An interesting feature of our data is the fact that *different* groups of aminoacyl-tRNA species are completely acylated by the heterologous enzymes. That is, the *E. coli* synthetases recognize all the species of cotton tRNA for arginine, histidine, lysine, and methionine, whereas the cotton synthetases which react with these homologous species do not recognize all the species of *E. coli* tRNA for these four amino acids. Rather, the cotton synthetases recognize all the species of *E. coli* tRNA for alanine, isoleucine, and valine, yet the bacterial synthetases for these amino acids do not react with the corresponding set from cotton. This observation suggests that the recognition interaction is not necessarily identical in homologous and heterologous systems, since the enzymes from both sources that recognize identical tRNA species from one source do not in turn recognize all the same tRNA species from the second source. Other examples of this phenomenon have been cited by Jacobson (1971), and it must be taken into account by any theory of tRNA-synthetase recognition.

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Protein Synthesis in Chicken Muscular Dystrophy†

Barbara-Anne Battelle‡ and James R. Florini*

ABSTRACT: Activities of ribosome preparations from muscles of normal and dystrophic chickens of various ages were compared. Ribosomes from breast muscles of newly hatched dystrophic chickens were significantly less active in protein synthesis than those from muscles of normal chickens, but this difference was reversed in older animals. The differences in activities could not be explained by elevated RNase activity or by differential loss of polysomes during the isolation procedure. We suggest that the lower activity of breast muscle ribosomes from young dystrophic chickens was caused by a defect in the ribosomes themselves. The basis for the increase in activity of muscle ribosomes from older animals is not clear, but it is not due to increased collagen synthesis. We conclude that the progressive loss of protein from dystrophic muscles cannot be explained by an inability of ribosomes from these muscles to efficiently synthesize protein. However, preliminary qualitative comparisons of proteins synthesized in normal and dystrophic chicken breast muscles showed that there may be changes in the types of soluble proteins synthesized in muscles of older dystrophic chickens.

Baieve and Florini (1970) have reported that a substantial decrease in RNA synthesis (measured in intact muscles or in isolated nuclei) occurs in young chickens with muscular dystrophy. On the basis of assays with Mg^{2+} and Mn^{2+} in the incubation medium, it was suggested that these differences could be attributed to changes in activity or amount of enzyme involved in synthesis of nonribosomal RNA; the enzyme for rRNA synthesis was relatively unaffected by the disease. If this apparent decrease in polymerase II activity leads to lesser availability of mRNA to the ribosomes, then it might be expected that ribosomes from dystrophic muscle would contain fewer polysomes and be less active in protein synthesis than ribosomes from normal muscle. However, studies in which the protein synthetic activity of normal and dystrophic chicken muscle were compared *in vivo* and in intact muscles *in vitro* (Weinstock *et al.*, 1969) showed dystrophic muscle to be more active in protein synthesis than normal muscle.

In an attempt to reconcile the apparently conflicting results of studies of RNA and protein synthesis in dystrophic muscle, we have compared the protein synthetic activity of isolated ribosome preparations (assayed in the transfer reaction) from breast muscles of normal and dystrophic chickens of various ages. We found that ribosomes from breast muscles of newly hatched dystrophic chickens were less active in protein synthesis than ribosomes from breast muscles of comparable normal chickens, but that this difference was reversed with ribosomes from older chickens. The lower activity of ribosomes from young dystrophic animals was not attributable to a lack of mRNA but rather to some defect in the ribosomes themselves. The cause of the increased activity of ribosomes from older dystrophic chickens was not clear, but it was not associated with an increased synthesis of collagen. Preliminary

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results of qualitative comparisons of the types of proteins synthesized in normal and dystrophic chicken breast muscle indicated, however, that the types of soluble proteins synthesized in dystrophic muscle differed from those synthesized in normal muscle.

Materials and Methods

Animals. Fertilized eggs of dystrophic chickens were supplied by Dr. Louis Pierro (Department of Animal Genetics, University of Connecticut, Storrs, Conn.) and by Dr. D. W. Peterson (Department of Avian Sciences, University of California, Davis, Calif.). The stock of dystrophic chickens maintained at the University of Connecticut has been bred with White Leghorn chickens and shall be referred to as "Leghorn dystrophic." The stock of dystrophic chickens maintained at the University of California at Davis is of the New Hampshire strain and shall be referred to as "New Hampshire dystrophics." All New Hampshire dystrophics used in this study were of line 304, which is characterized by early onset of muscle weakness and early muscle hypertrophy (Holliday *et al.*, 1968). Normal White Leghorn and normal New Hampshire controls were obtained from a local supplier (Ray Sachs Farms, Camillus, N. Y.). Eggs of normal and dystrophic chickens were incubated together at 37° and 55% humidity in a Petersime Model 1 incubator.

Reagents and Apparatus. Bovine serum albumin and DOC[1] were purchased from Mann Research Laboratories, ATP, GTP, and CTP from P-L Laboratories, GSH from General Biochemicals, lubrol WX from ICI/Organics, collagen and purified *Clostridium histolyticum* collagenase from Worthington Biochemical Corporation, and NCS from Amersham-Searle. [^{3}H]Leucine was prepared by the reduction of Δ^4-leucine as described by Florini (1964); [^{14}C]leucine and [^{3}H]-phenylalanine were purchased from Schwarz Bioresearch; POP (2,5-diphenyloxazole) and dimethyl-POPOP (1,4-bis[2-(4-dimethyl-5-phenyloxazole)]) for the preparation of toluene phosphor (Florini, 1970) were purchased from Fisher Scientific or Research Products International. The tissue press was purchased from Harvard Apparatus Co. and the Polytron 10 OD homogenizer from Brinkmann Instruments. Apparatus used in gel isoelectric focusing was purchased from Canalco and ampholites were from LKB. Acrylamide and bisacrylamide were purchased from Canalco or Eastman and purified in this laboratory (Loening, 1967).

Preparation of Ribosomes, Transfer Enzymes, and Aminoacyl-tRNA Charged with [^{3}H]Leucine or [^{3}H]Phenylalanine. Ribosomes were prepared from chicken muscle by a modification of the procedure described by Ionasescu *et al.* (1970). Chickens were decapitated and bled. The breast muscles were quickly removed and immersed in ice cold medium A (Heywood *et al.*, 1967) containing 0.25 M KCl, 0.01 M $MgCl_2$, and 0.01 M Tris-HCl, pH 7.4. All subsequent procedures were carried out at 0–4°. The tissue was passed through a Harvard Model 142 tissue press and then homogenized with the Polytron at low speed in three volumes (w/v) of medium A to give a uniform homogenate. Passing the muscle through a tissue press prior to homogenization with the Polytron reduced the time and force of homogenization necessary to give a uniform homogenate. The homogenate was centrifuged at 140,000*g* for 1.5 hr (A-147 rotor) in an international B-60 ultracentrifuge. The supernate was reserved for the preparation of transfer enzymes. The pellet was minced in medium A equal to three volumes of the original muscle sample, then resuspended by low speed homogenization with a Polytron. This homogenate was then treated with 0.1 vol of 10% aqueous DOC and 0.1 vol of 5% lubrol in medium A, diluted to 5 vol with medium A, stirred in the cold for 15 min, and centrifuged at 20,000*g* for 15 min in a Sorvall RC2-B. The resulting supernate was layered on 12 ml of 1 M sucrose containing medium A ions and centrifuged at 140,000*g* for 2.5 hr in the B-60 ultracentrifuge (A-147 rotor). The ribosomal pellet was rinsed in medium I (Florini and Breuer, 1965) which contained 0.09 M KCl, 0.016 M $MgCl_2$, and 0.08 M Tris-HCl, pH 7.6, resuspended in medium I with a Kontes glass-glass homogenizer, transferred to a stoppered centrifuge tube, and rotated gently overnight at 4° on a Fisher Roto-Rack. The next morning the suspension was clarified by centrifugation in a Sorvall RC2-B for 10 min at 10,000*g*. The concentration of ribosomes as RNA in the final supernate was estimated from the absorbance at 260 nm (1 mg/ml of ribosomes as RNA = 20 A_{260} units) (Breuer *et al.*, 1964; confirmed in this laboratory by orcinol analyses). The 260:280 and 235:260 absorbancy ratios of ribosome preparations used ranged from 1.65 to 1.89 and 0.54 to 0.76, respectively. (The 260:280 and 235:260 absorbancy ratios were usually between 1.70 and 1.80 and 0.60 and 0.68, respectively.) However, within any single experiment the variation in the 260:280 ratio did not exceed 0.12. The yield of ribosomes varied with the age of the chickens used and was in the range of 10–30% of the total muscle RNA as determined by the orcinol reaction (Colman, 1972[2]).

Transfer enzymes were prepared generally as described by Breuer *et al.* (1964). The initial 140,000*g* supernate was adjusted to pH 5.1 by dropwise addition of 1 M acetic acid, and the resulting precipitate pelleted by centrifugation at 10,000*g* for 10 min in a Sorvall RC2-B and discarded. The supernate was readjusted to pH 7.4 with 1 M KOH and fractionated by addition of solid ammonium sulfate. The 20–35% and 50–65% saturated ammonium sulfate fractions were combined, resuspended in medium I, and desalted by passage through a Bio-Gel P-6 column equilibrated with medium I ions. The protein concentration of the enzyme preparation was estimated from the absorbances at 260 and 280 nm using a nomograph prepared from the data of Warburg and Christian (1942).

For the preparation of pH 5 supernate in medium I, the supernate remaining after the removal of pH 5 precipitate was readjusted to pH 7.4 with 1 M KOH and dialyzed overnight against 100 vol of medium I. The protein and nucleic acid concentrations of the pH 5 supernate were also estimated from the nomograph.

Transfer RNA charged with [^{3}H]leucine was prepared from chicken liver pH 5 precipitate by the method of Bloemendal *et al.* (1962).

Analysis of Ribosome Preparations. Standard conditions used for *in vitro* transfer of labeled amino acids from aminoacyl-tRNA into protein were essentially those described by Florini and Breuer (1965). Each incubation tube contained 2.5 μmol of ATP, 0.8 μmol of GTP, 10 μmol of GSH, 1 mg of transfer enzymes, 0.1 mg of tRNA, and ribosomes, all in medium I. The final volume was 1.0 ml. In all assays of ribosome activity, transfer enzymes were from the breast muscles of normal (rather than from dystrophic) chickens. Reactions were started by the addition of ribosomes, allowed to proceed for 15 min at 37°, and stopped with the addition of 3 ml of

[1] Abbreviations used are: DOC, sodium deoxycholate; NCS, Nuclear Chicago solubilizer; GSH, reduced glutathione.

[2] J. T. Colman, unpublished results.

10% Cl_3CCOOH (w/v). After the mixtures were allowed to precipitate for at least 1 hr, they were heated in a 90° water bath for 30 min. The hot acid-insoluble precipitates, collected by centrifugation at 2600 rpm for 3 min in a Sorvall GLC-1 centrifuge, were washed by resuspension and pelleting two times with 5% Cl_3CCOOH (w/v), and once each with ethanol, ethanol–ether (1:1), and ether. To dissolve the final precipitate, 0.3 ml of NCS was added to each incubation tube. Toluene phosphor (10 ml) was added to each incubation tube in 5-ml aliquots, mixed with the solublized precipitates, and poured into glass vials. The vials were cooled and counted as described below. Incorporation was approximately ten times the blank for all experiments reported.

For assays of the transfer of [^{3}H]phenylalanine into protein by ribosomes in the presence of poly(U), conditions were identical with those described for [^{3}H]leucine except that 200 μg of poly(U) in medium I was added to each incubation tube.

Procedures used to determine collagen synthesis *in vitro* followed generally those described by Ionasescu *et al.* (1971). The transfer of [^{3}H]leucine from leucyl-tRNA into protein was allowed to continue for 15 min under conditions identical with those described by Florini and Breuer (1965) except that β-mercaptoethanol (4 μmol/tube) was substituted for GSH as the reducing agent. GSH reportedly inhibits the activity of collagenase (Seifter and Harper, 1970). At the end of the 15-min incubation period, 0.1 ml of medium I containing 8 μg/ml of collagenase was added to a set of tubes and 0.1 ml of medium I without collagenase was added to a set of control tubes. The tubes were then allowed to incubate at 37° for another 15 min. The reaction was stopped at the end of the 30-min total incubation time by the addition of 3 ml of 10% (w/v) Cl_3CCOOH. The precipitates were washed either as described previously using a hot Cl_3CCOOH step or they were washed as described below using NaOH to hydrolyze unincorporated aminoacyl-tRNA.

Cl_3CCOOH-insoluble material was allowed to precipitate in the cold at least 1 hr and then collected by centrifugation for 3 min at 2600 rpm in a Sorvall GLC-1 centrifuge. The Cl_3CCOOH-insoluble pellet was then dissolved in 1 ml of 0.4 M NaOH and allowed to stand at room temperature for 1 hr. At the end of this hour 4 ml of 10% Cl_3CCOOH was added to each tube and the Cl_3CCOOH insoluble material was allowed to reprecipitate in the cold for at least 1 hr. Following reprecipitation, the Cl_3CCOOH-insoluble material remaining after treatment with NaOH was washed and prepared for counting in the same manner as the hot Cl_3CCOOH-insoluble material.

That collagenase was active under the conditions used in this assay was verified using the suspension method described by Seifter and Gallop (1962). Briefly, collagen was suspended in medium I and β-mercaptoethanol and incubated for 30 min at 37°. At the end of the incubation the amount of collagen digested by collagenase was estimated by measuring hydroxyproline released into the collagen filtrates. Hydroxyproline was measured using the procedures of Leach (1959).

Ribosome preparations were analyzed in 12-ml linear 10–40% sucrose gradients prepared with RNase-free sucrose and medium I ions; the gradients were centrifuged for 1 hr at 280,000g (SB-283 rotor) in an International B-60 ultracentrifuge. Gradients to be compared contained equal amounts of A_{260} material layered in approximately the same volume of solution. Routinely 1.0 A_{260} unit of ribosomes was layered on each gradient in 0.05–0.3 ml of solution.

The effect of RNase on polysome profiles was tested by incubating 1 A_{260} unit of ribosomes with RNase (final concentration 50 μg/ml) in medium I for 4 min at 0°. Ribosomes (0.5 A_{260} unit) treated with RNase were layered on sucrose gradients which were prepared and developed as described in the preceding paragraph. The gradients were scanned at 254 nm with an ISCO Model UA-2 ultraviolet analyzer at a flow rate of 6.6 ml/min and a chart speed of 3 in./min.

Determinations of Polysome Degradation. Normal chick embryos (16–18 days) were injected in the leg muscle with 2 mCi of [^{3}H]leucine in saline solution and incubated in the eggs for 5–10 min. The leg muscles were then quickly removed, placed in ice-cold medium A, and forced through the tissue press. Ribosomes were prepared from the embryo muscle and the isolated ribosomes (carrying labeled peptide chains) were mixed with breast muscle homogenates from normal and dystrophic chickens to be carried through a second isolation procedure. Ribosomes were analyzed on sucrose gradients and 0.9-ml fractions were collected. After the addition of 0.2 mg of bovine serum albumin to each sample as carrier, the fractions were precipitated with 20% Cl_3CCOOH (w/v). The samples were allowed to precipitate in the cold and precipitates were collected by centrifugation at 2600 rpm for 3 min in a Sorvall GLC-1 centrifuge. One milliliter of 5% Cl_3CCOOH (w/v) was then added to each tube and the tubes were heated for 30 min in a 90° water bath. The hot Cl_3CCOOH-insoluble precipitates were collected on 2.5-cm filter paper disks in a Millipore apparatus and washed five times with 5% (w/v) Cl_3CCOOH and two times with ethanol and ether. After the filters were air dried, they were placed in counting vials and 0.4 ml of NCS was added to each vial to dissolve the precipitates. Samples were counted in 10 ml of toluene–phosphor.

To test the effect of RNase on these labeled ribosome preparations, aliquots were incubated with pancreatic RNase at a final concentration of 50 μg/ml for 4 min at 0°. The distribution of label in sucrose gradients of these RNase-treated preparations was determined as described in the preceding paragraph.

Double-Label Mixed-Fractionation Procedures. The procedures used to label chick breast muscle proteins were those described by Florini (1970). Isoelectric focusing of soluble proteins was done in 5% polyacrylamide gels and 6 M urea by the technique described by Catsimpoolas (1969). Isoelectric focusing of residue proteins in 9 M urea was performed using the technique developed by Florini *et al.* (1971) for myosin. Both soluble and residue proteins were focused for 8 hr, and the pH range of ampholites used in all experiments described was 3–10. Techniques used in cutting and counting isoelectric focusing gels were described by Florini (1970).

Determination of Radioactivity. Radioactivity in all samples was determined using a Nuclear-Chicago Unilux II liquid scintillation counter equipped with an external standard. The counting efficiency of each sample was determined using the external standard-channels ratio technique, and net disintegrations per minute were computed using a Programma 101 or 602 computer.

Results

Characterization. The assay system used in this investigation showed all requirements typical for the transfer reaction. Maximal activity depended on the presence of ribosomes, transfer enzymes, and an energy source. There was no incorporation of label above the zero-time blank if ribosomes were excluded from the reaction mixture, and the addition of 10 μg of pancreatic RNase completely inhibited incorporation. The addition of 200 μg/ml of cycloheximide caused a

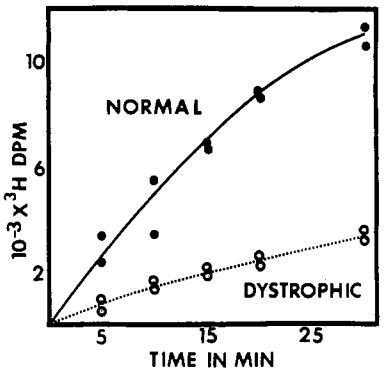

FIGURE 1: Time course of *in vitro* transfer of amino acids into protein. Ribosomes were prepared from 7-day old New Hampshire chickens. Conditions used for the transfer of amino acids from aminoacyl-tRNA into protein have been described in Methods. Between 50 and 100 μg of rRNA were used to determine the time course of transfer of [^{3}H]leucine from leucyl-tRNA into protein; results have been normalized to dpm of [^{3}H] incorporated per 100 μg of rRNA. Zero-time blanks have been subtracted from all values.

70% inhibition of amino acid incorporation, but an equal quantity of chloramphenicol had no effect on the activity of the muscle ribosome system.

As seen in Figure 1, amino acid incorporation was linear for at least 20 min. Similar time courses of incorporation were obtained for muscle ribosomes from all ages and strains of chickens investigated. Assays of ribosome activity were routinely done for 15 min, well within the linear range. In all assays of ribosome activity, ribosomes were the only variable in the reaction mixtures and at least five separate determinations at three different ribosome concentrations were done to assure that ribosomes were the only limiting component in every assay (Figure 2).

Comparisons: Normal vs. Dystrophic. Preliminary experiments were done to test the reproducibility of our ribosome isolation and assay procedures. In these experiments, the activities of four ribosome preparations isolated and assayed in parallel from aliquots of the same muscle sample differed by less than 10%. Polysome profiles were sensitive to minor variations in the isolation procedure; indeed, differences in the activity of ribosome preparations from normal and dystrophic muscle were not always consistent with apparent differences in polysome content (Battelle, 1972). We conclude that our assays reliably measure ribosome activity, but that results of sucrose gradient analyses must be interpreted with some caution.

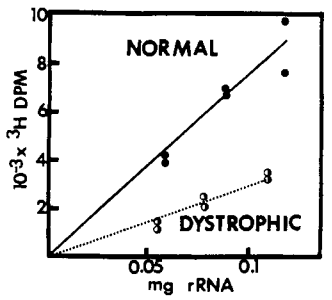

FIGURE 2: Effect of ribosome concentration on the transfer of amino acids into protein. Ribosomes were prepared from 4-day old normal and dystrophic Leghorns. Ribosomes were assayed using the standard procedures described in Methods, and blanks were subtracted from all values.

TABLE I: Average Relative Activity of Ribosome Preparations from Breast Muscles of Normal and Dystrophic Chickens.[a]

Age (Days)	Average Relative Activity of Ribosome Preparations, Dystrophic/Normal	
	Leghorn	New Hampshire
3–7	0.565 ± 0.08* (8)	0.287 ± 0.02 (4)
18	1.22 (2)	
21		0.96 (2)
27–33	1.58 ± 0.08* (5)	1.49 ± 0.60 (3)
39–44	1.57 ± 0.28* (8)	1.34 (2)
49–56	1.40 ± 0.10* (5)	1.80 (2)

[a] The activity of each ribosome preparation was expressed as the average disintegrations per minute of [^{3}H]leucine transferred from leucyl-tRNA into protein per milligram of ribosomes incubated in at least five separate determinations using three different ribosome concentrations (see Figure 2). The number in parentheses indicates the number of experiments used in determinations of average relative activities. Each experiment consisted of at least one normal and one dystrophic ribosome preparation isolated and assayed in parallel. Statistical analyses were done using a two-way analysis of variance and the asterisks denotes $P < 0.05$. The average relative activities are presented ± the standard error of the mean when more than two determinations were made.

The activities of ribosome preparations from breast muscles of normal and dystrophic Leghorn chickens of various ages were compared. (Only those ribosome preparations isolated and assayed in parallel were compared directly.) The results are summarized in Table I. The significance of the differences observed was tested using a two-way analysis of variance (Simpson *et al.*, 1960; Snedocer and Cochran, 1967). Typical polysome profiles of ribosome preparations from newly hatched and older normal and dystrophic chickens are shown in Figure 3. Ribosome preparations from breast muscles of newly hatched Leghorn dystrophic chickens were significantly less active in protein synthesis than ribosome preparations from comparable normal chicken breast muscles. This difference was reversed, however, with ribosome preparations from older animals. Ribosome preparations from breast muscles of young dystrophic animals also consistently contained fewer polysomes than preparations from normal muscle; results of sucrose gradient analyses of ribosome preparations from breast muscles of older normal and dystrophic animals were more variable. The relatively large variation in results obtained with older animals was caused by biological variations, particularly among the normal chickens (Battelle, 1972). Changes in the relative activities of ribosome preparations from breast muscles of normal and dystrophic New Hampshire chickens (dystrophic line 304) of different ages were similar to those observed with Leghorn chickens.

Results of a single experiment with 20-day old chick embryos showed that the ribosome preparation from dystrophic embryo breast muscle was 57% as active as the preparation from normal embryo muscle. (Because of the large number of embryos required, this experiment was not repeated.)

Leg muscle of dystrophic chickens is reportedly less severely affected by the disease than breast muscle (Asmundson *et al.*, 1966). As an additional control, therefore, we did several

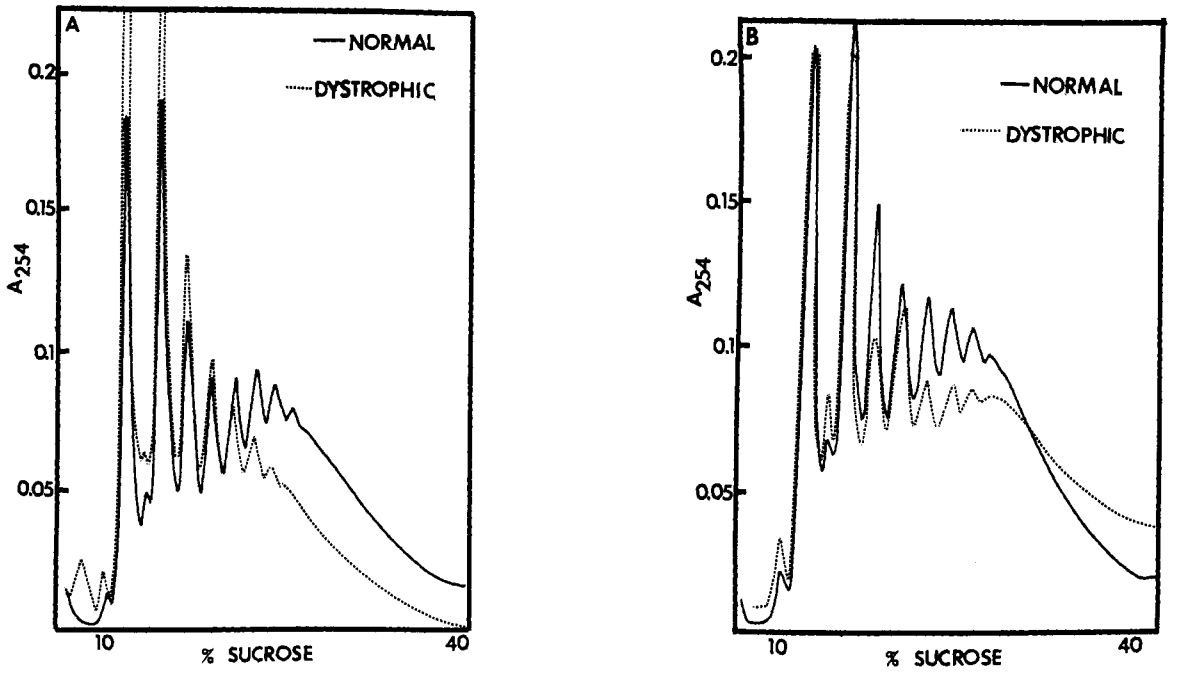

FIGURE 3: Sucrose gradient analyses of ribosome preparations from normal and dystrophic chicken muscle. One A_{260} unit of ribosomes was layered on 12 ml sucrose gradients, centrifuged, and scanned as described in Methods. Ribosomes were prepared from (A) 4-day old normal and dystrophic Leghorns and (B) 44-day old normal and dystrophic Leghorns.

comparisons of the activity of ribosomes prepared from leg muscles of normal and dystrophic chickens. The relative differences between activities of normal and dystrophic leg muscle ribosomes were less pronounced than those observed between activities of normal and dystrophic breast muscle ribosomes. The average relative activity of normal and dystrophic leg muscle ribosomes (dystrophic/normal) for three experiments with 3–7 day old Leghorn chickens was 0.829 ± 0.100. Ribosomes prepared from leg muscles of Leghorn dystrophic chick embryo were as active as parallel preparations from leg muscles of normal chick embryo.

Several experiments were also done to investigate the protein synthetic activity of pH 5 supernate from normal and dystrophic chicken breast muscles. Representative results are shown in Figure 4. The activities of pH 5 supernates from breast muscles of young normal and dystrophic chickens were similar (per milligram of pH 5 protein incubated), but pH 5 supernate from older dystrophic chickens was more active in protein synthesis than that from normal chickens. The activity of ribosomes was not affected by the source of pH 5 supernate under our standard conditions. When ribosomes were incubated with saturating pH 5 supernate from either normal or dystrophic chicken breast muscle (from old or newly hatched chickens), the incorporation per milligram of ribosome was the same in both cases. We conclude that the concentration of factors active in protein synthesis (per milligram of pH 5 supernate protein) was greater in the pH 5 supernate from older dystrophic chickens compared to normals. However, the pH 5 supernates from neither source contained detectable activators of ribosome activity.

We emphasize that these differences in activity of pH 5 supernates do not contribute to the differences in activity of ribosomes reported in Table I. The transfer enzymes used in assaying ribosome activities were prepared by ammonium sulfate fractionation of pH 5 supernate of normal (not dystrophic) muscle, and transfer enzymes were present in substantial excess in the reaction mixture.

Possible Explanations. One possible explanation for the lower activity and polysome content of ribosome preparations from young dystrophic chickens was a greater activity of RNase in dystrophic muscle. Increases in RNase activity in muscles of dystrophic chickens were reported by Tappel *et al.* (1962). In preliminary experiments, the reported RNase inhibitor polyvinyl sulfate was included in the isolation medium (60 μg/g of muscle). Subsequently we determined that polyvinyl sulfate caused an artifactual loss of ribosomes from the

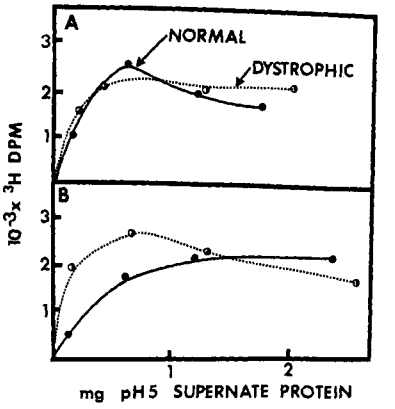

FIGURE 4: Activity of pH 5 supernate from normal and dystrophic chicken breast muscle. Ribosomes from dystrophic muscle (approximately 0.1 mg of rRNA) were incubated with different concentrations of pH 5 supernate proteins from normal and dystrophic chicken breast muscle. Assays were done for 15 min in medium I, stock and tRNA as described for standard assays in Methods. At the end of the incubation, bovine serum albumin was added to each tube so that the total amount of protein was the same in each tube. This was done to standardize conditions for the precipitation and washing procedures. Samples were washed using the NaOH procedure described in Methods. Ribosomes and pH 5 supernate were prepared from (A) 5-day old Leghorn and (B) 39-day old Leghorn chickens. Each point represents a single determination.

TABLE II: Assay of Degradation of Polysomes from Muscle of Normal and Dystrophic Chickens.[a]

Ribosome Preparation	Dpm × 10^{-4}[b]			RNase Treated[c]		
	Total[d]	Monosomes[e]	% in Polysomes	Total	Monosomes	% in Polysomes
Embryo	32.0	8.2	75	16.4	13.7	18
Normal + embryo	13.4	4.5	66	9.3	6.3	32
Dystrophic + embryo	15.6	4.3	73	7.5	5.9	21

[a] Ribosomes (carrying labeled peptide chains) isolated from the leg muscles of normal chick embryo were added to homogenates of breast muscle from normal and dystrophic 7-day old New Hampshire chickens. Ribosomes were isolated from these homogenates and the distribution of label in sucrose gradients of these preparations was determined. [b] Approximately 2 A_{260} units of embryo ribosomes and 5 A_{260} units of normal + embryo or dystrophic + embryo ribosomes were analyzed on gradients. [c] Approximately 1.5 A_{260} units of embryo ribosomes and 2.5 A_{260} units of normal + embryo or dystrophic + embryo ribosomes treated with RNase as described in Methods were analyzed on gradients. [d] Disintegrations per minute recovered in the entire polysome profile. [e] Disintegrations per minute recovered in the monosome region which also included dimers and some trimers.

muscle ribosome preparations. Furthermore, at the high ionic strength used in the isolation of muscle ribosomes, this polyanion also had no effect on the activity of pancreatic RNase (Battelle, 1972). Bentonite and heparin were also tried as RNase inhibitors in our preparations of muscle ribosomes, but each was found unsatisfactory.

Because no RNase inhibitor was used during the isolation of normal and dystrophic muscle ribosomes, experiments were designed to monitor polysome degradation during the isolation procedure. Normal chick embryos (16–18 day) were given a 5-min pulse of [^{3}H]leucine and ribosomes carrying labeled peptide chains were isolated from these muscles. These isolated labeled embryo ribosomes were then added to muscle homogenates of normal and dystrophic 7-day old New Hampshire chickens and ribosomes were isolated again. Fractions of sucrose gradients of the resulting ribosome preparations were collected, precipitated, and counted. Degradation of polysomes during the isolation procedure (either due to mechanical degradation or action of RNase) would be indicated by a shift in counts from polysomes to the monosome region of the gradient. Results of these experiments are summarized in Table II. As expected, treatment with pancreatic RNase shifted counts from the polysome region to that of monosomes. No such shift occurred during the preparation of ribosomes from normal and dystrophic muscle. From these results it was concluded that there was no substantial or differential degradation of polysomes from muscles of young normal and dystrophic chickens during the isolation procedure.

The activities of mixtures of ribosomes from normal and dystrophic muscle were sums of individual activities (Table III). Thus, it appeared that the differences in the activities of ribosomes from young normal and dystrophic chicken breast muscle could not have been caused by soluble RNase (or other soluble activators or inhibitors of protein synthesis) present in the purified ribosome preparations.

Another possible artifact that could have caused lower polysome content and activity of ribosome preparations from yound dystrophic animals was a differential loss of polysomes from dystrophic muscle preparations by coprecipitation with myosin. This possibility was investigated during the isolation of ribosomes from normal and dystrophic breast muscle homogenates seeded with ribosomes carrying labeled peptide chains (Table II). The recovery of label was determined at every step of the preparation and the per cent recovery of label throughout the isolation procedure was the same for preparations from normal and from dystrophic muscle.

The reduced polyribosome content of ribosome preparations from young dystrophic chickens was consistent with the

TABLE III: Activity of Mixtures of Ribosomes Prepared from Normal and Dystrophic Chicken Muscle.

Age (Days)	μg of Ribosomes Incubated	Activity[a]	
		Calcd[b]	Obsd
20E[c]	68 normal 57 dystrophic	17,600	16,100
3	46 normal 42 dystrophic	12,000	12,000

[a] Activity is expressed as disintegrations per minute of [^{3}H]leucine transferred from leucyl-tRNA into protein per milligram of ribosomes incubated. [b] The calculated activity was determined from the average activity of each ribosome preparation determined in assays described in Methods and in the legend to Table I and illustrated in Figure 2. [c] 20-day embryo.

TABLE IV: Activity of Ribosomes from Normal and Dystrophic Chicken Breast Muscle in Poly(U)-Directed Phenylalanine Incorporation.[a]

Conditions	Activity (dpm/mg of rRNA × 10^{-4})		Rel Activity Dystrophic/ Normal
	Normal	Dystrophic	
No Poly(U)	3.07	1.00	0.326
+ poly(U)	29.1	10.4	0.357

[a] Ribosomes were prepared from breast muscles of 4-day old normal and dystrophic Leghorn chickens. Assays were performed as described in Methods. Each tube contained 200 μg/ml of poly(U) as indicated and 0.1 mg of tRNA charged with [^{3}H]phenylalamine (3.4 × 10^6 dpm/mg of tRNA).

TABLE V: Determination of *in Vitro* Collagen Synthesis.[a]

Washing Procedure	Condition	dpm/mg of rRNA × 10^{-5} Normal	Dystrophic
Hot Cl_3C-COOH	− collagenase	2.96	5.10
	+ collagenase	2.93	5.95
NaOH	− collagenase	3.36	6.92
	+ collagenase	3.43	6.57

[a] Ribosomes were prepared from breast muscles of 43-day old normal and dystrophic Leghorn chickens. Conditions for the assay of collagen synthesis are described in Methods. Results are expressed as dpm of [^{3}H] in insoluble material remaining following a hot acid or NaOH wash. Each value is the mean of duplicate assays done either with or without added collagenase.

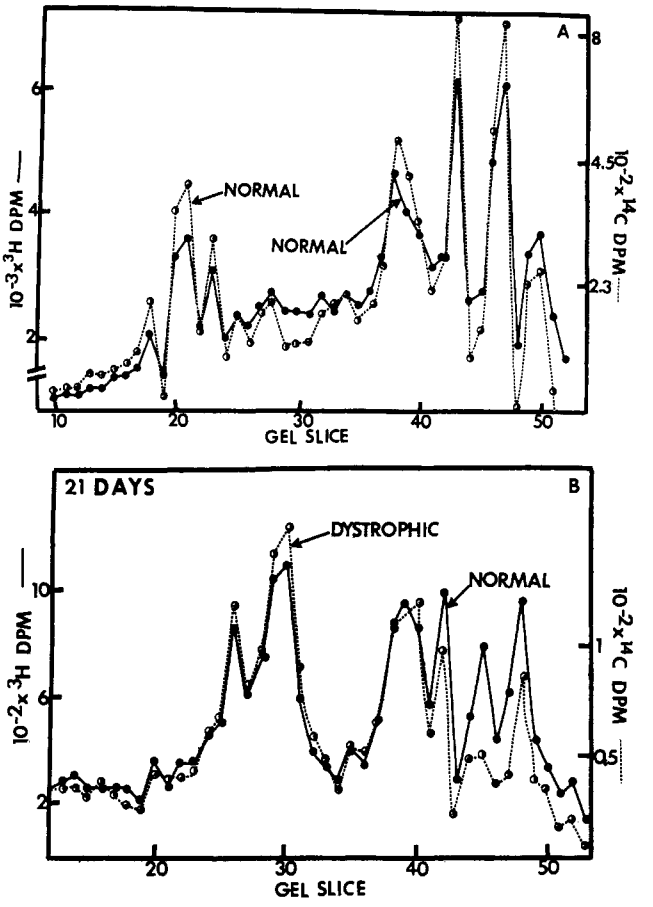

FIGURE 5: Qualitative comparisons of soluble proteins synthesized in chicken breast muscles. Pieces of chicken breast muscle were incubated *in vitro* as described in Methods, pooled, and homogenized together. Soluble and residue proteins were separated by ultracentrifugation. The soluble proteins were analyzed by isoelectric focusing (pH 3–10) in 5% polyacrylamide gels in 6 M urea. The top portions of the focused gels have not been included in the figures because they contained little radioactivity. (A) Proteins synthesized in breast muscles of the same 11-day old normal chicken; (B) proteins synthesized in breast muscles of 3-week old normal and dystrophic chickens.

suggestion of Baieve and Florini (1970) that a lack of mRNA might cause lower activity of ribosomes in dystrophic muscle. To test this possibility, we measured the activity of ribosome preparations from newly hatched normal and dystrophic chickens in the presence of the synthetic messenger, poly(U). The results of one such experiment are shown in Table IV. Similar results were obtained consistently with ribosomes from newly hatched normal and dystrophic Leghorn and New Hampshire chickens. As in all the assays reported herein, incorporation was linear with ribosome concentration, confirming that ribosomes were the limiting component of the assay. Poly(U) stimulated the incorporation of ^{3}[H]phenylalanine tenfold in both normal and dystrophic muscle ribosome preparations; the ratio of activities remained unchanged. Because poly(U) did not alter the relative activities of normal and dystrophic muscle ribosome preparations, we concluded that the low activity of ribosomes from breast muscles of newly hatched dystrophic chickens was not caused by a lack of mRNA, or by differences in the fraction of ribosomes bearing nascent polypeptide chains (a possible result of differences in transfer enzymes), but by some defect associated with the ribosomes themselves. Dystrophic muscle ribosomes either failed to attach to poly(U), translated poly(U) less efficiently, or both.

Ionasescu *et al.* (1971) found that the increased activity of ribosome preparations from human dystrophic muscle (as compared to normal) was due to increased collagen synthesis. The later stages of dystrophy in chickens as well as in humans is typified by increases in muscle connective tissue (Holliday *et al.*, 1968). Thus, it was desirable to investigate whether the increased activity of ribosome preparations from older dystrophic chickens observed in this investigation could also result from increases in collagen synthesis. Assays of collagen synthesis were done with ribosomes prepared from 40–56-day old normal and dystrophic Leghorn and New Hampshire chickens. Typical results are shown in Table V. To determine if collagen peptides were being solubilized during the hot acid wash routinely used to solubilize unincorporated aminoacyl-tRNA, NaOH was substituted for hot acid in the washing procedure. NaOH causes aminoacyl-tRNA to become acid soluble, but it does not affect the solubility of collagen. Results with both washing procedures were similar. The addition of collagenase to the *in vitro* assay did not cause a substantial solubilization of radioactivity. It was concluded that under the conditions used in this investigation, collagen synthesis made no significant contribution to the observed incorporation.

Preliminary studies for a more general qualitative comparison of proteins synthesized in normal and dystrophic muscle were also done using a double-label mixed-fractionation technique. Proteins were fractionated by isoelectric focusing in polyacrylamide gels. The reliability of the technique was tested by comparing the radioactivity patterns of proteins synthesized by pieces of breast muscle from the same normal chicken (Figure 5a). In this normal–normal comparison all major radioactive peaks coincided throughout the gel. We could not detect differences between the patterns of soluble or residue proteins synthesized in breast muscles of newly hatched normal and dystrophic chickens. Nor could we detect differences between the patterns of residue proteins synthesized in breast muscle from older normal and dystrophic chickens. However, a striking difference was seen between the radioactivity patterns of soluble proteins synthesized in breast muscles of older normal and dystrophic chickens (Figure 5b); at least one major radioactive protein peak was considerably reduced in

the pattern obtained from dystrophic muscle. We conclude that soluble proteins synthesized in breast muscles of older dystrophic chickens differed qualitatively from those synthesized in breast muscles of normal chickens of the same age. Because of the difficulty in obtaining adequate labeling of muscle proteins with whole pieces of muscle incubated *in vitro*, these qualitative comparisons of proteins synthesized by normal and dystrophic muscle are currently being extended in this laboratory using tissue culture techniques.

Discussion

Studies by Weinstock *et al.* (1969) on protein synthesis in dystrophic chicken breast muscle *in vivo* and *in vitro* with muscle minces showed that the rate of incorporation of labeled amino acids into protein (per milligram of protein) was greater in dystrophic muscle as compared to normal. Results reported here on the activity of ribosome preparations from normal and dystrophic chicken breast muscle are at least partially consistent with these earlier studies. Ribosome preparations from the muscles of Leghorn dystrophic chickens between the ages of 18 and 56 days of age were more active in protein synthesis than preparations from comparable normal chickens. We have shown that the increased protein synthetic activity of dystrophic muscle ribosomes could not be attributed to increased collagen synthesis, but our preliminary investigations of qualitative differences in proteins synthesized in normal and dystrophic muscles suggested that there were other differences in the populations of proteins synthesized in breast muscles of 3- and 5-week old normal and dystrophic chickens.

In contrast to the results of Weinstock *et al.* (1969) who found no differences in the rate of amino acid incorporation into protein of normal and dystrophic chick embryo breast muscle, we observed that ribosome preparations from breast muscles of dystrophic chick embryo and newly hatched dystrophic chickens were less active in protein synthesis than preparations from muscles of normal chickens. The lower activity of ribosome preparations from newly hatched dystrophic chickens could not be explained by increased RNase activity in dystrophic muscle or by a differential loss of polysomes from dystrophic muscle ribosome preparations. Nor could the lower activity and lower polysome content of these ribosome preparations be attributed simply to a lack of mRNA as suggested by Baieve and Florini (1970); the relative differences between the activities of ribosome preparations from newly hatched normal and dystrophic chickens were also observed in poly(U)-directed phenylalanine incorporation. Furthermore, the reduced synthesis of nonribosomal RNA observed by Baieve and Florini (1970) persisted in the muscles of dystrophic chickens up to about 42 days of age; ribosomes isolated from 42-day old dystrophic chicken breast muscle were substantially more active in protein synthesis than ribosomes from normal muscle. We hope that the inconsistencies between our results and those of Baieve and Florini (1970) can be resolved by specific analyses of the types of RNAs synthesized in normal and dystrophic muscle; these studies are now in progress.

We conclude that the lower activity of ribosome preparations from newly hatched dystrophic chicken breast muscle probably results from some defect in the ribosomes themselves; it may be caused by changes in ribosomal proteins or in the degree of phosphorylation of ribosomal proteins. Nihei and Tataryn (1971) have recently reported differences in proteins associated with ribosomes from normal and dystrophic mouse muscle, and reports from a number of laboratories (Kabat, 1970; Traught and Traut, 1972) have suggested that the activity of ribosomes may be altered by the phosphorylation of ribosomal proteins.

Our data on the activity of ribosomes from dystrophic chicken muscle correlate best with the morphological description by Ashmore and Doerr (1971) on increases in muscle fiber size which occur in dystrophic chicken breast muscle during the first month after hatching. This correlation makes it tempting to suggest that ribosomes from dystrophic muscle are actively engaged in the synthesis of proteins responsible for muscle fiber enlargement. It seems clear that dystrophic muscle degeneration cannot be explained by an inability of dystrophic muscle ribosomes to efficiently synthesize proteins.

From the data now available, it must be concluded that the muscular dystrophies of chickens, mice, and men differ substantially at the molecular level; it is possible that the muscular dystrophies, although pathologically similar, may result from different molecular lesions. Alterations in protein synthetic activity have been identified in chicken and human dystrophic muscle (this report; Weinstock *et al.* 1969; Ionasescu *et al.*, 1971), but the most reliable data on mouse dystrophy suggest that there is no change in the rate of protein synthesis in dystrophic mouse muscle as compared to normal (Watts and Reid, 1969). Furthermore, the increased protein synthetic activity observed with ribosomes from human dystrophic muscle seems associated with increased collagen synthesis (Ionasescu *et al.*, 1971); we have shown that the increased activity of dystrophic chicken muscle ribosomes was not associated with collagen synthesis. However, both Srivastava (1972) and Monckton and Nihei (1971) have concluded that there are differences in the types of proteins synthesized in normal and dystrophic mouse muscle, and on the basis of our initial qualitative comparisons of proteins synthesized in normal and dystrophic chicken breast muscle, we also suggest that there are differences in the types of proteins synthesized in normal and dystrophic muscle. These qualitative comparisons of proteins synthesized by normal and dystrophic chicken muscle are being continued in this laboratory using tissue culture techniques.

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A Stimulation by Phosphate of Malate Transport and Oxidation in Rat Adrenal Mitochondria†

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ABSTRACT: In isolated rat adrenal cortex mitochondria inorganic phosphate (P_i) stimulated the rates of the following malate-supported reactions: (1) large amplitude swelling (in isotonic ammonium malate); (2) intramitochondrial pyridine nucleotide reduction; (3) 11β steroid hydroxylation; and (4) pyruvate efflux into the medium. Mersalyl or *p*-iodobenzylmalonate, inhibitors of the P_i or malate exchange-diffusion carriers, respectively, either decreased or abolished the increments in these rates due to P_i. KCN inhibited the rate of P_i-stimulated 11β hydroxylation or pyruvate efflux by 60%. A portion of the rate of pyruvate formation which remained in the presence of KCN occurred as a result of a dismutation of malate to succinate and pyruvate. Rotenone inhibited this dismutation. The clear supernatant fluids obtained following centrifugation of sonicated mitochondrial suspensions contained $NADP^+$- and NAD^+-linked malic enzyme activities. It is concluded that rat adrenal cortex mitochondria contain P_i-stimulated malate carrier systems that increase the rate of malate entry to $NADP^+$- and NAD^+-linked malic enzyme(s) contained in the matrix space. The NADPH and NADH are available to steroid hydroxylation or oxidative phosphorylation, respectively, and the pyruvate leaves the mitochondria.

Several of the hydroxylations that occur during adrenal steroid hormone biosynthesis take place within the mitochondria. The NADPH required by the steroid hydroxylases can be generated inside the mitochondria either by the joint action of several NAD^+-linked dehydrogenases and a transhydrogenase or directly by $NADP^+$-linked dehydrogenases (Guerra *et al.*, 1966; Harding *et al.*, 1965; Cammer and Estabrook, 1967; Sauer and Mulrow, 1969). It is now known that specific carriers mediate the exchange of charged molecules, such as the substrate anions, across the inner mitochondrial membrane and it becomes of interest to determine: (1) which substrates provide the major source of the reducing equivalents for steroid hydroxylation; (2) how the substrates are generated outside of the mitochondria and enter the intramitochondrial space; and (3) how they are oxidized. In a previous report we demonstrated that inorganic phosphate (P_i) or arsenate stimulated the rate of malate-supported 11β hydroxylation in isolated rat adrenal cortex mitochondria (Sauer and Mulrow, 1969). P_i was also found to stimulate a malate-dependent formation of pyruvate. Since arsenate and P_i are known to stimulate malate entry *via* P_i–dicarboxylate anion-exchange-diffusion carriers (Chappell and Haarhof, 1967), it seemed likely that a study of the P_i effect would provide information on some of the points outlined above. We report here that P_i stimulates malate entry into rat adrenal cortex mitochondria *via* a P_i–malate exchange-diffusion carrier and that the malate is oxidized by malic enzyme (s) con-

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