INCORPORATION OF LABELED AMINO ACIDS INTO INTERIOR SITES OF PROTEIN BY A CELL-FREE SYSTEM FROM RAT SKELETAL MUSCLE

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Amino acid incorporation into protein by microsomal systems from bacteria, plants, and various mammalian tissues has been studied extensively, but thus far incorporation by similar systems from skeletal muscle has not been reported. (McLean et al. (1958) obtained low levels of incorporation into mitochondria from rat skeletal muscle; they did not report incorporation into microsomes in vitro.) However, when muscle microsomes* from young, rapidly growing rats were incubated with amino acids of extremely high specific activity**, it was possible to demonstrate amino acid incorporation into protein.

Experimental:

Young male rats (50-75 g) were decapitated and exsanguinated. Thigh muscles were removed, forced through a tissue press (Harvard Apparatus Company, Dover, Massachusetts), and homogenized in 2 volumes of a medium containing 0.25 M sucrose, 0.05 M tris(hydroxymethyl)aminomethane, pH 7.6, and KCl, NaCl and $MgCl_2$ as indicated in the figure and tables. The homogenate was centri-

*For purposes of this paper, microsomes are defined as those cellular constituents which are not sedimented at 10,000 x g (15 min.) but are sedimented at 105,000 x g (60 min.). There is reason to believe that such preparations contain mitochondrial debris as well as fragments of the endoplasmic reticulum (Siekevitz, 1962).

**L-leucine-U-C^{14}, 143.4 mc/mmole, was purchased from the New England Nuclear Corporation. L-Phenylalanine-U-C^{14}, 140 mc/mmole, was purchased from Schwarz BioResearch.

fuged at 10,000 x g for 15 minutes. (In some early experiments, centrifugation was at 5,000 x g.) Contents of the incubation media are listed in the tables. The reaction was stopped after 30 minutes by the addition of 5 ml of 5% (w/v) trichloroacetic acid, and the protein precipitate was washed with cold TCA, hot TCA, and organic solvents as outlined by Siekevitz (1952).

In order to eliminate the contribution of N-terminal acylation (see Results) to the apparent incorporation of labeled amino acids into protein, the dinitrofluorobenzene (DNFB) procedure of Sanger (1945) was adapted for routine use with small samples. The DNP-protein was hydrolyzed by heating overnight at 100° in a 1:1 mixture of HCl and acetic acid in a sealed tube. Dinitrophenyl derivatives of those amino acids which had occupied N-terminal positions were removed by extraction with ether; incorporation into internal positions was measured by determination of the specific activity of the amino acid mixture remaining in the aqueous phase. This was expressed as dpm (liquid scintillation counting results corrected for variations in counting efficiency by the internal standard method) per mg protein (ninhydrin analysis of the hydrolysate using a leucine standard curve).

Results:

Early studies on the incorporation of labeled amino acids into protein by the muscle microsome fraction gave results which differed in several important respects from those obtained with microsomes from other tissues. Appreciable labeling of the TCA precipitate occurred in the absence of microsomes - i.e., when the 105,000 x g supernate was incubated with labeled amino acid and the ATP-regenerating system. Incorporation in the presence of microsomes was not completely inhibited even by very high concentrations of RNAase (50 mcg/ml) or puromycin (10^{-3} M).

Analysis of radioactivity in N-terminal positions (Table I) revealed that nearly all of the leucine-C^{14} incorporated by the soluble fraction and an appreciable portion of that in the complete microsomal system was in

Table I

Dinitrofluorobenzene Analysis of Radioactive Protein from Rat Skeletal Muscle Preparations

Muscle Preparation	Total dpm	Ether Extractable dpm	% N-terminal
5,000 x g Supernatant	1940	535	28%
105,000 x g Supernatant	446	392	88%

Incubation tubes contained 1 μmole ATP, 20 μmoles creatine phosphate, 5 μc (0.035 μmoles) L-leucine-U-C^{14}, 60 μmoles NaCl, 5 μmoles $MgCl_2$, and 0.7 ml supernatant per ml of solution. These determinations were made on protein from several incubations. The protein was washed by the Siekevitz procedure and then pooled for DNFB analysis.

N-terminal positions. Ziodrou and Fruton (1959) and Moldave et al. (1959) reported non-enzymatic "incorporation" of labeled amino acids into protein by transacylation from synthetically prepared amino acid adenylates. Although Morgan et al. (1960) demonstrated that transacylation is of minor quantitative importance in purified rat liver systems, it appears that this reaction can contribute significantly to incorporation of labeled amino acids by relatively inactive systems such as that prepared from rat skeletal muscle.

When incorporation into interior sites of muscle protein was determined after removal of N-terminal amino acids by the DNFB procedure, it was possible to obtain almost complete inhibition of incorporation at high concentrations of ribonuclease or puromycin, and incorporation into the soluble fraction was very low (see Table II).

A comparison of the muscle and liver microsomal preparations was made in the experiment outlined in Table III. The liver system was much more active than that from muscle, even when incorporation was corrected for differences in content of free phenylalanine and of microsomal RNA. Results of incubations in which microsomes from one tissue were mixed with soluble fraction

Table II

Effects of "Microsomes", Ribonuclease, and Puromycin on Amino Acid Incorporation by a 10,000 x g Supernatant of Rat Muscle

Muscle Sup.	Addition	Incorporation dpm/mg protein
10,000 x g	none	83.9
105,000 x g	none	2.9
10,000 x g	10^{-3} M puromycin	1.9
10,000 x g	500 mcg/ml RNAase	4.5

Incubation tubes contained 1 μmole ATP, 20 μmoles creatine phosphate, 4 μc (0.028 μmoles) L-phenylalanine-U-C^{14}, 80 μmoles KCl, 10 μmoles $MgCl_2$, and 0.7 ml supernatant per ml of solution. The TCA precipitate was washed by the Siekevitz procedure followed by the DNFB procedure described under "Experimental".

from the other indicated that both fractions of liver were more active than the corresponding fractions of muscle; microsomes appeared to be the limiting component in the muscle system. Labeling of protein by the mixed fractions suggests that incorporation occurred in the muscle system by a mechanism similar to that in liver.

Calculations based on the hemoglobin content of typical muscle preparations indicated that less than 0.1 dpm/mg protein could be attributed to contamination by reticulocyte ribosomes. Bacterial contamination could be eliminated on the basis of the time course of the reaction; incorporation stopped in 20 to 30 minutes. The amount of incorporation was proportional to microsome content over a 25-fold concentration range.

Thus incorporation of labeled amino acids into interior sites of protein by a microsomal preparation from rat skeletal muscle has been demonstrated. The muscle system is much less active than but otherwise similar to that of rat liver.

Table III

Comparisons of Amino Acid Incorporation
by Rat Liver and Muscle Microsomal Systems

Origin of Tissue Fraction		Incorporation		
Micro-somes	Soluble	dpm/mg	moles phenylalanine / mole (100,000 g) protein	moles phenylalanine / mole protein / mg microsome RNA
Liver	Liver	7390	2.05×10^{-2}	7.22×10^{-3}
Liver	Muscle	2450		
Muscle	Liver	178		
Muscle	Muscle	116	2.64×10^{-4}	2.96×10^{-4}

Incubations and sample preparations were as described in Table II. For those cases in which both fractions were from the same tissue, the 10,000 x g supernate was incubated. In the mixed samples, the 105,000 x g pellet from 12.5 ml of 10,000 x g supernatant of one tissue was suspended by gentle homogenization in 12.5 ml of 105,000 x g supernate of the other tissue. Phenylalanine content of the tissues was determined by two-dimensional paper chromatography followed by ninhydrin analysis. RNA content of the washed 105,000 x g pellets was determined by the orcinol reaction.

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