Studies of a Factor from Dystrophic Mouse Muscle Inhibitory towards Protein Synthesis

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A substance inhibitory to protein synthesis was purified from mouse skeletal muscle by gel filtration and ion-exchange chromatography, as well as by centrifugation on sucrose gradients. The molecular weight of the inhibitor, determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, was 71 000. The inhibitory activity was insensitive to ribonuclease A, deoxyribonuclease I and phospholipase C. It was sensitive to Pronase treatment but insensitive to heat-treatment and trypsin degradation. The present results, taken together with previous studies, indicate that the site of action of the inhibitor is not on the initiation phase of protein synthesis but rather at a step after the binding of aminoacyl-tRNA to ribosomes. The increased inhibitor activity found in dystrophic muscle is discussed.

Previous studies in this laboratory have shown that the pH5-supernatant fraction, which contains most of the EF 1 activity of the cell, together with a relative excess of EF 2 activity, exhibits an increased capacity for peptide synthesis during hypertrophy and hyperplasia of rat kidney and liver (Girgis & Nicholls, 1971; Nicholls *et al.*, 1975, 1977; Cappon & Nicholls, 1974*a,b*; Chan *et al.*, 1977). In mice during cardiac hypertrophy resulting from genetic muscular dystrophy a similar situation was observed in the heart muscle (Petryshyn *et al.*, 1977). The increase in each instance depended upon an increased binding of aminoacyl-tRNA to ribosomes. This was due to an increase in the activity of purified EF 1 (Girgis & Nicholls, 1971; Nicholls *et al.*, 1974; Young & Nicholls, 1978) in the soluble cytosol fraction rather than to the presence or absence of stimulatory or inhibitory factors affecting EF 1.

In addition, in liver, a proteinaceous substance that was inhibitory to the aminoacyl-tRNA-binding step could be extracted from the ribosome fraction of the controls but not from hypertrophied liver (Goodchild & Nicholls, 1976; Tominaga *et al.*, 1975). Other ribosome-bound inhibitors of translation described in eukaryotes were also less active during growth and development (Scornik *et al.*, 1967; Metafora *et al.*, 1971; Rupniak & Quincey, 1973; Huang & Warner, 1974).

Abbreviations used: EF 1 elongation factor 1; EF 2 elongation factor 2; SDS sodium dodecyl sulphate.

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The soluble fraction of a number of tissues and species also contains inhibitors of translation. For example, a haemin-regulated inhibitor of translation (Rabinovitz *et al.*, 1969; Hunt *et al.*, 1972; Beuzard *et al.*, 1973), purified from rabbit reticulocytes and rat liver, is reported to be a protein of high molecular weight (Ranu & London, 1976; Delaunay *et al.*, 1977; Farrell *et al.*, 1977). Heywood *et al.* (1974) have isolated a low-molecular-weight single-stranded RNA from chick muscle that inhibits translation of heterologous mRNA. These substances apparently exert their effect upon the formation of the initiation complex. Recently an inhibitor of protein synthesis active against elongation has been purified from the high-speed supernatant fraction of wheat germ (Stewart *et al.*, 1977).

When the pH5-supernatant fraction of the hind-leg muscle of genetically dystrophic mice was studied in this laboratory, it exhibited a markedly decreased capacity for peptide synthesis compared with control preparations (Petryshyn & Nicholls, 1976). This decrease was not due to altered activity of the elongation factors, ribonuclease, proteolytic enzymes, GTP or thiol reagents. It was attributed to the presence of an activity that was inhibitory to protein synthesis. The present experiments were undertaken to elucidate the nature and the action of this inhibitory activity present in mouse skeletal muscle.

Materials and Methods

Experimental animals and chemicals

Male dystrophic mice (strain ReJ 129 dy/dy) and control littermate mice were obtained from the Roscoe B. Jackson Laboratory, Bar Harbor, ME,

U.S.A., and maintained for several weeks in the laboratory until they reached an average age of 72–78 days. The mice, and also rats of the Sprague–Dawley strain (180–220 g), had free access to food (fox cubes, Maple Leaf Milling Co., Georgetown, Ont., Canada) and water.

L-[U- 14 C]Phenylalanyl-tRNA (sp. radioactivity 385 Ci/mol, 0.56 μ Ci/mg of tRNA), containing 19 other unlabelled aminoacyl-tRNA species from *Escherichia coli* strain B, and other chemicals were obtained as described previously (Girgis & Nicholls, 1971, 1972; Bishay & Nicholls, 1973; Innanen & Nicholls, 1973; Nicholls *et al.*, 1974; Young & Nicholls, 1978). Myosin, actin and tropomyosin were gifts from Dr. A. Forer of York University.

Preparation of skeletal-muscle pH5-supernatant fraction and liver ribosome fraction

A 20% (w/v) homogenate of mouse muscle was prepared as previously described (Petryshyn & Nicholls, 1976) in buffer A containing 0.25 M-sucrose, 50 mM-Tris/HCl (pH 7.8 at 25°C), 80 mM-KCl, 6 mM-MgCl₂ and 10 mM-2-mercaptoethanol, and separated into a microsomal and postmicrosomal fraction. The pH5-supernatant fraction was obtained from the postmicrosomal supernatant fraction as described by Petryshyn & Nicholls (1976). Rat liver was used to prepare a microsomal fraction that was treated with sodium deoxycholate and salt-washed by the method of Girgis & Nicholls (1971) for the preparation of ribosomes.

Non-enzymic binding of [14 C]phenylalanyl-tRNA to ribosomes

Non-enzymically labelled ribosomes were prepared essentially as described by Heintz *et al.* (1968). The reaction was carried out in 1 ml of buffer B, which contained 50 mM-Tris/HCl (pH 7.5 at 25°C), 0.25 M-sucrose, 80 mM-KCl and 19.2 mM-MgCl₂. In addition, the reaction mixture contained 200 μ g of poly(U), 0.5 mM-GTP, 2 mM-dithiothreitol, 2 mg (RNA) of preincubated salt-washed ribosomes from rat liver and [14 C]phenylalanyl-tRNA (68 750 d.p.m.). The reaction mixture was incubated for 15 min at 37°C, and the reaction terminated by chilling on ice and diluting with cold buffer B. The ribosomes were collected by centrifugation at 0°C for 2 h at 105 000g in a Beckman type-40 rotor. The pellet was rinsed three times with buffer B, and then resuspended in buffer B. The ribosomal suspension was clarified by centrifuging at 15 000g for 10 min at 0°C. The amount of [14 C]phenylalanine bound to the ribosomes was determined by filtering the ribosomes on Millipore filters and washing as described in the legend to Fig. 5(a). Ribosomes that were labelled non-enzymically contained 10 c.p.m./ μ g of ribosomal RNA. These

preparations were used immediately or else frozen in small portions at -45°C.

Gel electrophoresis

Proteins were prepared for electrophoresis as described by Laemmli (1970). The proteins were analysed in the discontinuous buffer system described by Laemmli (1970), except that the gels were poured as slab gels in an apparatus that was essentially the same as described by Studier (1973). The gels were 2 mm thick composed of a resolving gel containing 10% (w/v) acrylamide and 0.1% (w/v) SDS. This was overlaid by a 2 cm spacer gel containing 4.5% (w/v) acrylamide and 0.1% (w/v) SDS.

Non-denaturing gels were prepared by using the buffering system described by Davis (1964), except they were poured as 2 mm-thick slab gels. Both types of gels were electrophoresed at 20 mA/slab, and then they were fixed and stained for 30–45 min in a staining solution containing 50% (w/v) trichloroacetic acid and 0.1% (w/v) Coomassie Brilliant Blue, and destained by diffusion with 7% (v/v) acetic acid.

Miscellaneous

Protein concentrations of the pH5-supernatant fractions were determined by the method of Lowry *et al.* (1951) or as previously described (Girgis & Nicholls, 1972), with bovine serum albumin as a standard.

RNA was determined from A_{260} ($A_{1\text{cm}}^{1\%} = 230$) after applying the ferritin correction as described by Wilson & Hoagland (1965). The ribosome preparations were used only when the A_{260}/A_{280} ratio gave a value of 1.7 or greater.

The points plotted in the Figures are the mean values of duplicate tubes. The results obtained are representative of those obtained in three or more separate experiments (10–12 pairs of mice per experiment).

Results

Preparation of the inhibitor

Fig. 1(a) shows that, when the pH5-supernatant fraction from dystrophic muscle was applied to a Sephadex G-75 column, the inhibitory activity was eluted near the void volume. This inhibitory activity was approximately 3 times greater, per mg of protein, than the inhibitory activity of the pH5-supernatant fraction that had not been subjected to gel filtration (Fig. 2). No inhibitory activity was detected in the control-muscle pH5-supernatant fraction or in the corresponding fractions when control-muscle pH5 supernatant was applied to a Sephadex G-75 column (Fig. 2). The fractions from Sephadex G-75 gel filtration that gave inhibitory activity were adsorbed on a DEAE-cellulose column.

Fig. 1. Sephadex G-75 chromatography of dystrophic-muscle pH5-supernatant preparation (a) and purification by chromatography on DEAE-cellulose of the inhibitor isolated (b)

(a) Sephadex G-75 was swollen and packed in a glass column (2 cm $\times$ 62 cm) with a bed volume of 120 ml and equilibrated with a buffer containing 50 mM-Tris/HCl (pH 7.8 at 25°C), 6 mM-MgCl₂, 80 mM-KCl, 0.1 mM-EDTA, 10 mM-2-mercaptoethanol and 10% (v/v) glycerol. The pH5-supernatant fraction from skeletal muscle of dystrophic mice (26 mg of protein) was applied to the gel and eluted with the equilibrating buffer at a flow rate of 0.93 ml/min; 35 ml of the eluting buffer was allowed to pass through, and then fractions (2 ml) were collected and assayed as described below. Fractions containing inhibitory activity were pooled and concentrated by ultrafiltration in an Amicon cell with an XM-50 filter. The concentrate was used immediately or frozen at -20°C. The inhibitory activity was assayed by adding 0.2 ml of the column fractions to the control-muscle pH5-supernatant fraction (675 μ g) and incubating for 10 min at 37°C in 0.40 ml of medium containing 50 mM-Tris/HCl (pH 7.8 at 25°C), 80 mM-KCl, 6 mM-MgCl₂, 2 mM-dithiothreitol, 0.5 mM-GTP, [¹⁴C]phenylalanyl-tRNA (13750 d.p.m.; 33 μ g of tRNA), excess salt-washed rat liver ribosomes (43 μ g of RNA) and 100 μ g of poly(U). The reaction was terminated, and the protein precipitated, washed and counted for radioactivity as described by Petryshyn & Nicholls (1976). Percentage inhibition is defined as the decrease in polypeptide synthesis from that when the control-muscle pH5-supernatant fraction was incubated with 0.2 ml of column buffer alone. The control incorporated 4676 d.p.m./tube. $\circ$, A_{280} ; $\blacksquare$, percentage inhibition. (b) DEAE-cellulose was prepared and packed in a glass column (2 cm $\times$ 28 cm) with a 50 ml bed volume. This column was extensively equilibrated with a buffer containing 50 mM-Tris/HCl (pH 7.8 at 25°C), 10 mM-2-mercaptoethanol and 10% (v/v) glycerol. The inhibitory material from the Sephadex G-75 step (9 mg of protein) was applied to the column, and the non-adsorbed material was eluted with the equilibration buffer. The material that was adsorbed was eluted with a 150 ml linear gradient of 0–1 M-KCl contained in a buffer of 50 mM-Tris/HCl (pH 7.8 at 25°C) and 10% (v/v) glycerol at a flow rate of 0.25 ml/min. Fractions of volume 2 ml were collected. The eluted protein was detected by measurement of A_{280} . The KCl concentration was determined by using a YSI model 31 conductivity bridge. Individual protein peaks were pooled and concentrated as described above. Samples containing high KCl concentration were desalted to a final KCl concentration of 80 mM with the equilibration buffer containing 6 mM-MgCl₂. Inhibitory activity was found only in the 280–375 mM-KCl fraction. (The pH5-supernatant fraction from skeletal muscle of control mice was subjected to an identical purification procedure.) The assay procedure was as described in (a). Inhibitory activity was found only in the pooled area eluted from fractions 34–42. The control incorporated 4700 d.p.m./tube. $\circ$, A_{280} ; —, KCl concentration (M).

Fractions were eluted from DEAE-cellulose with a 0–1 M-KCl gradient. Inhibitory activity was found in the 0.28–0.38 M-KCl fractions, the main peak being eluted at 0.34 M-KCl (Fig. 1b). This inhibitory activity was approximately 20 times greater, per mg of protein, than the inhibitory activity of the pH5-supernatant fraction from dystrophic muscle (Fig. 2).

Fractions of control-muscle preparations obtained

by gel filtration on Sephadex G-75 (fractions 10–16) were chromatographed on DEAE-cellulose and tested for inhibitory activity. An identical elution profile for inhibitory activity was found. The control fractions, which corresponded to those that gave inhibitory activity in the dystrophic-muscle preparations, exhibited a significant amount of inhibitory activity. This activity, however, was less than half of

that obtained from the DEAE-cellulose preparation of the dystrophic-muscle inhibitor, per mg of protein (Fig. 2).

The inhibitory activity purified by DEAE-cellulose chromatography (30 μ g of protein) was analysed by SDS/polyacrylamide-slab-gel electrophoresis. One major stained band and occasionally several lightly stained bands were visible. In order to see which of these proteins was responsible for the inhibitory activity, the fraction containing inhibitory activity, prepared by DEAE-cellulose chromatography, was analysed by sedimentation through linear sucrose gradients. Fig. 3(a) shows a typical profile of the inhibitory activity that was always obtained in this type of experiment. The active fractions obtained from the sucrose gradients were pooled and analysed by polyacrylamide-slab-gel electrophoresis. Only one stained band could be detected, and the proteins in this band were identical in mobility with the proteins in the major stained band found when the DEAE-cellulose preparation was subjected to the same electrophoretic step.

Fig. 2. Summary of the inhibitory activity from crude and partially purified fractions from control and dystrophic muscle preparations

Control muscle preparations were subjected to a purification procedure identical with that described for the dystrophic-muscle preparations. The resulting preparations were assayed for inhibitory activity as described for Fig. 1(a). ●, Control-muscle preparations; ■, dystrophic-muscle preparations.

Fig. 3. Sucrose gradient analysis of the inhibitor (a) and determination of the molecular weight of the inhibitor by SDS/polyacrylamide-gel electrophoresis (b)

(a) A volume of 500 μ l (32 μ g of protein) of the DEAE-cellulose-purified fraction which contained inhibitory activity was layered on a 5 ml linear sucrose gradient [10–30% (w/v) sucrose]. The sucrose gradient was contained in a buffer composed of 50 mM-Tris/HCl (pH 7.8 at 25°C), 80 mM-KCl, 6 mM-MgCl₂ and 10 mM-2-mercaptoethanol. The gradient was centrifuged at 0°C for 16 h in a Beckman SW 65 Ti rotor at 105 000g. The fractions (0.3 ml) were collected from the top of the tube by injecting 50% (w/v) sucrose into the bottom of the tube by using an ISCO model 182 density-gradient fractionator. The assay for inhibition was done by adding 0.15 ml fractions from the sucrose gradient to the control-muscle pH 5-supernatant fraction (675 μ g) and incubating as described in Fig. 1. The addition of the same volume of buffer containing sucrose in the same concentration range had no effect on polyphenylalanine synthesis. (b) Proteins to be used as standards (12 μ g) and inhibitor (12 μ g), purified by chromatography on DEAE-cellulose, were applied to the gels. The molecular weight of actin prepared from rabbit skeletal muscle, was determined by Dr. A. Forer of York University. All other standard proteins were given the molecular weights described by Weber & Osborn (1969).

The molecular weight of the major stained protein from the DEAE-cellulose preparation was approx.

71000, as judged by SDS/polyacrylamide-slab-gel electrophoresis (Fig. 3b).

Electrophoresis of the inhibitor (purified by DEAE-cellulose chromatography) on non-denaturing gels showed one major stained band that exhibited 'fast' migration to the anode. The same 'fast' migrating band was found when the inhibitory activity from the sucrose gradient was analysed on non-denaturing gels.

Fig. 4 shows the two-dimensional gel analysis of the inhibitor fraction prepared by DEAE-cellulose chromatography. One heavily stained spot of mol.wt. about 70000 is clearly visible. Several other spots are also detectable in the preparation. The two major spots, representing proteins of lower molecular weight, on the lower right of the slab, could be removed from the DEAE-cellulose fraction by further chromatography on Sephadex G-75 without any loss of inhibitory activity.

Characterization of the inhibitor

It was decided to determine if the inhibitor obtained from muscle had RNA, DNA or phospholipid components that could be involved in its activity. Treatment of the partially purified inhibitor (purified by Sephadex G-75 or DEAE-cellulose chromatography) with ribonuclease A, phospholipase C or deoxyribonuclease I did not affect the activity. Treatment

of the inhibitor with Pronase for 10 min at 25°C was sufficient to remove all the inhibitory activity. However, the inhibitor was resistant to trypsin digestion. The inhibitory activity remained, even when the trypsin/protein ratio was increased from 1:50 to 1:12. Heat-treatment of the crude or of the partially purified fractions containing inhibitor showed that the inhibitor is resistant to 90°C for 5 min. Since the inhibitory activity from skeletal muscle is resistant to treatment with phospholipase C, trypsin and heating, it is unlikely that it is the same as the inhibitor found in the salt-wash from rat liver ribosomes (Goodchild & Nicholls, 1976). To determine if any carbohydrate moiety was associated with the inhibitor, the periodic acid/Schiff reaction was carried out on non-denaturing gels as described by Clarke (1964). The band containing inhibitor did not exhibit any staining for carbohydrate. The inhibitor purified by DEAE-cellulose has been stored in small portions (0.1–0.2 mg/ml) for up to 1 month at –20°C in a buffer containing 50 mM-Tris/HCl (pH 7.8), 6 mM-MgCl₂, 80 mM-KCl and 10 mM-2-mercaptoethanol. This resulted in only a slight loss of activity. However, continuous freezing and thawing decreased the inhibitory activity considerably.

Mode of action

A number of considerations made it unlikely that the inhibitory activity was directed against the initiation of protein synthesis. At low Mg²⁺ concentrations, initiation factors are required to initiate polypeptide formation. However, at higher concentrations the initiation-factor requirement is diminished, and the translational cycle is catalysed by the soluble elongation factors (Shafritz & Anderson, 1970). Since the Mg²⁺ concentration for optimal activity was the same in pH 5-supernatant preparations from both control and dystrophic mice (6–7 mM) and not shifted downwards, it appears most unlikely that soluble initiation factors play a role in the activity of the preparations. Furthermore, the preparations from control and dystrophic mice exhibited similar responses to the addition of aurintricarboxylic acid, which, at least at low concentrations, affects initiation. In these experiments, it was essential to use liver ribosomes bearing endogenous mRNA rather than poly(U). These results contrast with the effects of aurintricarboxylic acid (and other inhibitors of initiation) that were observed in the liver of diabetic animals and in liver after microsomal enzyme induction, situations where initiation is affected (Pain, 1973; Cappon & Nicholls, 1974a).

The decreased activity of the pH 5 supernatant from dystrophic muscle cannot be explained by the presence of the haemin-reversible inhibitor that has been widely studied in reticulocytes (Farrell *et al.*, 1977), since the addition of haemin does not affect

Fig. 4. Two-dimensional gel electrophoresis of the inhibitor derived from the DEAE-cellulose-chromatographic step

The inhibitor was applied to the gel and subjected to isoelectric focusing in the first dimension and SDS/polyacrylamide-slab-gel electrophoresis in the second dimension by the method of O'Farrell (1975). The second dimension was electrophoresed at 0–4°C with a current of 20 mA/gel. Gels were fixed and stained overnight in a solution containing 10% (w/v) trichloroacetic acid, 7% (v/v) acetic acid, 50% (v/v) ethanol and 0.02% (w/v) Coomassie Brilliant Blue. The gels were further treated for 2 h in a solution containing 10% trichloroacetic acid, 7% acetic acid and 0.02% Coomassie Brilliant Blue. The gels were destained by diffusion using 7% acetic acid.

the activity of this supernatant (Table 1). Since initiation is required for the action of the haemin-reversible inhibitor, either initiation is not involved or the haemin-reversible inhibitor is not involved, or both. It is possible that the decreased activity of the preparation from dystrophic muscle could be due to the formation of an inhibitor(s) during the incubation step, such as reported for the haemin-reversible inhibitor. Table 2 shows that preincubation of the pH5-supernatant fraction from dystrophic mice before addition of the other reactants resulted in no change in the results for peptide synthesis. Furthermore, the addition of 0.2 µg of purified inhibitor to rabbit reticulocyte lysates, prepared and incubated as described by Delaunay *et al.* (1977), induced a 50% inhibition of protein synthesis (R. A. Petryshyn & D. M. Nicholls, unpublished experiments), but it was not accompanied by a disaggregation of polyribosomes into 80S ribosomes as in haem deficiency. All the above observations make it most unlikely that initiation is involved.

Previous results indicated that the addition of EF 1 could not overcome the lower activity of pH5-supernatant fraction from the dystrophic muscle (Petryshyn & Nicholls, 1976). This made it unlikely that the inhibitory material was interfering with the EF-1-catalysed binding of the aminoacyl-tRNA. A direct measurement of aminoacyl-tRNA binding was carried out with pH5-supernatant fractions from control and dystrophic mice (Fig. 5a). There was an

insignificant decrease (less than 6%) in binding activity in the pH5-supernatant fraction from dystrophic mice at protein concentrations that showed 36–48% decreases in polyphenylalanine synthesis. Thus the binding of aminoacyl-tRNA appears not to be affected by the inhibitor. Furthermore, the antibiotic chartreusin, which has been shown to inhibit the binding of aminoacyl-tRNA (Gregg & Heintz, 1972; Nicholls *et al.*, 1974), exhibited the same degree of inhibition of incorporation in the two preparations (Fig. 5b). This result would not be expected if an inhibitor in dystrophic-muscle preparations exerted its effect on aminoacyl-tRNA binding. If binding were involved, it is likely that less chartreusin would be needed in the dystrophic-muscle preparations than in the control preparations in order to achieve the same degree of inhibition of peptide synthesis. In other words, a differential effect would be observed at low chartreusin concentrations. Indeed such a difference was found in regenerating rat liver, where increased aminoacyl-tRNA binding occurs (Nicholls *et al.*, 1974, 1977). At high chartreusin concentrations, as with other inhibitors such as fusidate (Fig. 5c), the total incorporation of radioactivity is very low and hence significance cannot be attached to the small differences noted (less than 15%) in Figs. 5(b), 5(c) and 5(d) at high antibiotic concentrations.

It was observed previously that the addition of purified EF 2 did not restore peptide synthesis in the pH5-supernatant fraction from dystrophic mice (Petryshyn & Nicholls, 1976). This result suggested that the inhibitory material was not active directly against EF 2, since one would expect to overcome the inhibitory activity by saturating the mixture with EF 2.

Owing to the formation of a fusidic acid-GDP-ribosome-EF 2 complex, the effect of fusidic acid as an inhibitor of elongation can be decreased by

Table 1. *Effect of haemin on the incorporation of [¹⁴C]-phenylalanine*

pH5-supernatant fractions (600 µg) from control and dystrophic mice were incubated as described in Fig. 1. Samples were incubated for the times indicated either with or without haemin (50 µM). Haemin solution was prepared by dissolving haemin in a buffer containing 50 mM-Tris/HCl (pH 9.9), 80 mM-KCl and 6 mM-MgCl₂.

$$\text{Percentage decrease} = \left(\frac{\text{control} - \text{dystrophic}}{\text{control}} \right) \times 100$$

pH5-supernatant fraction	Incubation time (min)	...	10 ⁻³ × Incorporation (d.p.m./mg of ribosomal RNA)		
			10	20	40
Control					
+ Haemin			72.4	77.0	77.1
– Haemin			74.9	79.9	78.8
Dystrophic					
+ Haemin			46.4	50.1	48.2
– Haemin			50.5	51.7	52.1
			Percentage decrease		
+ Haemin			39.5	34.9	37.5
– Haemin			32.6	35.3	33.9

Table 2. *Effect of preincubation on incorporation of [¹⁴C]-phenylalanine*

pH5-supernatant fractions (660 µg) from control and dystrophic mice were preincubated at 37°C for the time indicated. Immediately after preincubation the tubes were chilled and then incubated as described in Fig. 1. Percentage decrease was calculated as shown in Table 1.

pH5-supernatant fraction preincubation time (min)	10 ⁻³ × Incorporation (d.p.m./mg of		ribosomal RNA) Percentage decrease
	Control	Dystrophic	
0	116.5	65.1	44.1
10	117.0	66.6	43.1
20	126.5	73.7	41.7

Fig. 5. Binding of $[^{14}\text{C}]$ phenylalanyl-tRNA to ribosomes (a) and the effects of chartreusin (b), fusidic acid (c) and puromycin (d) on the incorporation of $[^{14}\text{C}]$ phenylalanyl-tRNA

(a) The assay was carried out in the same buffer as described for the incorporation assay. An excess of preincubated salt-washed ribosomes ($100\mu\text{g}$ of RNA) was incubated with pH5 supernatant from control or dystrophic muscle for 5 min at 37°C in a 0.4ml volume containing excess poly(U) ($200\mu\text{g}$), 0.2mM -GTP, 4mM -sodium fusidate (Malkin & Lipmann, 1969) and excess $[^{14}\text{C}]$ phenylalanyl-tRNA (13750d.p.m. ; $33\mu\text{g}$). The reaction was terminated by chilling on ice and the addition of ice-cold buffer. The samples were filtered on to Millipore filters by using a gentle vacuum and washed three times (5ml each) with the same buffer. The filters were dried under a heat lamp and then left in 1ml of 2-ethoxyethanol for 30 min. Scintillation mixture (12ml) was added and the samples were counted for radioactivity. Values were corrected for blank tubes that contained no pH 5 supernatant. (b) Incubations were carried out as described in Fig. 1. Control- and dystrophic-muscle pH5-supernatant fractions ($600\mu\text{g}$) were incubated with various amounts of chartreusin. Results are expressed as percentages of the values for the tubes to which no chartreusin was added. (c) Control- and dystrophic-muscle pH5-supernatant fractions ($650\mu\text{g}$) were incubated in the reaction mixture described in Fig. 1, except that various concentrations of fusidic acid were added. The results are expressed as described in (b). (d) Control- and dystrophic-muscle pH5-supernatant fractions ($650\mu\text{g}$) were incubated in a reaction mixture as described in Fig. 1, except that various concentrations of puromycin were added. The results are expressed as described in (b). ●, Control; ■, dystrophic.

saturating concentrations of EF 2 (Carrasco & Vazquez, 1973). Fig. 5(c) shows that increasing the concentration of fusidic acid inhibits the pH5-supernatant fraction from dystrophic mice to the same degree as that from the control mice. If the inhibitory protein present in dystrophic material is inactivating EF 2, then it might be expected that less fusidic acid would be required in the preparations from dystrophic mice to achieve the same degree of inhibition as the control. However, no such differential effect was observed.

Puromycin is a potent inhibitor of protein synthesis in eukaryotic and prokaryotic organisms and causes the premature release of polypeptides from ribosomes (Traut & Monro, 1964). Puromycin has been used to study the peptidyltransferase reaction of the 60S ribosomal subunit (Heintz *et al.*, 1968; Innanen & Nicholls, 1974). Fig. 5(d) shows the inhibition of polyphenylalanine synthesis by puromycin. The pH5-supernatant fraction from dystrophic mice was inhibited to a much lesser extent than the control pH5-supernatant fraction at low puromycin concentrations (e.g. 16% compared with 31% at 0.02 mM). Thus there is a 50–30% decrease in the 'response' to 0.02–0.05 mM-puromycin. This result could be explained if the inhibitor inactivated the ribosomes, thus making them less sensitive to puromycin inhibition, or if some other component of the peptidyltransferase activity was inhibited.

Inactivation of ribosomes by the inhibitor was unlikely, since increasing the ribosome concentrations to excess quantities does not decrease the difference in incorporation between the pH5 supernatant from control and dystrophic mice (Petryshyn & Nicholls, 1976). Also, preincubating the ribosomes with pH5-supernatant fraction from dystrophic mice or partially purified inhibitor, with or without puromycin (0.025 mM), neither decreased nor increased the inhibitory effect. Thus it seemed unlikely that ribosomes themselves are inactivated by the inhibitor. Furthermore, the inhibitor could not be extracted from the ribosomal salt-wash, as might be expected if it exerted its effect directly against aminoacyl-tRNA binding to ribosomes (Goodchild & Nicholls, 1976).

In order to examine peptidyltransferase activity, the release of phenylalanylpuromycin was measured in the presence of preparations from control or dystrophic mice (Table 3). When ribosomes were prepared as described by Heintz *et al.* (1968), labelled phenylalanyl-tRNA was bound non-enzymically to the 'A' site. Thus the addition of puromycin alone does not result in the release of labelled material (Table 3). The addition of preparations from control or dystrophic mice containing comparable quantities of EF 2 activity promotes the translocation of the bound aminoacyl-tRNA to the 'P' site, in the two-site model of protein synthesis. Puromycin binds to

Table 3. *Release of phenylalanylpuromycin by pH5-supernatant fractions from control and dystrophic mice*
The pH5-supernatant fractions (625 µg) were incubated in a 0.4 ml of reaction mixture containing 50 mM-Tris/HCl (pH 7.8), 80 mM-KCl, 6 mM-MgCl₂ and 1 mM-puromycin (adjusted to pH 7.0). The reaction was initiated by the addition of ribosomes (74 µg of RNA) that had [¹⁴C]phenylalanyl-tRNA bound non-enzymically, prepared as described in the Materials and Methods section. Incubations were for 20 min at 37°C. The reaction was terminated and the [¹⁴C]phenylalanylpuromycin formed was extracted by the method of Heintz *et al.* (1968). Ethyl acetate phase (1 ml) was mixed with Bray's (1960) solution (10 ml) and counted for radioactivity with an efficiency of 66%. All assays were done in duplicate and corrected for blank tubes containing no pH5-supernatant fraction. The latter tubes gave the same low values for release as those without puromycin. Values are expressed as percentage release of label previously bound to ribosomes (673 c.p.m./tube).

Additions	Release of bound [¹⁴ C]Phe-puromycin (%)
Puromycin	6.7
Control pH5-supernatant fraction + puromycin	48.6
Dystrophic pH5-supernatant fraction + puromycin	24.1

the ribosomal 'A' site and acts with ribosomal peptidyltransferase activity to produce aminoacylpuromycin (Pestka, 1971). However, the preparation from dystrophic mice catalysed the release of a much lower quantity of phenylalanylpuromycin than did the control preparation (24% compared with 48%). This result, admittedly indirect, suggests that the inhibitory activity in the dystrophic-muscle preparation may be directed toward the peptidyltransferase step of elongation. The inhibition is certainly acting at a step subsequent to aminoacyl-tRNA binding.

Discussion

The results show that a substance that is inhibitory to protein synthesis can be prepared from muscle of control or dystrophic mice by gel filtration and ion-exchange chromatography. The inhibitory activity in the soluble postmicrosomal fraction and in the pH5 supernatant obtained from dystrophic muscle is detectable in assays where protein synthesis is directed by poly(U) and also by endogenous mRNA bound to ribosomes from either mouse muscle or rat liver (Petryshyn & Nicholls, 1976). The inhibitory activity in control-muscle preparations could only be detected when the cytosol fractions were purified by ion-exchange chromatography. The control preparation was approximately half as active, per mg of protein, as the preparation from dystrophic mice.

Since there was a possibility that some of the control mice might carry the dystrophic trait, homozygous normal mice were studied. In addition, mice bearing a different and milder form of dystrophy (C57B1/6J¹ dy^{2J}/dy^{2J}) were studied. In both cases the results resembled those for the control mice used in the experiments reported here. When the pH 5-supernatant preparations from control and dystrophic mice were subjected to isoelectric focusing and gel electrophoresis, as described for Fig. 4, it was not possible to detect any difference in the pattern of polypeptides. All these results suggest that the inhibitor does not arise from synthesis *de novo* in dystrophic muscle.

The inhibitor might be a substance not normally present in amounts sufficient to block protein synthesis. Its presence could be due to increased entry from the extracellular compartment, or from overproduction or decreased degradation within the muscle itself. In this regard, Ionasescu *et al.* (1971) noted a decreased capacity of the postmicrosomal supernatant fraction for protein synthesis in muscle preparations derived from humans with Duchenne muscular dystrophy.

Another possibility is that there might be decreased activity of an 'anti-inhibitor' in preparations from dystrophic mice. Such a substance might be removed by DEAE-cellulose chromatography. Lee-Huang *et al.* (1977) have reported an 'anti-inhibitor' that regulates an inhibitor of protein synthesis in developing *Artemia salina*. It is unlikely that a similar situation exists in dystrophy, since combining preparations of control-muscle extracts (as a source of 'anti-inhibitor') with dystrophic-muscle extracts (or with inhibitor purified from dystrophic muscle) and incubating them together at 37°C, before the addition of labelled aminoacyl-tRNA, resulted in no decrease in the inhibitory activity.

Previous results from this laboratory showed that the decreased capacity for protein synthesis in the preparations from dystrophic mice could not be attributed to alkaline ribonuclease activity, which might decrease the amount or activity of the mRNA, tRNA or ribosome during the incubation procedure. Furthermore, the decreased protein synthesis could not be attributed to a GTP- or thiol-sensitive inhibitor, as in liver preparations, nor to increased proteolytic activity (Petryshyn & Nicholls, 1976). Thus the elongation and/or initiation phases of protein synthesis were thought to be involved.

The results do not support the view that the inhibitor has any effect on initiation, but rather that the inhibitor exerts its effect on the elongation phase of protein synthesis, at some step of elongation occurring after aminoacyl-tRNA binding. There are limitations to the conclusions that can be made from the results obtained with protein-synthesis inhibitors,

such as aurintricarboxylic acid, chartreusin and fusidic acid. For example, the muscle inhibitor and the exogenous inhibitors may have very different affinities for the target site, the exogenous inhibitors may affect more than one target site at high concentrations, and, in some cases, their precise mode of action is still not clear. The mode of action of puromycin is well established, however. Thus the different response to low concentrations of puromycin with preparations of muscle inhibitor, taken together with the results from the present and the previously reported experiments, show that the inhibitor acts at a step that is subsequent to aminoacyl binding and that may involve the peptidyltransferase step.

It seems unlikely that a direct inactivation of ribosomes or EF 2 is taking place, since the addition of excess amounts did not alleviate the inhibition and since ribosome-associated inhibitors, unlike the one studied here, can be obtained in a ribosomal salt-wash, even after they have exerted an inhibitory effect in the cell-free assay system (Gambino *et al.*, 1973; Goodchild & Nicholls, 1976). It might be possible that peptidyl-tRNA and EF 2 have to be bound to ribosomes for the inhibitor to function. A modified EF 2 is thought to act as an inhibitor of elongation in brine-shrimp embryos (Warner *et al.*, 1977), and EF 2, and its prokaryotic counterpart, under certain conditions, inhibit the functions of EF 1 (Richter, 1973; Nolan *et al.*, 1975; Lee-Huang *et al.*, 1974). Before the observations made here, no inhibitor occurring in mammals had been reported to interfere with steps following aminoacyl binding and translocation, although a number of substances produced by fungi have been shown to inhibit peptidyltransferase activity in mammalian systems (Vazquez, 1974; Innanen & Nicholls, 1974).

The sensitivity of the muscle inhibitor to Pronase indicates that its activity depends on protein structures, but that it is distinct from the heat- and trypsin-sensitive inhibitor in salt-wash preparations of rat liver ribosomes (Goodchild & Nicholls, 1976) and the heat-labile inhibitor in rat liver microsomal fractions (Hoagland *et al.*, 1964). The muscle inhibitor, moreover, has different properties from the inhibitors of protein synthesis described for unfertilized sea-urchin eggs and dormant embryos from *Artemia salina*, since both of these inhibitors are destroyed by trypsin treatment (Metafora *et al.*, 1971; Huang & Warner, 1974).

The results of ribonuclease A, deoxyribonuclease I and Pronase digestion of the inhibitor suggest that it is unlikely that the inhibitor is the same as the translational-control RNA reported by Heywood *et al.* (1974), or the RNA oligonucleotide found in dormant and developing *Artemia salina* (Lee-Huang *et al.*, 1977). The possible association of double-stranded RNA and/or double-stranded DNA with

the inhibitor are not excluded, since these oligonucleotides are resistant to the nuclease treatment that was used. Legon *et al.* (1974) and Farrell *et al.* (1977) have shown that there is an inhibition of initiation by double-stranded RNA that is due to the formation of an inhibitor that appears to be different from the haemin-regulated inhibitor. The haemin-regulated inhibitor, double-stranded RNA, oxidized glutathione and the inhibitor found in lysates prepared from leukaemic cells all inhibit initiation of protein synthesis (Legon *et al.*, 1974; Cimadevilla & Hardesty, 1975) and thus are different from the inhibitor found in muscle. Moreover the haemin-regulated inhibitor has no effect on poly(U)-directed protein synthesis (Clemens *et al.*, 1974). Furthermore, the addition of haemin to our incubation assay does not remove the inhibitory activity. Thus the muscle inhibitor is unlikely to be related to previously reported substances that inhibit protein synthesis.

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