquid nitrogen, indicating a stabilizing effect of the ribosomes on the bound enzyme.

TABLE II: Complementarity of Supernatant Fractions in Amino Acid Polymerization.[a]

Additions	cpm Incorpd into Protein
Binding enzyme (50 μg)	553
60–70 $(NH_4)_2SO_4$ fraction[b] (100 μg)	104
Binding enzyme (50 μg) plus 60–70 $(NH_4)_2SO_4$ fraction (100 μg)	1599

[a] The polymerization reaction was carried out as described in Methods, except that salt-washed ribosomes were used and the supernatant fractions were varied as indicated. [b] Material precipitating between 60 and 70% saturation with ammonium sulfate.

TABLE III: Noncomplementarity of Supernatant Fractions in Binding AA-tRNA to Ribosomes.[a]

Additions	cpm Bound to Ribosomes
Binding enzyme (56 μg)	801
60–70 $(NH_4)_2SO_4$ (40 μg)	24
Binding enzyme (56 μg) plus 60–70 $(NH_4)_2SO_4$ (40 μg)	554

[a] The binding reaction was carried out as described in Methods, except that the supernatant fractions were varied as indicated and salt-washed ribosomes were used in place of sucrose-washed ribosomes. The 60–70 $(NH_4)_2SO_4$ fraction represents material which precipitates between 60 and 70% saturation with ammonium sulfate.

Complementarity of Supernatant Fractions in Polymerization. The stable supernatant factor which is required by *Drosophila* sucrose-washed ribosomes for amino acid polymerization has been partially purified and shown to catalyze the binding of AA-tRNA to ribosomes (Pelley and Stafford, 1970). Further evidence of a requirement for supernatant factors other than the binding enzyme fraction in the polymerization reaction is shown in Table II. The activity obtained in the presence of both the binding enzyme fraction and the fraction from the 230,000*g* supernatant which precipitated between 60 and 70% saturation with ammonium sulfate was clearly greater than the sum of their activities when assayed separately. This indicated that one or more factors different from the binding enzyme were present in the 60–70 ammonium sulfate fraction and that these factors were required for polymerization.

The relatively high activity of the binding enzyme fraction added alone was probably due to trace contamination of the salt-washed ribosomes with the unstable factor. In other experiments the binding enzyme fraction alone showed progressively less activity as the ribosomes were more exten-

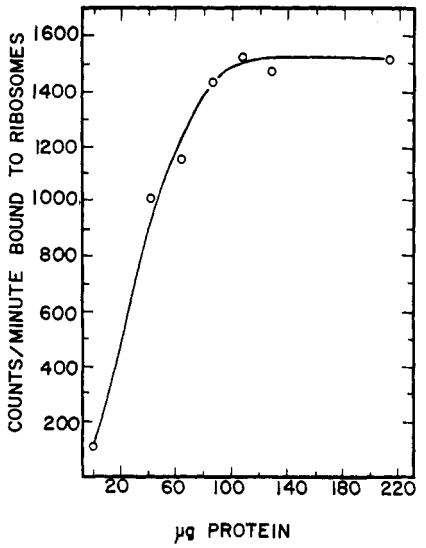

FIGURE 1: AA-tRNA binding as a function of binding enzyme fraction added. The binding reaction was carried out as described in Methods with protein from the binding enzyme fraction added as indicated.

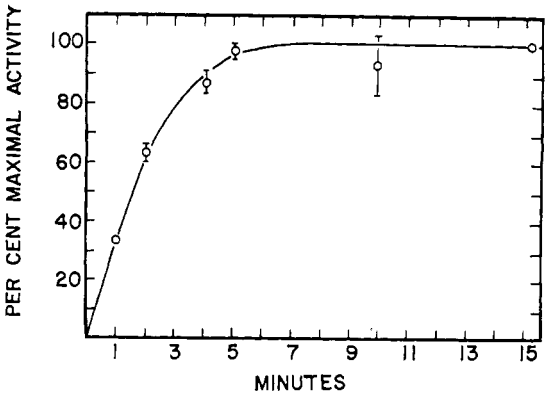

FIGURE 2: Time course of AA-tRNA binding. The binding reaction was carried out as described in Methods for the time intervals indicated. The maximal activity at 100% was 1280 cpm bound to ribosomes. Values for nonenzymatic controls have been subtracted.

sively washed. A similar finding has been reported by Felicetti and Lipmann (1968). Also, in other systems the full complement of transfer enzymes was absolutely required for activity when extensively washed ribosomes were used (Arlinghaus *et al.*, 1968; Gasior and Moldave, 1965; Lucas-Lenard and Lipmann, 1966; Skoultchi *et al.*, 1968; Ravel, 1967). The low levels of activity of the 60–70 ammonium sulfate fraction were probably due to a small contamination with the binding enzyme.

The possibility existed that the complementarity of these two fractions was a property of the binding reaction alone. As shown in Table III, the 60–70 ammonium sulfate fraction had no significant binding activity when tested alone, nor did it act synergistically with the binding enzyme fraction. This indicated that the enzyme, or enzymes, present in the 60–70 ammonium sulfate fraction function in a reaction subsequent to AA-tRNA binding during amino acid polymerization.

Requirement for Binding Enzyme. Figure 1 shows that the binding reaction occurred at low levels in the absence of the binding enzyme fraction, while addition of the binding enzyme fraction increased the binding activity up to 15-fold. A similar dependence of AA-tRNA binding on the addition of a binding enzyme fraction has been reported in rat liver (Ibuki and Moldave, 1968), rabbit reticulocytes (Lin *et al.*, 1969), *Bacillus stearothermophilus* (Skoultchi *et al.*, 1968), and *E. coli* (Ravel, 1967). The amount of binding enzyme fraction required for maximal binding rates varied from 100 to 130 μg depending on the preparation used. All other binding experiments employed saturating levels of the binding enzyme fraction.

Control binding reaction mixtures, with the binding enzyme fraction present at saturating levels, were completely inactive in incorporating amino acids into polypeptides when assayed for polymerization activity by the procedure described in Methods.

Time Course of Reaction. As shown in Figure 2, the binding reaction under optimum conditions is linear with time for the first 2.5 min and 70% of the maximal activity is bound during this period. The reaction is complete after 5 min with no decline in the amount of AA-tRNA bound up to 15-min incubation.

Requirement for Ribosomes. Figure 3 shows that the reaction had a linear dependence on the ribosome concentration up to the point of saturation of the filters. The conclusion that the decline from linearity was due to saturation of the filters was based on the fact that all other components required for binding were present in excess for each point tested.

Requirement for AA-tRNA and Polyuridylic Acid. Binding was proportional to the level of AA-tRNA added and saturation of the ribosomes with 200 μg of this substrate was observed (Figure 4).

The amount of poly U required for saturation of the system was 50 μg or less, while levels above 100 μg progressively inhibited the reaction. Poly U stimulated the reaction above endogenous levels (100–150 cpm bound to ribosomes) about tenfold, while substitution of poly A or rRNA suppressed the reaction below endogenous levels. The enzymatic poly U directed binding of Phe-tRNA to ribosomes has been

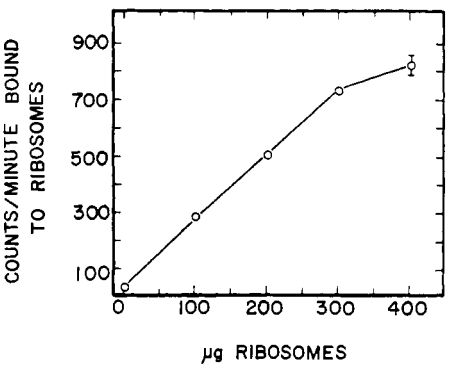

FIGURE 3: AA-tRNA binding as a function of ribosome concentration. The binding reaction was carried out as described in Methods with sucrose-washed ribosomes added as indicated. Values for nonenzymatic controls have been subtracted.

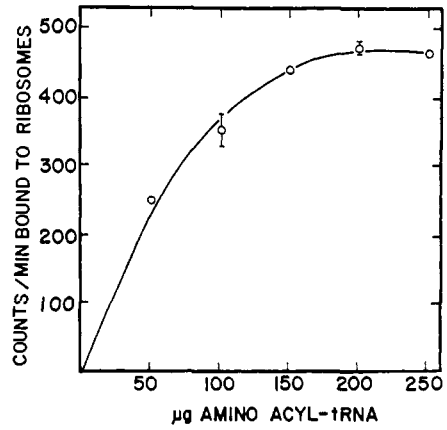

FIGURE 4: AA-tRNA binding as a function of AA-tRNA added. The binding reaction was carried out as described in Methods with AA-tRNA added as indicated. Values for nonenzymatic controls have been subtracted.

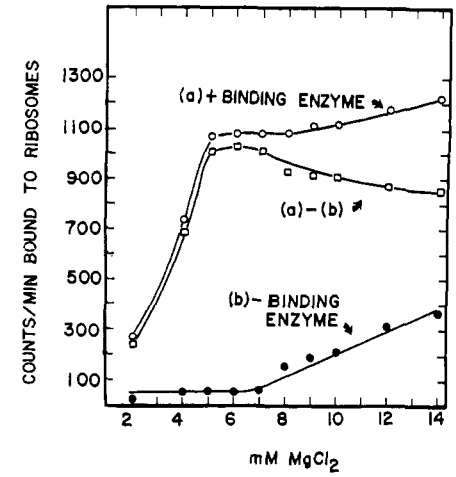

FIGURE 6: AA-tRNA binding as a function of $MgCl_2$ added. The binding assay was carried out as described in Methods with $MgCl_2$ added as indicated. The amount of $MgCl_2$ indicated has been corrected for the amount added with the ribosomes.

studied in systems derived from rabbit reticulocytes (Lin *et al.*, 1969), rat liver (Siler and Moldave, 1969), *E. coli* (Ravel, 1967; Lucas-Lenard and Haenni, 1968), *B. stearothermophilus* (Ono *et al.*, 1969), and yeast (Ayuso and Heredia, 1968).

Requirement for GTP. The enzymatic binding of AA-tRNA to ribosomes was significant in the absence of added GTP; however, the reaction was stimulated over fourfold by the addition of GTP. The binding activity in the absence of added GTP was somewhat variable, indicating that GTP might have been added as a contaminant of one or more of the reaction components.

The GTP analog, GMD, introduced by Hershey and Monro (1966), is a competitive inhibitor for reactions involving GTP hydrolysis. When GMD and GTP were compared for their ability to stimulate enzymatic AA-tRNA binding, it was found that GMD could substitute for GTP (Figure 5). Although GMD was required at somewhat higher concentrations than GTP for maximal binding, it gave a 1.5-fold higher stimulation than GTP.

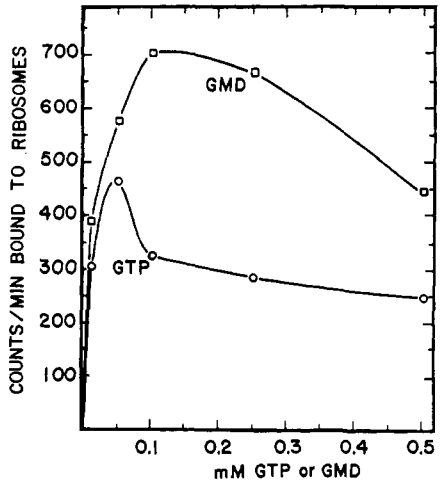

FIGURE 5: AA-tRNA binding as a function of GTP or GMD. The binding assay was carried out as described in Methods with either GTP or GMD added as indicated. Binding activity measured without added GTP or GMD was subtracted.

Requirement for Magnesium and Potassium. The effect of increasing levels of magnesium on both nonenzymatic and net enzymatic binding in the *Drosophila* system is shown in Figure 6. Nonenzymatic binding was unaffected by up to 7 mM magnesium while it was increased linearly with higher concentrations. Net enzymatic binding was calculated by subtracting the nonenzymatic binding from the total binding. This requires the assumption that nonenzymatic binding could take place equally as well in the presence of enzymatic binding. The inhibition of net enzymatic binding at higher magnesium levels was very gradual. A definite optimum for net enzymatic binding was evident between 5 and 7 mM.

In similar experiments designed to test the effect of potassium on the binding reaction it was observed that increasing the concentration of KCl from 10 to 130 mM resulted in a 60% linear decrease of net enzymatic binding. Under these conditions nonenzymatic binding remained constant at about 20% of the net enzymatic levels.

Characterization of the Polymerization Reaction. The use of the binding enzyme fraction with sucrose-washed ribosomes obviated the purification of the unstable supernatant factor and allowed a partial characterization of the polymerization reaction. Figure 7 shows that polymerization on sucrose-washed ribosomes was proportional to the level of the binding enzyme fraction added. The maximum level of [^{14}C]phenylalanine polymerized into polypeptide was about sixfold greater than the maximum amount of [^{14}C]phenylalanine which could be bound to an equivalent amount of ribosomes in the absence of polymerization.

The polymerization reaction was also proportional to limiting amounts of AA-tRNA and polyuridylic acid, although levels of 500 and 100 μg, respectively, did not saturate the system. In contrast to the stimulation by poly U, the system was completely inert to added poly A or rRNA. Endogenous levels of polymerization generally did not exceed incorporation of 150 cpm while poly U stimulated the system to incorporation levels as high as 6000 cpm.

GTP was limiting in this system up to the highest levels tested (1 mM), unless the system was supplemented with phosphoenolpyruvate (10 mM) in which case minimal amounts of GTP (0.1 mM) gave maximal rates. Polymerization was completely dependent on added GTP.

Both magnesium and potassium showed well-defined optimal values for polymerization at 7 and 60 mM, respectively, and ammonium ions could be substituted equally for potassium ions. A marked decrease in polymerization occurred at levels of magnesium lower than 5 mM and higher than 10 mM, and also at levels of potassium lower than 40 mM and higher than 100 mM.

The kinetics for polymerization showed an initial lag phase which could be eliminated by preincubation of the ribosomes with phosphoenolpyruvate, dithiothreitol, KCl, and $MgCl_2$. Neither poly U nor AA-tRNA was required during the preincubation.

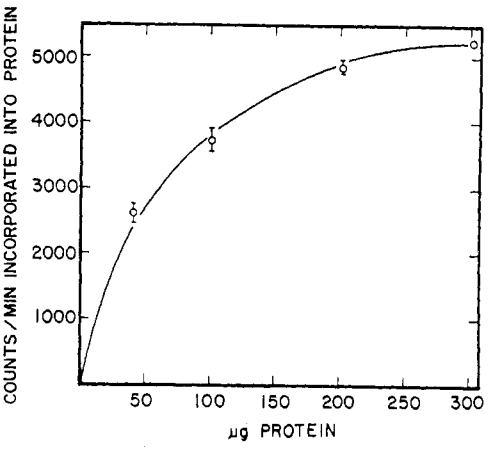

FIGURE 7: Amino acid polymerization as a function of binding enzyme fraction added. Protein from the binding enzyme fraction was added to the polymerization assay in the amounts shown and the polymerization activity was measured as described in Methods.

Discussion

Amino acid polymerization on *Drosophila* ribosomes clearly requires at least two complementary supernatant factors, one of which we have partially purified in the binding enzyme fraction. Both fractions can be distinguished by their differential precipitation with ammonium sulfate, by their differential degree of adherence to the ribosomes, and by their catalysis of different reactions during polymerization.

The enzymatic binding of AA-tRNA to *Drosophila* ribosomes requires the partially purified binding enzyme fraction along with optimal levels of magnesium, GTP or GMD, and poly U. No requirement for reduced thiols is evident since the reaction takes place in the presence of the alkylating reagent, *N*-ethylmaleimide.

In most systems in which the binding reaction has been studied, some enzymatic binding occurred in the absence of added GTP. However, the reactions were stimulated from 3.5- to 12-fold when GTP was added (Ayuso and Heredia, 1968; Ibuki and Moldave, 1968; Lin *et al.*, 1969; Lucas-Lenard and Haenni, 1968). An absolute requirement for GTP has been shown for enzymatic binding in *E. coli* (Ravel, 1967), and in rat liver (Siler and Moldave, 1969). The occurrence of binding in the *Drosophila* system in the absence of added GTP cannot be interpreted on the basis of the data available. It has been suggested by other investigators that contamination of the reaction components with GTP may have given spurious enzymatic binding in the absence of exogenous GTP (Arlinghaus *et al.*, 1964; Ibuki and Moldave, 1968; Lin *et al.*, 1969). The stimulation of the *Drosophila* binding reaction by GMD may have been due either to its direct substitution for GTP in the binding step, or to its inhibition of nonspecific GTPase activity resulting in the availability of more endogenous GTP. The use of more highly purified (washed) ribosomes in the *Drosophila* system might reveal an absolute requirement for GTP and clarify the function of GMD during the binding reaction.

A comparison of the binding reaction and the polymerization reaction shows that the former required less of the binding enzyme fraction, GTP, AA-tRNA, and poly U per unit of ribosomes. The magnesium optimum for both reactions appears to be very similar while the potassium requirements are very different. The progressive inhibition of the binding reaction with increasing potassium concentrations has not been previously observed in other systems.

The data presented here indicate a definite potential for more detailed studies on the events which take place during *in vitro* amino acid polymerization on *Drosophila* ribosomes.

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Structures and Activities of Oligosaccharides Produced by Alkaline Degradation of a Blood Group Substance Lacking A, B, H, Le^a, and Le^b Specificities*

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ABSTRACT: Alkaline degradation of an "inactive" blood group substance OG with NaOD–NaBD$_4$ gave a number of compounds which were characterized and each was shown to be identical with one previously isolated and characterized from Le^a substance and from A, B, and H substances. These include 3-hexenetetrol(s), D-galactitol, 2-acetamido-2-deoxy-D-galactitol, and three oligosaccharides β-D-Gal-(1→4)-β-D-GNAc-(1→6?)-3-hexenetetrol(s), β-D-Gal-(1→4)-β-D-GNAc-(1→6)-1,2,4,5,6-hexanepentol(s), and β-D-Gal-(1→4)-β-D-GNAc-(1→6)-[β-D-Gal-(1→3)]-2-acetamido-2-deoxy-D-galactitol. In addition β-D-Gal-(1→3)-2-acetamido-2-deoxy-D-galactitol was isolated and characterized; it corresponds to the small amount of R_L 0.83 previously reported with Le^a substance.

The chromatographic pattern on charcoal from the dialyzable material formed by alkaline borodeuteride degradation of OG was simpler than that previously seen with the A, B, H, and Le^a substances. The amounts of the various compounds isolated from OG were compared with those previously reported from Le^a substance in relation to the mechanism of alkaline degradation and the overall structure proposed for these substances. The data for OG are consistent with the proposed overall structure and indicate that OG can reasonably be considered a precursor of the blood group A, B, H, Le^a, and Le^b substances as well as of the new determinant in which an L-fucosyl residue is linked to C-3 of the GNAc of the type 2 β-D-Gal-(1→4)-β-D-GNAc- chain.

About 1% of individuals have secretions lacking A, B, H, Le^a, and Le^b blood group activities (*cf.* Kabat, 1956; Race and Sanger, 1968). From the secretions of such individuals a glycoprotein similar to the A, B, H, Le^a, and Le^b substances but devoid of these activities has been reported (Watkins and Morgan, 1959; Vicari and Kabat, 1969). According to the genetic scheme advanced by Ceppellini (1959) and by Watkins and Morgan (1959) this "inactive" glycoprotein or precursor should contain both basic types of antigenic determinants, β-D-Gal-(1→3)-D-GNAc (type 1) and β-D-Gal-(1→4)-D-GNAc (type 2), and all groupings that confer A, B, H, Le^a, or Le^b activity should be missing. These two types of determinants are joined by β-(1→3) and β-(1→6) linkages to a galactose which is in turn linked to several additional sugars; an overall structure for the oligosaccharide moiety has been advanced (Lloyd *et al.*, 1968; Lloyd and Kabat, 1968). The genetic scheme considers only the four gene systems (ABO, Hh, Lele, Sese) responsible for the synthesis of the outer terminal sequences of the carbohydrate chains in the blood group glycoproteins of

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