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Amino Acid Incorporation into Protein by Cell-free Preparations from Rat Skeletal Muscle. I. Properties of the Muscle Microsomal System

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Rat skeletal muscle contains the enzymes, cofactors, and particulate fractions required for amino acid incorporation into protein by the classical pathway involving amino acid adenylates and amino acyl s-RNA (soluble RNA) as intermediates. The muscle system requires a monovalent cation (NH_4^+, K^+, or Rb^+), and Mg^{++}, an ATP-generating system, GTP, and both microsomes and soluble fraction or *p*H 5 precipitate. Incorporation of phenylalanine is stimulated by the addition of polyuridylic acid. The muscle system is much less active than similar preparations from normal rat liver; this difference could be explained only in part by the large differences in RNA content of the microsome fractions of these tissues, and is not attributable to the presence of inhibitors in the muscle fractions.

The incorporation of labeled amino acids into submitochondrial preparations from bacteria, plants, and various mammalian tissues has been studied extensively in recent years (Simpson, 1962), but thus far no detailed report on incorporation by similar systems from mammalian skeletal muscle has been published.[1] This paper presents a description of the requirements for amino acid incorporation into protein by microsome–*p*H 5 precipitate preparations from rat skeletal muscle and the inhibition or stimulation of this system by various additions to the medium. The muscle system is similar in many respects to systems in which labeling of specific proteins has been demonstrated (Allen and Schweet, 1962; Campbell and Kernot, 1962). However, comparison of the muscle system with similar liver preparations indicates that muscle microsomes are very much less active than liver microsomes even when allowance is made for the rather large differences in RNA[2] content of these fractions. This difference in activity is not attributable to the presence of an inhibitor in muscle microsomes.

[1] A preliminary report of this work was presented at the 145th annual meeting of the American Chemical Society, New York, September, 1963, and a report on the incorporation of labeled amino acids into crude muscle preparations has been published (Florini, 1962).

[2] Abbreviations used in this work: RNA, ribonucleic acid; s-RNA, soluble RNA; ATP, adenosine triphosphate; GTP, guanosine triphosphate; poly-U, polyuridylic acid; Tris, tris(hydroxymethyl)aminomethane; DNFB, dinitrofluorobenzene; DNP, dinitrophenyl.

EXPERIMENTAL

Materials.—L-Leucine-4,5-H^3 was prepared by the catalytic reduction of Δ^4-L-leucine in the presence of carrier-free H_2^3 gas by a procedure similar to that of du Vigneaud *et al.* (1962).[3] Exchangeable tritium was removed by repeated lyophilization from dilute acid, base, and aqueous solution; the leucine-H^3 was then separated from unreduced starting material by paper chromatography in butanol–acetic acid–water (4:1:5). The purity of the radioactive material eluted from the paper strip was established by paper chromatography and extensive countercurrent distribution. The specific activity of the final product was 16 c/mmole. To minimize radiation decomposition, the purified L-leucine-4,5-H^3 was dissolved in a small quantity of water and diluted by ethanol followed by benzene to a final solvent ratio of 1:20:80; the concentration of tritium was 0.5–1.0 mc/ml. This solution was divided into convenient aliquots and stored at −10°. Fresh preparations were made from the original reaction mixture (similarly frozen in water-ethanol-benzene) every 4–6 months.

Coenzymes and other biochemicals were purchased from the following suppliers: L-phenylalanine-U-C^{14} (142 mc/mmole) and GTP, Schwarz BioResearch; ATP, Pabst Laboratories; creatine phosphate (Na salt) and creatine phosphokinase, California Corp. for Biochemical Research or Mann Laboratories;

[3] Δ^4-L-Leucine was generously provided by Dr. R. Purdy, Boston City Hospital.

creatine phosphate (Tris salt), General Biochemicals; ribonuclease, Pentex; polyuridylic acid (poly-U), Miles Laboratories.

Methods.—Muscle from young rats (40–60 g) was used on the assumption that it would be more active in protein synthesis and because it was easier to homogenize than muscle from older animals. In a typical preparation, muscle from the rear legs of 10–12 normal male rats was forced with minimum pressure through a tissue press (Harvard Apparatus Co.) which had been adapted for use with a hydraulic automobile jack. The mince was weighed (usually 25–30 g) and homogenized in 2 volumes of medium containing 0.01 M $MgCl_2$, 0.08 M KCl, 0.25 M sucrose, and 0.05 M tris(hydroxymethyl)-aminomethane adjusted to *p*H 7.6 with HCl. The homogenization was conducted in two stages: first, a coarse homogenization with a loose-fitting Teflon pestle (clearance, tapered from 0.025 to 0.015 in.); second, a finer homogenization with a tighter pestle (clearance, 0.005 in.). At each stage, the pestle was passed up and down three times using minimum pressure. The homogenizer was immersed in an ice-water bath throughout this operation. The homogenate was centrifuged at 15,000 × *g* for 15 minutes and the rather large pellet was discarded. The mitochondria-free supernatant was filtered through cheesecloth to remove lipid aggregates and recentrifuged at 105,000 × *g* for 1 hour. The microsome pellet was resuspended in a volume of the homogenizing solution equal in ml to the weight in g of the muscle mince. The supernatant was adjusted to *p*H 5 with 0.1 M acetic acid, centrifuged at 10,000 × *g* for 10 minutes, and resuspended in 0.4 volume of homogenizing solution. In those experiments in which ion effects were determined, the tissue fractions were suspended in medium from which $MgCl_2$ and KCl were omitted. Both fractions were filtered through three layers of cheesecloth to remove aggregates. All operations were conducted in a cold room or a refrigerated centrifuge at 4°. Liver fractions were prepared by the same general procedure; the coarse homogenization was omitted. Protein content of tissue fractions was determined by the procedure of Lowry *et al.* (1951).

With the exceptions specified in the tables and figures, incubation tubes contained ATP, 10^{-3} M; creatine phosphate, 2×10^{-2} M; GTP, 10^{-4} M; 18 unlabeled amino acids, 10^{-6} M each; creatine phosphokinase, 25–50 μg/ml; sucrose, 0.25 M; Tris-HCl, 0.05 M, *p*H 7.3; $MgCl_2$, 0.01 M; KCl, 0.08 M; 3.12 mμmoles (50 μc) L-leucine-4,5-H^3 or 14 mμmoles (2 μc) L-phenylalanine-U-C^{14}; microsomes (1.0–1.6 mg protein) from 0.5 g muscle; and *p*H 5 precipitate (0.3–0.5 mg protein) from 0.25 g muscle in a final volume of 1.0 ml. In general, incubations were started by adding the microsome–*p*H 5 preparation (at 0°) at zero time to tubes which contained all the other constituents of the reaction mixture. In the *p*H-activity determination, 0.1 ml of HCl or Tris (of a concentration calculated from a titration curve to give the desired *p*H) was added to each incubation tube 10 seconds before addition of the tissue preparation at zero time. Incubations were conducted under air at 37° for 15 minutes. The reactions were stopped by addition of 5 ml of 5% trichloroacetic acid which contained approximately 1 mg/ml of unlabeled leucine or phenylalanine, as appropriate. Precipitates were washed twice with 5 ml cold trichloroacetic acid, extracted with 5 ml trichloroacetic acid in a boiling-water bath for 15 minutes, washed with 5 ml ethanol-ether (1:1), and extracted overnight with chloroform-methanol (3:2) to remove possible amino acid–lipid complexes (Mokrasch and Manner, 1962). The precipitates were suspended in 2 ml 2% aqueous $NaHCO_3$, 2 ml 2% dinitrofluorobenzene (DNFB) was added, and the tubes were capped and placed on a vertically rotating turntable at room temperature for 2 hours. The DNP-proteins were washed with 5 ml 25% ethanol, 95% ethanol, and ethanol-ether (1:1), and then hydrolized in 4 ml HCl-acetic acid (1:1) by heating overnight at 90–95° in tightly-capped tubes.[4] Acid was removed under an air stream in a boiling-water bath, and the residue was dissolved in 2 ml H_2O. The aqueous solution was extracted twice with ether to remove DNP-amino acids, and 0.5-ml aliquots of the aqueous phase were added to 20 ml of polyether 611 phosphor (Davidson and Feigelson, 1957) and counted in a Packard Tri-Carb liquid scintillation spectrometer. Internal standards were added to all samples, counting efficiencies were calculated, and the counting results were thus expressed as disintegrations per minute. Other aliquots of the aqueous phase were diluted as required and analyzed for amino acid content by the ninhydrin reaction (Yemm and Cocking, 1955). Zero-time incorporation (see Results) was subtracted from all incorporation data. Previously reported observations (Florini, 1962) that significant N-terminal labeling occurred in crude muscle preparations indicated the necessity that this modification of the protein-washing procedure of Siekevitz (1952) be followed by the DNFB reaction and extraction (Sanger, 1945) as outlined above. When any steps except the liquid extractions were omitted, the apparent specific activity of the final protein hydrolysate was 20% (or more) higher than that of comparable controls. The lipid extractions were included in the procedure because removal of lipids facilitated subsequent handling of the protein precipitates. Suitable controls were run to establish that dinitrophenylation and protein hydrolysis were essentially complete under the conditions specified, and that less than 10% of DNP-leucine decomposed on acid hydrolysis. Analysis of a pooled sample of the final amino acid mixture by countercurrent distribution followed by paper chromatography demonstrated that all the incorporated radioactivity corresponded to free leucine.

Results

Optimum Conditions for Amino Acid Incorporation.—The muscle system requires the presence of both a monovalent and a divalent cation for maximal incorporation. The specificity of the monovalent cation requirement was determined in the experiments summarized in Figure 1. (Similar results were obtained with liver microsome–*p*H 5 precipitate preparations; Rb^+ and Cs^+ were relatively less active and Na^+ and Li^+ more active than in the muscle system.) The optimum Mg^{++} concentration was 10^{-2} M; no activity was obtained when Ca^{++} or Mn^{++} were substituted for Mg^{++}. Indeed, these cations were strongly inhibitory when added at 0.005 M to tubes containing Mg^{++} at 0.005 M. Similarly, Na^+ and Li^+ were inhibitory when added to replace half of the K^+ requirement. Thus those cations which did not meet the requirements of the muscle system were inhibitors of incorporation when added under conditions in which the total ionic strength of the medium was kept constant.

The *p*H optimum of the muscle system was at neutrality; this is slightly lower than that reported by Sachs (1957) for similar preparations from rat liver.

[4] Heavy-walled screw-capped centrifuge tubes (Kimball No. 45196) were used for all steps in the incubation and washing procedures to avoid transferring the small protein precipitates from one tube to another.

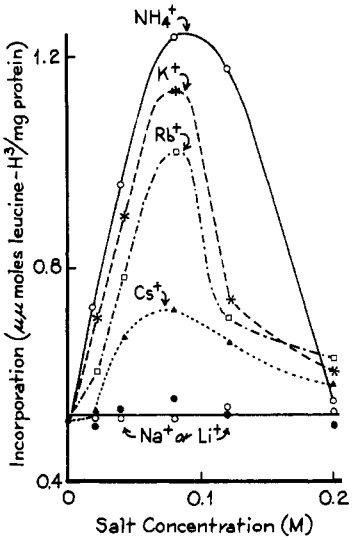

FIG. 1.—Effects of various ions on leucine-H^3 incorporation into protein by rat muscle microsome–*p*H 5 precipitate preparations. Incubation conditions and sample preparations were as described under Methods. ATP and creatine phosphate were added as Tris salts. Thus, except as indicated in the figure, the only monovalent cations present were 4×10^{-4} M Li^+ (added with GTP) and approximately 0.1 M Tris cation.

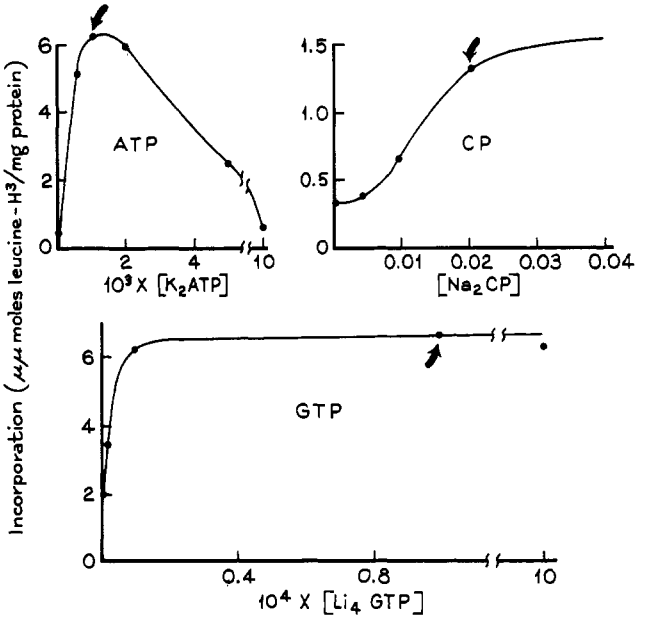

FIG. 2.—Effect of concentrations of coenzymes and energy sources on leucine-H^3 incorporation into protein by rat muscle microsome–*p*H 5 precipitate preparations. Incubation conditions and sample preparations were as described under Methods. ATP, GTP, and creatine phosphate were diluted in solutions of KCl, LiCl, and NaCl, respectively, at concentrations calculated to keep the ion composition of the incubation medium constant as concentrations of coenzyme or energy source were varied. Arrows indicate concentrations used in all other experiments described in this paper.

TABLE I

EFFECTS OF PUROMYCIN, RIBONUCLEASE, AND CHLORAMPHENICOL ON LEUCINE-H^3 INCORPORATION INTO PROTEIN BY RAT MUSCLE MICROSOME–*p*H 5 PRECIPITATE PREPARATIONS[a]

Additions (final concn in incubation medium)	Incorporation ($\mu\mu$moles leucine/mg protein)	Incorporation (% of control)
None (control)	1.85	100
Puromycin		
10^{-3} M	0.32	17
10^{-4} M	0.37	20
10^{-5} M	0.76	41
10^{-6} M	1.54	83
Ribonuclease		
500 mcg/ml	0.34	18
5 mcg/ml	0.30	16
0.5 mcg/ml	0.38	21
0.05 mcg/ml	1.22	66
Chloramphenicol		
10^{-3} M	1.55	84
10^{-4} M	1.87	101
10^{-5} M	1.81	97

[a] Incubation conditions were as described under Methods. The reactions were initiated by adding the microsome *p*H 5 enzyme preparations to tubes containing all other reaction constituents, i.e., there was no preincubation of tissue preparations and inhibitors.

Determinations of the optimum concentrations for various constituents of the reaction mixture gave the results presented in Figure 2. Requirements for an energy-yielding system and GTP are apparent; similar optima were observed with liver microsome–*p*H 5 preparations. The inhibition at 10^{-2} M ATP could be almost completely reversed by increasing the Mg^{++} concentration to 0.02 M. A similar relationship between Mg^{++} and ATP concentration was reported for the liver system (Sachs, 1957). No requirement for unlabeled amino acids could be demonstrated under the conditions of these experiments; presumably sufficient quantities of amino acids were trapped in the microsome pellets. A similar failure to demonstrate a requirement for unlabeled amino acids has been reported in liver preparations (Keller and Zamecnik, 1956).

The muscle system was very sensitive to puromycin and ribonuclease but resistant to chloramphenicol, as shown in Table I.

Mechanism of Amino Acid Incorporation.—The observation that incorporation occurred when liver and muscle fractions were intermixed (see below) strongly suggested that incorporation proceeded by a similar mechanism in these tissues. The existence of amino acid–activating enzymes in mouse skeletal muscle has been reported by Pennington (1960), and we have been able to demonstrate hydroxamate formation from leucine-H^3 in rat skeletal muscle *p*H 5 precipitate preparations using the chromatographic procedure of Loftfield and Eigner (1959). Phenol extraction by the procedure of Hoagland *et al.* (1958) of pooled muscle *p*H 5 precipitates left over from several of the incorporation experiments resulted in the isolation of a typical s-RNA fraction with an ultraviolet absorption maximum at 259 mμ, a minimum at 230 mμ, and a 260/280 ratio of 1.90. Analytical centrifugation in the Spinco Model E ultracentrifuge of a 0.27% solution of muscle s-RNA in 0.1 M NaCl gave a single peak with a sedimentation coefficient of 4.5 S. Incubation of muscle *p*H 5 precipitate, leucine-H^3, and the ATP-regenerating system resulted in the incorporation of radioactivity into the s-RNA fraction reisolated by the phenol procedure (Table II). This radioactivity was released upon mild alkaline hydrolysis of the s-RNA fraction. Hoagland (1960) has reported that s-RNA isolated from mammalian tissues is saturated with amino acids. Thus the data of Table II represent exchange between labeled and unlabeled leucine on leucyl-s-RNA; it is not possible to calculate the extent

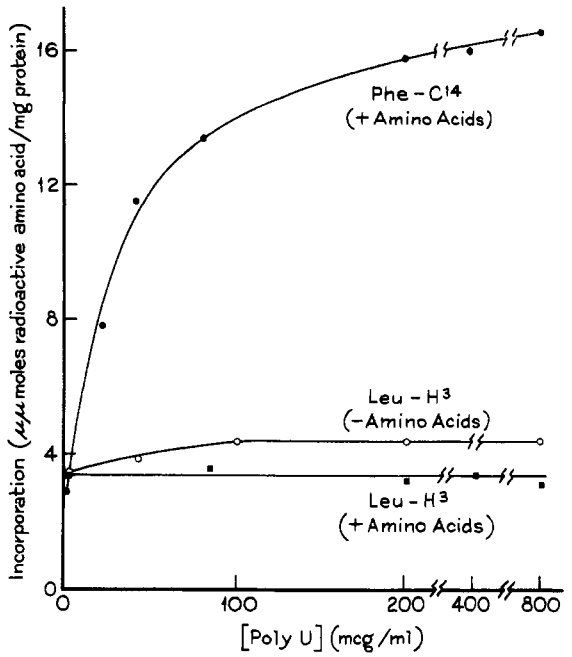

FIG. 3.—Effect of polyuridylic acid concentration on phenylalanine-C^{14} and leucine-H^3 incorporation into protein by rat muscle microsome–*p*H 5 precipitate preparations. Incubation conditions and sample preparations were as described under Methods. Reactions were started by adding the tissue preparations to ice-cold tubes containing all the other components of the incubation mixture; tissue fractions were not preincubated at 37° either with or without polyuridylic acid.

TABLE II

INCORPORATION OF LEUCINE-H^3 INTO S-RNA OF RAT MUSCLE *p*H 5 PRECIPITATE[a]

Incubation Time (minutes)	mMoles Leucine-H^3/Mole s-RNA: Dialyzed Ethanol Precipitate	mMoles Leucine-H^3/Mole s-RNA: After Alkaline Hydrolysis
0	1.1	0.37
5	11.7	0.35
10	12.9	0.48
15	14.1	0.43

[a] Incubation tubes contained 10^{-3} M ATP, 2×10^{-2} M creatine phosphate, 50 μg creatine phosphokinase, 12.5 mμmoles (200 μc) L-leucine-4,5-H^3, and 1.8 ml *p*H 5 enzyme (1.5 mg protein and 150 μg RNA) in a final volume of 2.0 ml. Incubations were conducted at 37°. Reactions were stopped by the addition of 2 ml 88% phenol and s-RNA was isolated by the ethanol-precipitation technique of Hoagland *et al.* (1958). Aliquots of the labeled s-RNA were hydrolyzed in 0.01 N KOH for 20 minutes at 37°, dialyzed, and recounted. Concentration of s-RNA was determined from the OD at 260 mμ assuming an extinction coefficient of 23 ml/mg·cm and a molecular weight of 24,000 (Luborsky and Cantoni, 1962).

of charging of the s-RNA on the basis of these data.

Table III presents the results of an investigation into the transfer of radioactivity from leucyl-H^3-s-RNA (rat liver) to protein when incubated with muscle microsome–*p*H 5 precipitate preparations. The transfer reaction in muscle exhibited many of the characteristics of that in liver (Hoagland *et al.*, 1958); it required GTP and both tissue fractions, but exhibited only a slight decrease in incorporation when the energy system was omitted. The failure of added unlabeled leucine to decrease labeling of protein indicated that leucine-H^3 was not released from the s-RNA and then incorporated by some other pathway.

TABLE III

TRANSFER OF LABEL FROM LEUCYL-H^3-S-RNA TO PROTEIN IN MUSCLE MICROSOME–*p*H 5 PREPARATIONS[a]

Description	Incorporation (net dpm/mg protein)
Control	5.31×10^{-3}
− Energy (ATP, creatine phosphate)	4.63
− GTP	1.39
− Microsomes	0.23
− *p*H 5 fraction	3.13
+ 2×10^{-6} M unlabeled leucine	5.27

[a] *Incubation conditions:* ATP, 10^{-3} M; creatine phosphate 0.02 M; GTP, 10^{-4} M; creatine phosphokinase, 50 μg/ml; 18 unlabeled amino acids, 10^{-6} M each; rat liver leucyl-H^3-s-RNA, 1.4 mg (1.1 μc); muscle microsomes, 1.2 mg protein; muscle *p*H 5 precipitate, 0.6 mg protein; Tris-HCl, *p*H 7.3, 0.05 M; sucrose, 0.25 M; KCl, 0.08 M; $MgCl_2$, 0.01 M. Tubes were incubated 10 min at 25°. Trichloroacetic acid precipitates were prepared for counting as described under Methods.

TABLE IV

REQUIREMENTS AND INHIBITORS OF POLYURIDYLIC ACID-STIMULATED INCORPORATION OF PHENYLALANINE BY RAT MUSCLE MICROSOME–*p*H 5 PRECIPITATE PREPARATIONS[a]

Additions or Omissions	Incorporation (μμmoles phenylalanine/mg protein)
Control	27.1
− ATP, creatine phosphate	3.7
− GTP	6.6
− *p*H 5 precipitate	6.7
− Microsomes	4.5
− Unlabeled amino acids	49.7
+ 200 μg/ml Rat liver s-RNA	27.6
+ 50 μg/ml Ribonuclease	3.3
+ 0.05 μg/ml Ribonuclease	16.7
+ 10^{-3} M Puromycin	1.7
+ 10^{-5} M Puromycin	15.9

[a] Incubation conditions were as described under Methods, except that 200 μg/ml poly-U was added to each tube.

Stimulation of Incorporation by Polyuridylic Acid.—The incorporation of phenylalanine-U-C^{14} into protein by the muscle microsome–*p*H 5 precipitate system was stimulated about 5-fold upon the addition of polyuridylic acid (Figure 3). Maximal response occurred at poly-U levels of 200 mcg/ml. The stimulation of phenylalanine incorporation was not a result of a change in the time course of the reaction, which was essentially complete in 15 minutes whether or not poly-U was added. As shown in Table IV, the poly-U-stimulated system was similar to the unstimulated system in its requirements and response to inhibitors (see Figure 2 and Table II).

Stimulation by poly-U was considerably less than that reported in preparations from *E. coli* (Nirenberg and Matthei, 1961), *Chlamydomonas* (Sager *et al.*, 1963), or some animal tissues (Weinstein and Schechter, 1962); this difference was apparently not attributable to a lack of s-RNA, as the addition of liver s-RNA had no effect on incorporation. More rapid degradation of added polynucleotide by the muscle preparation could not account for the difference; the data of Figure 3 show that addition of a 4-fold excess of poly-U had little effect on incorporation of phenylalanine.

In Figure 3, data are also presented on another interesting aspect of the poly-U-stimulated incorporation of amino acids—that of the leucine "ambiguity."

TABLE V

RELATIONSHIP BETWEEN MICROSOME COMPOSITION AND LEUCINE-H^3 INCORPORATION INTO PROTEIN BY RAT MUSCLE AND LIVER MICROSOME–*p*H 5 PRECIPITATE PREPARATIONS

Microsomes from	mg Microsomal Protein/g Tissue[a]	μg Microsomal RNA/g Tissue[a]	Incorporation of Leucine-H^3[b] ($\mu\mu$moles/g tissue)	($\mu\mu$moles/mg microsomal protein)	($\mu\mu$moles/μg microsomal RNA
Liver	12	336	678	56.1	2.01
Muscle	4.2	41	2.07	5.12	0.53
Ratio Liver/Muscle	2.9	8.2	31.2	11.0	3.80

[a] Biuret analysis (Gornall *et al.*, 1949) of protein and orcinol analysis (Volkin and Cohn, 1954) of the nucleic acid obtained by fractionation of 5 ml (equivalent to 5 g tissue) of microsome suspension by the procedure of Schneider (1945). [b] Incorporation data from Figure 5; liver microsome–liver *p*H 5 precipitate and muscle microsome–muscle *p*H 5 precipitate incubations.

It has been reported that in preparations from other tissues poly-U stimulates the incorporation of leucine as well as phenylalanine (Sager *et al.*, 1963; Matthei *et al.*, 1962; Lengyel *et al.*, 1961). This phenomenon was observed in the muscle system only when the unlabeled amino acids were omitted, and even then the stimulation of leucine incorporation was so small as to be barely detectable.

Comparisons of Liver and Muscle Preparations.—We have reported previously that crude preparations from muscle were much less active than similar liver systems (Florini, 1962). This difference has been examined in greater detail in the more purified preparations described herein. The close similarities in requirements of the liver and muscle systems allowed their incubation under identical conditions with assurance that these conditions were optimal for both preparations. Incubations of various mixtures of the liver and muscle fractions at a series of dilutions gave the results presented in Figure 4. It was essential to establish that incorporation was linear with microsome concentration in the range under study because inhibition by high microsome concentrations has previously been observed in muscle preparations.[5] When comparisons were made on the basis of an equal wet weight of tissue, the liver system was approximately thirty times as active as that prepared from muscle. It was apparent from the results obtained when fractions from the two tissues were intermixed that the observed differences in incorporation were attributable to differences in activity of the microsome fractions. Thus, the muscle *p*H 5 preparation could catalyze incorporation of fifteen times as much leucine-H^3 when incubated with liver microsomes as when incubated with muscle microsomes, whereas replacement of muscle *p*H 5 fraction by liver *p*H 5 fraction did not increase incorporation by muscle microsomes.

The microsome content of rat muscle is considerably less than that of rat liver. Table V presents the results obtained when the observed incorporation was adjusted for differences in protein or RNA content of the microsome preparations used in these experiments. The differences in incorporation could not be explained simply on the basis of the RNA or protein content of the microsomes.

The muscle microsome fraction contained appreciable adenosine triphosphatase activity. This activity in muscle microsomes was four times that in liver microsomes when assayed by the technique of Ernster *et al.* (1962) and expressed on the basis of equal tissue weights. However, the differences in amino acid–incorporation activity are not attributable to more rapid hydrolysis of ATP in the muscle preparation because addition of more ATP did not increase labeling in the muscle system (Figure 2), and addition of muscle

[5] J. R. Florini, unpublished experiments.

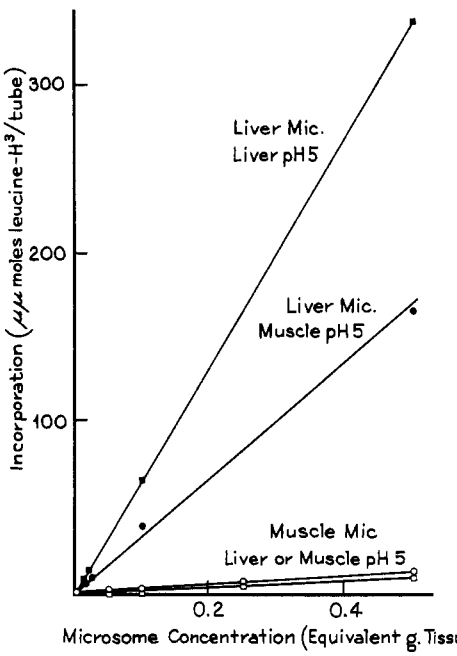

FIG. 4.—Effects of liver or muscle microsome concentrations on leucine-H^3 incorporation into protein. Incubation conditions and sample preparations were as described under Methods, except that tissue fractions were mixed in the incubation tubes at 0° and the reactions were initiated by adding a stock solution containing the other constituents of the incubation mixtures.

microsomes to a complete liver system did not inhibit incorporation by the liver preparation (Table VI). The data of Table VI also demonstrate that muscle fractions did not contain other inhibitors of the liver system. In addition, these data indicate that the differences in activity are not a result of greater dilution of added leucine-H^3 by free unlabeled leucine in the muscle preparations.

Efficiency of Removal of Leucine-H^3 by the Washing-Extraction Procedure.—Comparison of the incorporation data of Figure 5 (12 $\mu\mu$moles leucine-H^3 incorporated/tube in the muscle microsome preparations) with the amount of radioactivity initially present in the tubes (50 μc or 3.12 mμmoles) demonstrates that less than 0.4% of the added radioactivity was incorporated into protein. In order to obtain useful data on variations in the amount of incorporation as reaction conditions were varied, it was essential that the blank be less than 10% of control incorporation, i.e., that at least 99.96% of the leucine-H^3 be removed by the washing procedure. In the experiments reported herein, the zero-time blank varied from 0.14 to 0.43 $\mu\mu$moles of leucine-H^3/incubation tube, and was quite consistent within individual experiments. Thus the washing procedure removed

TABLE VI

EFFECT OF ADDED MUSCLE FRACTIONS ON INCORPORATION OF LEUCINE-H^3 INTO PROTEIN BY LIVER MICROSOME–*p*H 5 PRECIPITATE PREPARATIONS[a]

Muscle Fraction Added	Incorporation ($\mu\mu$moles leucine-H^3/tube)
None	174
0.5 mg *p*H 5 ppt	177
1.0 mg *p*H 5 ppt	181
1.5 mg *p*H 5 ppt	173
0.2 mg microsomes	193
0.4 mg microsomes	191
0.6 mg microsomes	187

[a] Incubation conditions and sample preparations were essentially as described under Methods, except muscle fractions were added as indicated 10 seconds before addition of the liver preparation at zero time. Each tube contained 3.0 mg microsomal protein (from 0.3 g liver) and 1.0 mg *p*H 5 precipitate protein (from 0.15 g liver).

at least 99.987% of the incubated radioactivity. The appropriate zero-time blank values have been subtracted from all results presented in this paper.

DISCUSSION

The foregoing results demonstrate that amino acids can be incorporated into protein by rat skeletal muscle preparations which are similar in all respects except activity to the microsomes and *p*H 5 precipitates prepared from a variety of other sources. The requirement for an ATP-generating system, the charging of and transfer from s-RNA, and the inhibition by puromycin and ribonuclease indicate that incorporation occurs via the classical pathway in which amino acid adenylates and amino acyl–s-RNA are intermediates in the sequence. Stimulation of phenylalanine incorporation upon addition of polyuridylic acid suggests that messenger RNA may be involved in protein synthesis in muscle.

The ion requirements of the muscle system have been defined in detail. The Mg^{++} requirement appears to be specific, whereas any of several monovalent cations will serve. Until recently, K^+ was used almost universally in amino acid–incorporating systems. The inhibitory effect of Na^+ and Li^+ has been reported (Sachs, 1957; Lubin, 1963). No determinations of the effects of Rb^+ and Cs^+ have been published. The greater activity of systems in which NH_4^+ was substituted for K^+ has recently been observed by Lubin (1963) and by Nakamoto *et al.* (1963). Thus the effects of monovalent cations on the muscle system are similar to those which have been published for other systems of cytoplasmic origin, and which we have observed with analogous liver preparations. The sodium requirement and potassium inhibition of incorporation by nuclear ribosomes (Frenster *et al.*, 1960) is in sharp contrast to the observations with cytoplasmic preparations.

It has been reported that labeling of muscle protein by radioactive amino acids *in vivo* occurs almost equally in microsomes and in mitochondria (McLean *et al.*, 1958; Winnick and Winnick, 1960). In exploratory experiments we found that skeletal muscle mitochondria were about one-fifth as active as microsomes from the same weight of tissue. This observation must be regarded as preliminary, however, because the incubation conditions[6] which were optimum for beef heart mitochondria are not necessarily optimum for rat skeletal muscle mitochondria. Furthermore, no attempt was made to correct for differences in isotope dilution by unlabeled leucine in mitochondria as compared to microsomes.

The difference in activity between muscle and liver micrososomes is a potentially fruitful field for further investigation. There are several reasonable explanations for the differences in activity of the two preparations. Differences in isotope dilution or the presence of an inhibitor in the muscle preparations can be dismissed on the basis of the results presented in Table VI. If muscle microsomes contain an appreciable quantity of nonribosomal RNA, then this would cause differences in incorporation when expressed on the basis of RNA content (Table V). Probably the most likely explanation is that differences in activity between muscle and liver ribosomes are a result of differences in their content of messenger RNA. A direct experimental determination of the activities of muscle vs. liver ribosomes has been deferred because of difficulties in the preparation of muscle ribosomes in sufficient quantities. The lower RNA-protein ratio of muscle microsomes suggests that the ribosome content of muscle is lower than that of liver, i.e., that muscle endoplasmic reticulum is relatively "smooth" (Siekevitz, 1962). In preliminary experiments we have prepared ribonucleoprotein particles by deoxycholate treatment of muscle microsomes; these were active in amino acid–incorporation experiments, but yields were very low and RNA-protein ratios were variable. Wool (1963) found approximately one-thirtieth as much labeling in muscle as in liver ribosomes, but interpreted his results as indicating that the muscle preparation was inactive. Further studies on the preparation and properties of muscle ribosomes are now in progress in this laboratory.

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The Reaction of Indole and T2 Bacteriophage*

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Many aromatic compounds bind to bacteriophage T2 and prevent adsorption of the virus to its host cell. Indole and iodobenzene are the most active of a large number of substances tested. All active compounds are either π- or n-electron donors, and their ability to inhibit adsorption is correlated with their ability to form molecular charge-transfer complexes. Indole and other inhibitors cannot react with all of the alternative forms which T2 particles can assume in solution. The phage particles normally participate in a rapid equilibrium between a state in which they can adsorb to *E. coli* B ("active state"), and a state in which they cannot adsorb ("inactive state"). Indole reacts only with the nonadsorbing form of phage, and fixes the particles in the inactive form. The change from active to inactive phage results from a change in the tail-fiber configuration of the T2 particle from an extended to a nonextended state. This alteration of the fibers also affects the hydrodynamic behavior of T2. Bacteriophage T2H in the active state has a velocity sedimentation coefficient 10–15% smaller than it has in the inactive state.

Anderson (1945, 1948) has shown that a medium containing L-tryptophan is required for the attachment of T4 bacteriophage to its host, *Escherichia coli*. The ability of a number of tryptophan derivatives and analogs to substitute for tryptophan was studied (Anderson, 1946), and it was concluded that the NH_3^+, COO^-, and indole moieties of the molecule were all involved in the reaction with T4. Delbrück (1948) subsequently found that the activation of T4 by L-tryptophan was prevented if indole were also present, presumably by competition of the indole with the corresponding part of the tryptophan molecule.

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Bacteriophage T2 does not require tryptophan for adsorption, but indole was observed to inhibit adsorption of a strain of T2 known as T2H (Hershey and Davidson, 1951). We have found that indole will also inhibit T2L, another strain of T2.

The reaction of T2 with indole (like that of T4 with tryptophan) affects the initial attachment of the phage to *E. coli* B. The indole reaction must involve the tail fibers of the phage, since these have been shown in two types of experiments to contain the sites of attachment to the host: (1) Isolated fibers were found to adsorb to host cells and retain their host specificity (Williams and Fraser, 1956); and (2) adsorption of T2 to *E. coli* B was found to be interrupted by a serum which was largely antifiber in activity (Franklin, 1961).

The phage-indole or phage-tryptophan reaction also has been shown by immunological experiments to involve another component of the virus, the contractile sheath-protein. After reaction of phage with sheath antisera, T4 no longer has a tryptophan requirement for adsorption (Jerne, 1956), and indole no longer inhibits the adsorption of T4 or T2 (Jerne, 1956, Brenner *et al.*, 1962).

These observations on the role of both sheath and tail fibers have led to the hypothesis by Brenner *et al.* (1962) that: (a) T2 particles in their normal state and T4 particles which have reacted with tryptophan