Influence of Protein and Calorie Deficiencies in the Rat on the Energy-Transfer Reactions of the Striated Muscle

BY KUSUM TASKAR AND P. G. TULPULÉ

Nutrition Research Laboratories, Indian Council of Medical Research, Tarnaka, Hyderabad 7, India

(Received 21 October 1963)

Although muscle contributes the largest amount of protein during depletion, this organ is relatively neglected in experimental studies on protein malnutrition because it is difficult to assess directly the total muscle mass of the body at any stage. Holmes, Jones, Lyle & Stanier (1956) observed that the protein content of the muscle of malnourished adults was slightly decreased as compared with that of healthy normal animals. Waterlow and his associates attempted to study the extent of nitrogen loss of muscle in protein-deficient rats (Mendes & Waterlow, 1958) as well as in malnourished infants (Waterlow & Weisz, 1956; Waterlow & Mendes, 1957), and observed that the muscle nitrogen:DNA phosphorus ratio was considerably decreased in the protein-deficiency state. During recovery on high-protein diets, the total muscle nitrogen of the body increased much faster than the nitrogen:DNA phosphorus ratio in muscle. These experiments, however, did not establish whether the sarcoplasmic or fibrillar proteins contributed more to the cytoplasmic protein lost during protein depletion.

Histological and biochemical differences between red and white striated muscles have been investigated by various workers (Szent-Gyorgyi, 1953; Lawrie, 1953; Ogata, 1958, 1960), and it has been demonstrated that white muscle contains more of the soluble anaerobic enzymes and hence that its contractile matter is loosely packed. On the other hand, in red muscle the sarcosomal bodies containing the less soluble oxidative enzymes are far more in number. In the present investigation, the quantitative differences between red-muscle and white-muscle fibres with regard to their important labile constituents and metabolic activities have been studied. It was also decided to extend the studies of Mendes & Waterlow (1958) on the biochemical effects of protein deficiency, calorie deficiency or both to determine if the changes in protein fractions of the muscle would be correlated with their effects on specific respiratory activity and specific aspects of energy-rich phosphate metabolism.

MATERIALS AND METHODS

Animals. Four-week-old albino rats of either sex, weighing 45–60 g., were divided into four groups, care being taken to distribute the litter mates equally. Animals

in group 1 (control) received a basal diet consisting of 65 parts of corn starch, 25 parts of casein, 5 parts of sesame oil, 4 parts of salt mixture (Wesson, 1932) and 1 part of vitamin mixture (Tulpulé & Kshirsagar, 1958). To each rat were administered weekly 100 i.u. of vitamin A and 1.5 i.u. of vitamin D in sesame oil. Group 2 (calorie-restricted) differed from group 1 in that the casein in the diet was increased to 50% at the expense of corn starch, but the rats were allowed only one-half the quantity of the ration consumed by the rats in group 1. Group 3 (protein-restricted) received a diet containing only 2% of rice protein, the remaining protein component being replaced by corn starch in the basal diet. For group 4 (protein- and calorie-restricted) a diet containing 4% of rice protein was given in amounts equal to half of that eaten by the animals in group 3. The daily food intake of protein-deficient animals (group 3) was nearly 80% of that of control animals, and the lowered intake of food could be attributed to loss of appetite due to acute protein deficiency.

The diets ensured comparable intakes of protein in groups 1 and 2 on the one hand and in groups 2 and 4 on the other, and the calorie intake was restricted in groups 2 and 4 to 50% of that for the animals of groups 1 and 3 respectively.

Half the number of animals in each group were killed after 4 weeks and the remaining animals were rehabilitated on the complete diet containing 20% of protein given *ad lib.*, so as to study the effect of a preceding episode of protein deficiency, calorie deficiency or both on the chemical composition of muscle.

Muscle homogenates. The rats were killed by a blow on the neck and rapidly exsanguinated. Two separate pieces of muscle, representing red and white muscle respectively, were dissected out in the cold room at -3° . The group of muscles of the lower leg and of the flexor surface of the foot, including muscle soleus, muscle gastrocnemius and muscle tibialis anterior, represented red muscle, and vastus medialis adductor magnus and adductor longus brevis, taken from the frontal surface of the thigh, represented white muscle. The weighed portions of muscles, usually 100–150 mg., were cut up finely with scissors and then homogenized in a glass homogenizer fitted with a Teflon pestle driven at 1000 rev./min. The fluids used for extraction depended on the experiment to be performed and they are described below. For creatine phosphate determinations and creatine-phosphokinase assay procedures, the minced tissue was disintegrated in a Mickel cell disintegrator (H. Mickel, Gomshall, Surrey).

Fractionation of muscle proteins. The method described by Robinson (1952) for the quantitative fractionation of intracellular and extracellular proteins of muscle was followed in the present investigation. Weighed samples of red muscle and white muscle were separately homogenized as described above, in two different salt solutions:

(a) dilute salt solution (0.1 M-KCl-potassium phosphate buffer, pH 7.1 and I 0.2; and (b) concentrated salt solution (1.25 M-KCl-66 mM- K_2HPO_4 , pH 8.5 and I 1.45). Intracellular sarcoplasmic protein fraction (fraction 1), consisting of myogen, myoalbumin and globulin X, was extracted from the homogenized muscle by dilute salt solution and separated further by selectively precipitating out the other intracellular proteins by diluting the extract to I 0.1 with 30% (v/v) ethanol. The intracellular fibrillar proteins (fraction 2) were extracted in concentrated salt solution and then precipitated by dilution of the solution to I 0.12 with 19% (v/v) ethanol. The protein fraction insoluble in concentrated salt solution but soluble in dilute alkali formed a complex of intracellular denatured fibrillar proteins (fraction 3), and the proteins of the residue, insoluble in dilute alkali, were extracellular stroma proteins (fraction 4). The protein content of a sample of each fraction was determined by a micro-Kjeldahl procedure. For the determination of intracellular protein content of the muscle (fractions 1 and 2) the proteins of the soluble extract were precipitated with 20% (w/v) trichloroacetic acid before nitrogen was estimated. The non-protein nitrogen of the muscle was estimated separately. The total nitrogen of fresh samples of red muscle and white muscle was also determined and the total protein content, as well as the protein content of different fractions, was calculated by multiplying the nitrogen value by 6.25.

Assay of succinate dehydrogenase. The method of Tulpulé & Patwardhan (1952) was employed. The assay was carried out in a glass cuvette of 3.0 ml. capacity and 1 cm. light-path. A sample (0.1 ml.) of muscle homogenate in 0.9% NaCl solution, containing 10 mg. of fresh tissue, was added to the enzyme reaction mixture containing 0.3 ml. of 0.5 M-sodium succinate, 1.0 ml. of 0.1 M-sodium-potassium phosphate buffer, pH 7.4, and 0.5 ml. of a freshly prepared solution of 2,6-dichlorophenol-indophenol, containing 0.5 mg. of the dye, and the total volume was made to 3.0 ml. with water. The decrease in extinction at 660 m μ was recorded at 1 min. intervals for 4 min. and the activity was expressed as the change in extinction/mg. wet wt. of tissue/hr.

Assay of cytochrome c oxidase. The manometric method of Schneider & Potter (1943) was used with muscle suspensions prepared in 0.1 M-phosphate buffer, pH 7.4. Measurements were made at 10 min. intervals during the first 30 min. and the activities were expressed as μ l. of O_2 uptake/mg. wet wt./hr. at 37°.

Assay of lactate dehydrogenase. The spectrophotometric method of Zimmerman & Weinstein (1956) was employed. A portion of 10% muscle homogenate in 0.1 M-phosphate buffer, pH 7.4, was further diluted so that 1.0 ml. contained the equivalent of 10 mg. wet wt. of muscle. The reaction was started by the addition of 0.1 ml. of the diluted homogenate to a 3.0 ml. quartz cuvette containing 0.5 ml. of 0.5 M-sodium lactate, 0.1 ml. of 0.02 M-NAD and 2.0 ml. of 0.1 M-glycine buffer, pH 10.0, and the increase in extinction at 340 m μ was followed at 30 sec. intervals for 2 min. Lactate-dehydrogenase activity was expressed as the change in extinction/mg. wet wt. of tissue/min.

Assay of aldolase. The enzyme activity was estimated by the method of Taylor, Green & Cori (1948), which is based on the estimation of the alkali-labile phosphate of triose phosphate formed in the aldolase reaction. The enzyme reaction mixture contained 0.1 ml. of 0.01 M-fructose 1,6-

diphosphate, 0.1 ml. of 0.1 M-potassium cyanide and 0.2 ml. of enzyme preparation. After 10 min. at 37° the reaction was stopped with 1.0 ml. of N-NaOH. The inorganic phosphate liberated from triose phosphate by incubating the mixture with alkali for 30 min. at 37° was estimated by the method of Fiske & Subbarow (1925). Blanks without substrate were run together with the samples.

Assay of phosphoglucumutase. This was measured from the disappearance of glucose 1-phosphate from the enzymic reaction mixture (Najjar & Pullman, 1954). The enzymic reaction mixture consisted of 0.1 ml. of 0.02 M-glucose 1-phosphate, 0.1 ml. of 6 mM-MgSO₄, 0.1 ml. of freshly prepared 0.1 M-cysteine and 0.1 ml. of 10% muscle preparation. The reaction was carried out at 37° for 10 min., and the enzyme was inactivated with 2.0 ml. of 2.5 N-H₂SO₄ and the volume made to 5.0 ml. The acid-labile phosphate of glucose 1-phosphate was determined before and after enzymic reaction. The activities of phosphoglucumutase and aldolase were expressed as mg. of inorganic phosphate liberated/g. wet wt. of tissue/hr.

Measurement of endogenous respiration. The respiration of weighed slices (100–150 mg.) of the tissue of uniform thickness was measured in Ringer phosphate solution, pH 7.4 (Kleinzeller, 1940), for 30 min. at 37° in the presence of air in the conventional Warburg apparatus. Respiration was linear and was expressed as μ l. of O_2 uptake/g. wet wt./hr.

Measurement of anaerobic glycolysis. Liberation of CO₂ was measured in Warburg manometers at 37° with nitrogen as the gas phase. The reaction mixture contained 0.2 ml. of 7.5 mM-fructose 1,6-diphosphate, 1.0 ml. of 36 mM-sodium-potassium phosphate buffer, pH 7.4, 0.1 ml. of 0.2 M-MgSO₄, 0.1 ml. of 2.0 mM-ATP, 0.1 ml. of 5 mM-NAD, 0.1 ml. of 0.6 M-nicotinamide, 0.2 ml. of 36 mM-NaHCO₃ and muscle homogenate equivalent to 50 mg. wet wt. of tissue, in a total volume of 3.0 ml. Enzyme activity was expressed as μ l. of CO₂ liberated/g. wet wt./hr.

Assay of adenosine triphosphatase. The assay of Ca²⁺ ion-activated adenosine triphosphatase was carried out by the method of Perry (1952) by estimating colorimetrically the inorganic phosphate liberated by enzyme from ATP in a reaction mixture containing 1.0 ml. of 0.2 M-glycine buffer, pH 9.1, 0.2 ml. of 0.1 M-CaCl₂, 0.3 ml. of 0.05 M-ATP, 0.1 ml. of 10% muscle homogenate in water, and distilled water to bring the volume up to 2.0 ml. The contents of the tubes were incubated at 37° for 30 min. and the reaction was stopped by the rapid addition of 1.0 ml. of 18% (w/v) trichloroacetic acid. For the estimation of Mg²⁺ ion-activated adenosine triphosphatase, the procedure of Lowry, Roberts, Wu, Hixon & Grawford (1954) was used. The concentrations of ATP and the enzyme preparation in the reaction mixture were similar to those used in the studies on Ca²⁺ ion-activated adenosine triphosphatase. The inorganic phosphate released was measured by the method of Fiske & Subbarow (1925). The adenosine-triphosphatase activity was expressed as mg. of inorganic phosphate released/g. wet wt./hr.

Assay of creatine kinase. The enzyme activity was determined by measuring the phosphate transferred from ATP to creatine in a reaction mixture consisting of 0.4 ml. of 0.08 M-creatine, 0.1 ml. of 1 mM-MgSO₄, 0.3 ml. of 5 mM-ATP, 0.4 ml. of 0.4 M-glycine buffer, pH 9.0, and 0.3 ml. of 5% muscle homogenate in water. The other details of the assay were as set out by Kuby, Noda & Lardy (1954).

Separation of acid-soluble phosphorus compounds of muscle. Phosphorus was determined in all fractions by the method of Fiske & Subbarow (1925). The weighed sample of muscle (100–150 mg.) was dispersed in a Teflon homogenizer at 2°, and ice-cold trichloroacetic acid was added to give a final concentration of 10% (w/v). The sample was once again homogenized to ensure the complete extraction of acid-soluble phosphorus compounds and centrifuged at 2°. The clear supernatant was used for the measurement of total acid-soluble phosphorus, ATP and ADP. The total acid-soluble phosphorus was determined in a sample that had been digested in a micro-Kjeldahl flask with 1.0 ml. of 15N-H₂SO₄ and 0.2 ml. of 30% (w/v) H₂O₂. The chemical determination of ATP and ADP was carried out according to the procedure of LePage (1946). All the steps in the quantitative separation of ATP in the barium-insoluble fraction were made below 5°. Finally, washed barium salts of ATP and ADP were analysed for ribose (Kerr & Serai-dasian, 1945) and acid-labile phosphorus.

The quantitative assessment of the total skeletal muscle protein of the body was done in one batch of 48 rats belonging to the four experimental groups. The animals were killed by injecting air into the heart. The carcass was out open and the heart, liver, spleen, kidneys, lungs, brains, sex organs and all the viscera, together with the skin and subcutaneous fat, were removed. The total muscular portion of the body and bones was chopped into small pieces, dried at 100°, powdered and weighed. A portion of the homogeneous dry sample was taken for nitrogen estimation. Protein was calculated as the nitrogen content multiplied by 6.25.

RESULTS

Body weight of rats and muscle nitrogen. The effect of the four diets on the mean body weights and on the estimated body composition with regard to the muscle protein of the rats that were killed at the end of the period of calorie and protein deprivation and after rehabilitation on 'complete' diet are shown in Table 1.

The total nitrogen content of the muscular portion of the body was generally proportional to the body weight in control as well as calorie-deficient groups. In rats given the high-protein

calorie-deficient diet (group 2) for 4 weeks, an average decrease of 32% in body weight and muscle nitrogen was observed when compared with the control group, whereas superimposition of protein deficiency on calorie deficiency (group 4) showed a decrease of about 60%. Protein depletion alone (group 3), however, resulted in a more severe decrease in muscle protein. Although these animals showed a 62% decrease in body weight after 4 weeks, their muscle nitrogen was depleted by nearly 72% during this period. Thus calorie restriction seemed to have given some protection against loss of muscle protein.

After the rehabilitation of the animals in groups 2, 3 and 4 on 'control diet' for 2 weeks, the total body proteins showed a rise commensurate with the rapid gain in body weight. However, the animals in the combined protein-deficient and calorie-deficient group (group 4) gained body weight at a relatively faster rate than that at which they laid down the muscle protein. These observations agree with those of Widdowson & McCance (1957) and do not support the view that animals given low-protein diets store extra amounts of intracellular proteins after rehabilitation, by the removal of water and potassium, as postulated by Holmes, Jones & Stanier (1954).

Changes in muscle protein fractions. Tables 2 and 3 show the total protein content, moisture, non-protein nitrogen and distribution of nitrogen in each protein fraction of red muscle and white muscle respectively in the experimental rats. The protein values are given as g./100 g. wet wt. of muscle and the results of different protein fractions are also expressed as percentages of the total protein of the muscle.

The total protein contents of red muscle and white muscle remained constant within about 20% when protein and calories, either singly or together, were deficient in the diets. The total protein content of white muscle did not differ significantly

Table 1. *Total muscle protein and body weights of experimental rats*

Experimental details are given in the Materials and Methods section. Each value in the Table is an average ($\pm$ S.E.M., where appropriate) of six observations.

Group of rats	Diet	Period of diet (weeks)	Body wt. when killed (g.)	Total muscle N (g.)	Muscle protein (g./100 g. body wt.)
1 (control)	20% protein; <i>ad lib.</i> feeding	4	128	2.01 $\pm$ 0.124	9.89 $\pm$ 0.509
	20% protein; <i>ad lib.</i> feeding	6	189	2.87 $\pm$ 0.163	9.52 $\pm$ 0.168
2 (calorie-deficient)	40% protein; semi-starved	4	87	1.50 $\pm$ 0.082	10.73 $\pm$ 0.409
	Rehabilitation on control diet	2	151	2.22 $\pm$ 0.113	9.25 $\pm$ 0.155
3 (protein-deficient)	2% protein; <i>ad lib.</i> feeding	4	47	0.62 $\pm$ 0.037	8.22 $\pm$ 0.293
	Rehabilitation on control diet	2	101	1.36 $\pm$ 0.062	8.47 $\pm$ 0.141
4 (protein- and calorie-deficient)	4% protein; semi-starved	4	53	0.83 $\pm$ 0.042	9.91 $\pm$ 0.543
	Rehabilitation on control diet	2	108	1.40 $\pm$ 0.069	8.12 $\pm$ 0.557

Table 2. *Effect of dietary deficiency of protein and calories on the protein fractions of red muscle of rat*

Experimental details are given in the Materials and Methods section. Each value in the Table is an average ($\pm$ s.e.m., where appropriate) of six observations; the values in parentheses indicate the results in terms of percentages of total muscle protein.

Group of rats	Diet	Period of diet (weeks)	(g./100 g. wet wt. of muscle)			Muscle protein fractions (g./100 g. wet wt. of muscle)			
			Total protein	Non-protein N	Moisture	Fraction 1	Fraction 2	Fraction 3	Fraction 4
						(intracellular sarcoplasmic protein)	(intracellular fibril protein)	(intracellular denatured protein)	(stroma protein)
1 (control)	20% protein; <i>ad lib.</i> feeding	4	21.4 $\pm$ 1.21	0.607	72.7	8.17 $\pm$ 0.416 (39.4%)	6.45 $\pm$ 0.282 (30.4%)	4.27 $\pm$ 0.688 (19.6%)	2.47 $\pm$ 0.153 (11.6%)
	20% protein; <i>ad lib.</i> feeding	6	22.7 $\pm$ 0.82	—	—	8.61 $\pm$ 0.358 (38.0%)	6.77 $\pm$ 0.424 (29.6%)	4.96 $\pm$ 0.312 (22.0%)	2.43 $\pm$ 0.216 (10.6%)
2 (calorie-deficient)	40% protein; semi-starved	4	21.8 $\pm$ 0.71	—	—	7.53 $\pm$ 0.304 (34.6%)	6.81 $\pm$ 0.135 (31.4%)	4.77 $\pm$ 0.504 (21.8%)	2.68 $\pm$ 0.213 (12.3%)
	Rehabilitation on control diet	2	23.3 $\pm$ 0.79	—	—	8.44 $\pm$ 0.456 (36.2%)	7.25 $\pm$ 0.288 (31.3%)	4.45 $\pm$ 0.255 (19.3%)	3.20 $\pm$ 0.213 (13.8%)
3 (protein-deficient)	2% protein; <i>ad lib.</i> feeding	4	19.2 $\pm$ 1.18	0.581	73.5	6.62 $\pm$ 0.358 (35.2%)	5.14 $\pm$ 0.549 (26.6%)	4.01 $\pm$ 0.698 (20.2%)	3.41 $\pm$ 0.377 (18.0%)
	Rehabilitation on control diet	2	22.5 $\pm$ 0.89	—	—	8.39 $\pm$ 0.165 (37.5%)	6.51 $\pm$ 0.172 (29.5%)	4.75 $\pm$ 0.481 (20.9%)	2.76 $\pm$ 0.346 (12.6%)
4 (protein- and calorie-deficient)	4% protein; semi-starved	4	20.1 $\pm$ 0.71	—	—	6.52 $\pm$ 0.203 (32.4%)	5.93 $\pm$ 0.329 (29.5%)	4.68 $\pm$ 0.380 (23.1%)	3.71 $\pm$ 0.158 (18.4%)
	Rehabilitation on control diet	2	22.5 $\pm$ 0.50	—	—	8.35 $\pm$ 0.425 (37.1%)	6.71 $\pm$ 0.156 (29.9%)	4.18 $\pm$ 0.360 (18.7%)	3.01 $\pm$ 0.141 (13.4%)

Table 3. *Effect of dietary deficiency of protein and calories on the protein fractions of white muscle of rat*

Experimental details are given in the Materials and Methods section. Each value in the Table is an average ($\pm$ s.e.m., where appropriate) of six observations; the values in parentheses indicate the results in terms of percentages of total muscle protein.

Group of rats	Diet	Period of diet (weeks)	(g./100 g. wet wt. of muscle)			Muscle protein fractions (g./100 g. wet wt. of muscle)			
			Total protein	Non-protein N	Moisture	Fraction 1	Fraction 2	Fraction 3	Fraction 4
						(intracellular sarcoplasmic protein)	(intracellular fibril protein)	(intracellular denatured protein)	(stroma protein)
1 (control)	20% protein; <i>ad lib.</i> feeding	4	24.0 $\pm$ 1.43	0.665	74.0	9.22 $\pm$ 0.949 (38.0%)	7.49 $\pm$ 0.264 (31.4%)	5.06 $\pm$ 0.416 (21.1%)	2.27 $\pm$ 0.536 (9.75%)
	20% protein; <i>ad lib.</i> feeding	6	23.7 $\pm$ 0.54	—	—	8.63 $\pm$ 0.323 (36.4%)	7.40 $\pm$ 0.057 (31.3%)	5.67 $\pm$ 0.303 (23.6%)	1.87 $\pm$ 0.298 (8.5%)
2 (calorie-deficient)	40% protein; semi-starved	4	23.7 $\pm$ 1.30	—	—	8.48 $\pm$ 0.611 (35.7%)	7.50 $\pm$ 0.462 (31.8%)	5.22 $\pm$ 0.262 (22.1%)	2.49 $\pm$ 0.516 (10.4%)
	Rehabilitation on control diet	2	23.2 $\pm$ 0.33	—	—	8.52 $\pm$ 0.189 (36.8%)	7.64 $\pm$ 0.363 (32.9%)	4.39 $\pm$ 0.345 (18.9%)	2.61 $\pm$ 0.090 (11.2%)
3 (protein-deficient)	2% protein; <i>ad lib.</i> feeding	4	19.1 $\pm$ 0.82	0.669	74.5	5.58 $\pm$ 0.326 (30.1%)	5.02 $\pm$ 0.410 (27.1%)	4.66 $\pm$ 0.321 (23.0%)	3.56 $\pm$ 0.508 (18.5%)
	Rehabilitation on control diet	2	22.6 $\pm$ 0.51	—	—	8.14 $\pm$ 0.423 (35.9%)	6.87 $\pm$ 0.219 (30.3%)	4.87 $\pm$ 0.254 (21.6%)	2.74 $\pm$ 0.180 (12.2%)
4 (protein- and calorie-deficient)	4% protein; semi-starved	4	20.3 $\pm$ 0.42	—	—	6.50 $\pm$ 0.113 (32.2%)	6.14 $\pm$ 0.300 (30.4%)	4.48 $\pm$ 0.231 (22.1%)	3.11 $\pm$ 0.369 (15.4%)
	Rehabilitation on control diet	2	21.9 $\pm$ 1.05	—	—	8.19 $\pm$ 0.352 (37.5%)	7.00 $\pm$ 0.359 (32.0%)	4.14 $\pm$ 0.400 (19.2%)	2.55 $\pm$ 1.054 (11.7%)

from that of red muscle. Also, the moisture content and non-protein nitrogen of the muscle were not affected by protein deficiency. There was evidence of a qualitative change in the protein composition of red muscle and white muscle in the protein-deficient groups. The intracellular sarcoplasmic fraction (fraction 1), consisting of the genuinely soluble proteins, was significantly decreased in protein-deficient (group 3) and protein- and calorie-deficient (group 4) animals. The extracellular stroma proteins, on the other hand, showed a considerable increase in groups 3 and 4. These changes, which were seen in protein fractions 1 and 4 in the protein-deficient group (group 3), were not accentuated in the protein- and calorie-deficient group (group 4). The proteins of the myofibrils and deoxyribonucleoproteins of the cell nuclei, isolated in fraction 2 (Robinson, 1952), and unextracted denatured actomyosin in fraction 3, were not influenced by the deficiency in dietary protein and calories, either singly or together. It appeared that the deficiency of dietary calories alone did not alter the protein composition of the muscle. After rehabilitation of the animals on an adequate diet the composition of the muscle protein fractions returned to normal.

Enzyme studies. For the purpose of clarification and convenience the results of enzyme studies have been considered under three broad groups, namely glycolytic enzymes, respiratory enzymes and phosphate-transfer enzymes. Since the changes in the intracellular active protein components were observed only in the protein-deficient animals and the superimposed calorie deficiency did not accentuate the effect, the studies on the enzyme pattern of the muscle were carried out only in the 'control' (group 1) and protein-deficiency (group 3) rats. As mentioned above, the dietary deficiency of protein did not affect the relative proportion of the protein content of the muscle, and hence the results of enzyme activities are expressed on the basis of wet wt. of the muscle.

Glycolytic enzymes. When the rats were given a protein-deficiency diet for 4 weeks, no appreciable changes were observed in the glycogen content of the muscle or in the glycolytic activity of this

tissue. The activities of the various glycolytic enzymes investigated, namely aldolase, phosphoglucumutase and lactate dehydrogenase, did not show any difference between the control group and the protein-deficient group. No attempt was made in the present studies to assess the contribution of the plasma proteins to the soluble sarcoplasmic proteins of the muscle, to which fraction the glycolytic enzymes belong. If these smaller contributions of the plasma proteins had been omitted by perfusing the muscle before the enzyme studies were made, the concentrations of the glycolytic enzymes in the sarcoplasmic fraction of the muscle would have indicated some increase in the protein-depleted rats as compared with the control animals, owing to the decrease in the serum protein content of the former.

Respiratory enzymes. Activities of succinate dehydrogenase and cytochrome *c* oxidase and measurement of endogenous respiration in muscle tissue are presented in Table 4. A marked difference was observed between red muscle and white muscle with regard to the response of the activities of respiratory enzymes to protein depletion. The activities of cytochrome *c* oxidase and succinate dehydrogenase of the homogenate of red muscle were 2–3 times as high as those of white muscle in the control group. In the protein-deficiency state the activities of these enzymes were diminished significantly only in the red muscle, and little or no change was observed in white muscle, with the result that there was very little difference in the enzyme activities of red muscle and white muscle of these animals. It was also observed that the endogenous respiration without substrate was significantly decreased in the red muscle of protein-deficient rats.

Adenosine triphosphatase and creatine phosphokinase. The Ca^{2+} ion-activated myosin adenosine-triphosphatase activity of the muscle and the Mg^{2+} ion-activated ATP-splitting power of actomyosin and sarcosomes were measured separately. It was observed (Table 5) that, under the conditions of the experiment, the activity of Mg^{2+} ion-activated adenosine triphosphatase was far more than that of Ca^{2+} ion-activated adenosine triphosphatase.

Table 4. *Activities of respiratory enzymes in muscle of experimental rats*

Experimental details are given in the Materials and Methods section. Each value in the Table is an average ($\pm$ S.E.M.) of six observations.

Group of rats	Type of muscle	Endogenous respiration ($\mu\text{l. of O}_2/\text{g. wet wt./hr.}$)	Succinate-dehydrogenase activity (decrease in $E_{660\text{m}\mu}/\text{mg. wet wt./hr.}$)	Cytochrome <i>c</i> oxidase activity ($\mu\text{l. of O}_2/\text{mg. wet wt./hr.}$)
1 (control, with adequate dietary protein)	Red	407 ± 29.3	0.287 ± 0.0105	24.1 ± 0.89
	White	—	0.102 ± 0.0148	13.5 ± 0.43
3 (protein-deficient)	Red	103 ± 13.5	0.152 ± 0.0110	10.8 ± 0.71
	White	—	0.102 ± 0.0080	8.4 ± 0.56

Table 5. *Adenosine-triphosphatase and creatine-phosphokinase activities in muscle of experimental rats*

Experimental details are given in the Materials and Methods section. Each value in the Table is an average ($\pm$ s.e.m.) of six observations.

Group of rats	Type of muscle	Adenosine-triphosphatase activity (mg. of inorganic P released/g. wet wt./hr.)		Creatine-phosphokinase (mg. of P released/g. wet wt./hr.)
		Mg ²⁺ ion-activated	Ca ²⁺ ion-activated	
1 (control, with adequate dietary protein)	Red	25.7 $\pm$ 1.77	12.3 $\pm$ 0.81	34.0 $\pm$ 2.97
	White	41.7 $\pm$ 2.71	25.1 $\pm$ 1.37	36.9 $\pm$ 2.42
3 (protein-deficient)	Red	16.3 $\pm$ 0.83	6.8 $\pm$ 0.46	16.2 $\pm$ 1.26
	White	28.8 $\pm$ 0.80	11.3 $\pm$ 0.56	14.6 $\pm$ 3.21

Table 6. *Distribution of acid-soluble phosphates, adenosine triphosphate and adenosine diphosphate in striated muscle of experimental rats*

Group of rats	Diet	Period of diet (weeks)	(mg. of P/100 g. wet wt. of muscle)			ADP:ATP ratio
			Total acid-soluble P	ADP P	ATP P	
1 (control)	20% protein; <i>ad lib.</i> feeding	4	217	5.60	46.0	0.151
			$\pm$ 8.28	$\pm$ 1.197	$\pm$ 3.34	$\pm$ 0.028
	20% protein; <i>ad lib.</i> feeding	6	224	3.84	43.2	0.115
			$\pm$ 24.89	$\pm$ 0.240	$\pm$ 4.17	$\pm$ 0.042
2 (calorie-deficient)	40% protein; semi-starved	4	217	3.50	41.7	0.105
			$\pm$ 20.20	$\pm$ 0.639	$\pm$ 3.35	$\pm$ 0.008
	Rehabilitation on control diet	2	191	3.38	39.9	0.107
			$\pm$ 8.63	$\pm$ 0.740	$\pm$ 2.71	$\pm$ 0.074
3 (protein-deficient)	2% protein; <i>ad lib.</i> feeding	4	214	8.62	38.3	0.278
			$\pm$ 19.31	$\pm$ 2.175	$\pm$ 3.59	$\pm$ 0.082
	Rehabilitation on control diet	2	219	7.61	27.2	0.390
			$\pm$ 17.59	$\pm$ 0.329	$\pm$ 3.13	$\pm$ 0.091
4 (protein- and calorie-deficient)	4% protein; semi-starved	4	226	14.76	41.1	0.446
			13.83	$\pm$ 2.102	$\pm$ 2.37	$\pm$ 0.087
	Rehabilitation on control diet	2	189	10.36	29.3	0.468
			$\pm$ 13.47	$\pm$ 2.639	$\pm$ 3.57	$\pm$ 0.058

phatase in both the red-muscle and the white-muscle preparations used in the studies. Presumably the actomyosin complex was largely intact in the muscle homogenates prepared with the Teflon homogenizer at pH 7.4 and used immediately after preparation. In both the systems adenosine-triphosphatase activity was greater in white muscle than in red muscle. Protein depletion produced a significant decrease in muscle adenosine-triphosphatase and creatine-phosphokinase activities.

Acid-soluble phosphates of muscle. Table 6 shows the changes in ATP, ADP and total acid-soluble phosphate compounds of muscle that occurred during protein deficiency, calorie deficiency or both. In the absence of adequate protein in the diet there was a significant rise in the ADP content of the muscle, and ATP showed a slight but statistically insignificant decrease. The elevated content of ADP caused an alteration in the ADP:ATP ratio from the mean ratio of 0.151 in the control group to 0.278 in the protein-deficient group. In combined deficiency of protein and calorie, the ADP concentration of the muscle was further increased with little or no change in ATP,

and a significant rise (to 0.446) in the ADP:ATP ratio was observed. The calorie deficiency alone, however, did not influence the concentrations of ADP and ATP in the muscle. The total acid-soluble phosphates of the muscle remained constant in spite of deficiency of protein or calories or both.

After supplementing the missing nutrient in the diets of these animals for 2 weeks the ADP:ATP ratio remained higher than normal in groups 3 and 4, and the ATP content of the muscle actually showed a decrease after rehabilitation on 'complete diet'. With calorie deficiency alone the pattern of the phosphates in muscle was similar to that of normal muscle.

DISCUSSION

The results may be interpreted to suggest that in protein-deficient animals the intracellular sarcoplasmic proteins of the muscle undergo appreciable change and alter the metabolic pattern of the tissue. The oxidative enzymes of red muscle associated with sarcosome granules (Harman & Feigelson, 1952; Cleland & Slater, 1953) and the glycolytic enzymes associated with soluble sarco-

plasm (Bailey & Marsh, 1952) showed different responses to protein deprivation. The activities of oxidative enzymes were generally lower in the red muscle of protein-deficient animals, suggesting that the sarcosomes of red muscle are more sensitive to protein deficiency than the sarcosomes of white muscle. In protein-deficient muscle, the diminished activity of oxidative enzymes appeared to be responsible for the shift in the balance of ATP and ADP. On the other hand, as indicated by the stability of the glycolytic enzymes, the soluble proteins of sarcoplasm seemed not to be affected under the stress of protein depletion.

The significant decrease observed in the activity of adenosine triphosphatase in the muscle of protein-deficient rats cannot be explained easily, since the quantity of myofibrillar proteins was not affected in the protein-deficiency state. A large proportion of the total adenosine-triphosphatase activity of the whole homogenate of striated muscle in the presence of Mg^{2+} or Ca^{2+} ions has been shown to be associated with the myofibril fraction that could be centrifuged down in 15 min. at 1500g (Perry, 1951; Mommaerts & Seraidarian, 1947). Kielley & Meyerhof (1948), on the other hand, suggested that the Mg^{2+} ion-activated adenosine triphosphatase of the granular component of the muscle was capable of splitting appreciable amounts of ATP. The observations presented in Table 5 do not support this view, since the white muscle, which is poorer in its sarcosomal content than red muscle, had a higher activity of Mg^{2+} ion-activated adenosine triphosphatase. A relatively smaller proportion of Ca^{2+} ion-dependent adenosine-triphosphatase activity in red muscle and white muscle indicated the predominance of the actomyosin complex in the muscle preparations in these studies.

SUMMARY

1. The metabolic activity of striated muscle of young albino rats given diets deficient in protein or calories or both has been studied. As far as possible, red-muscle and white-muscle fibres were separately analysed for: (a) intracellular and extracellular protein fractions, (b) acid-soluble phosphate compounds and (c) certain aerobic, anaerobic and phosphate-transferring enzyme systems. The response of the metabolic activity of the muscle after return to a complete diet was also investigated during the rehabilitation period.

2. Dietary deficiency of protein produced qualitative changes in the protein composition of muscle. The intracellular sarcoplasmic protein fraction was significantly decreased but extracellular proteins were significantly increased. These changes were less pronounced when calorie deficiency was superimposed on protein deficiency.

3. In protein-deficient rats the activities of aldolase and phosphoglucumutase, and the overall glycolytic activity of the muscle, were almost the same as those in the control animals. On the other hand, a marked decrease in the activities of oxidative enzymes (cytochrome *c* oxidase, succinate dehydrogenase and endogenous respiration) was observed in red muscle only in the protein-deficiency state, whereas the adenosine-triphosphatase and creatine-phosphokinase activities were significantly decreased in red muscle as well as in white muscle.

4. The total acid-soluble phosphate content of the muscle was not affected in protein depletion. The ADP content of the muscle was significantly increased, but the ATP content remained unchanged; as a consequence, the ADP:ATP ratio was raised in protein deficiency. When deficiency of calories was superimposed on that of protein a further rise in the ADP:ATP ratio was observed.

5. The results are discussed in relation to energy-transferring reactions in muscle.

We thank Dr C. Copalan, M.D., Ph.D., Director of the Nutrition Research Laboratories, Hyderabad, India, for his interest and encouragement in this work.

REFERENCES

- Bailey, K. & Marsh, B. B. (1952). *Biochim. biophys. Acta*, **9**, 133.
- Cleland, K. W. & Slater, E. C. (1953). *Biochem. J.* **53**, 547.
- Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
- Harman, J. W. & Feigelson, M. (1952). *Exp. Cell Res.* **3**, 47.
- Holmes, E. G., Jones, E. R., Lyle, M. D. & Stanier, M. W. (1956). *Brit. J. Nutr.* **10**, 198.
- Holmes, E. G., Jones, E. R. & Stanier, M. W. (1954). *Brit. J. Nutr.* **8**, 173.
- Kerr, S. E. & Seraidasian, K. (1945). *J. biol. Chem.* **159**, 211.
- Kielley, W. W. & Meyerhof, O. (1948). *J. biol. Chem.* **176**, 591.
- Kleinzeller, A. (1940). *Biochem. J.* **34**, 1241.
- Kuby, S. A., Noda, L. & Lardy, H. A. (1954). *J. biol. Chem.* **209**, 191.
- Lawrie, R. A. (1953). *Nature, Lond.*, **171**, 1069.
- LePage, G. A. (1946). *Amer. J. Physiol.* **146**, 267.
- Lowry, O. H., Roberts, N. R., Wu, M. L., Hixon, W. S. & Grawford, E. J. (1954). *J. biol. Chem.* **207**, 19.
- Mendes, C. B. & Waterlow, J. C. (1958). *Brit. J. Nutr.* **12**, 74.
- Mommaerts, W. F. H. M. & Seraidarian, K. (1947). *J. gen. Physiol.* **30**, 401.
- Najjar, V. A. & Pullman, M. E. (1954). *Science*, **119**, 631.
- Ogata, T. (1958). *Acta med. Okayama*, **12**, 228.
- Ogata, T. (1960). *J. Biochem., Tokyo*, **47**, 726.
- Perry, S. V. (1951). *Biochem. J.* **48**, 257.
- Perry, S. V. (1952). *Biochim. biophys. Acta*, **8**, 499.
- Robinson, D. S. (1952). *Biochem. J.* **52**, 621.
- Schneider, W. C. & Potter, V. R. (1943). *J. biol. Chem.* **149**, 217.

- Szent-Gyorgyi, A. G. (1953). *Arch. Biochem. Biophys.* **42**, 305.
- Taylor, J. F., Green, A. A. & Cori, G. T. (1948). *J. biol. Chem.* **173**, 591.
- Tulpule, P. G. & Kshirsagar, S. G. (1958). *Indian J. med. Res.* **46**, 452.
- Tulpule, P. G. & Patwardhan, V. N. (1952). *Arch. Biochem. Biophys.* **39**, 450.
- Waterlow, J. C. & Mendes, C. B. (1957). *Nature, Lond.*, **180**, 1361.
- Waterlow, J. C. & Weisz, T. (1956). *J. clin. Invest.* **35**, 346.
- Wesson, L. G. (1932). *Science*, **75**, 339.
- Widdowson, E. M. & McCance, R. A. (1957). *Brit. J. Nutr.* **11**, 198.
- Zimmerman, M. J. & Weinstein, H. G. (1956). *J. Lab. clin. Med.* **48**, 607.

Biochem. J. (1964), **92**, 398

Inhibition of Adenylosuccinate Synthetase and Adenylosuccinate Lyase from Ehrlich Ascites-Tumour Cells by 6-Thioinosine 5'-Phosphate

By M. R. ATKINSON, R. K. MORTON* AND A. W. MURRAY

Departments of Agricultural Chemistry and Biochemistry, University of Adelaide, South Australia

(Received 11 November 1963)

6-Mercaptopurine inhibits the conversion of hypoxanthine and of non-cyclic precursors into the guanine and adenine of nucleic acids in a number of micro-organisms and animal tumours (for recent reviews see Davidson, 1960; Carey & Mandel, 1961; Balis, Pecora & Salser, 1963). In an attempt to identify the site or sites of action of 6-mercaptopurine in Ehrlich ascites-tumour cells its nucleotide thio-IMP† was tested as an inhibitor of IMP-NAD oxidoreductase (IMP dehydrogenase; EC 1.2.1.14) from these cells, and was found to be a powerful competitive inhibitor (K_i 3.6 μ M) of oxidation of IMP to xanthosine 5'-phosphate (Atkinson, Morton & Murray, 1963). Thio-IMP has been reported to inhibit formation of adenylosuccinate from IMP with IMP-L-aspartate ligase (GDP) (adenylosuccinate synthetase; EC 6.3.4.4) from *Streptococcus faecalis* (Salser, Hutchison & Balis, 1960). Hampton (1960) reported inhibition of adenylosuccinate synthesis with the enzyme from *Escherichia coli* and found that conversion of adenylosuccinate into adenylyate by adenylosuccinate AMP-lyase (EC 4.3.2.2) from *E. coli* was also inhibited by thio-IMP. The values of K_i and the nature of the inhibitions were not reported by these workers; Hampton (1962) has discussed unpublished results indicating non-competitive inhibition of adenylosuccinate lyase by thio-IMP.

The present paper describes the preparation of adenylosuccinate synthetase and adenylosuccinate lyase of adequate purity for kinetic studies from Ehrlich ascites-tumour cells. The results show that thio-IMP is a non-competitive inhibitor of the conversion of IMP into adenylosuccinate and a

competitive inhibitor of the conversion of adenylosuccinate into adenylyate. In neither case was K_i as low as that found with IMP dehydrogenase from the same cells.

EXPERIMENTAL

Preparation of nucleotides

6-Thioinosine 5'-phosphate. A 25 mM solution of the triethylammonium salt in 1 mM-mercaptoethanol, pH 4, was prepared as described by Atkinson *et al.* (1963); no hydrolysis or oxidation of the nucleotide could be detected after 3 months at -15° . At its final concentration in assay solutions the mercaptoethanol had no inhibitory activity.

Guanosine 5'-triphosphate. Most of the GDP in a commercial sample of GTP (Pabst Laboratories, Milwaukee, Wis., U.S.A.) was removed by chromatography on DEAE-cellulose with a linear gradient (0.50–0.4 M) of NH_4HCO_3 . GTP was eluted between 0.21 M- and 0.28 M- NH_4HCO_3 , and freed of bicarbonate by evaporation. On electrophoresis in 0.04 M-sodium citrate buffer, pH 4.2, the product was found to contain 0.02 mol.prop. of GDP. The resultant concentration of GDP in the assay solution for adenylosuccinate synthetase was 3.5 μ M. The addition of GDP to increase the concentration of this compound in the assay solution to 7 μ M caused no inhibition of the initial rate of formation of adenylosuccinate.

Adenylosuccinate. The nucleotide was prepared from adenylyate and fumarate with adenylosuccinate lyase from yeast and was purified by anion-exchange chromatography as described by Carter & Cohen (1956). Only one component that absorbed light at 254 m μ could be detected on chromatography in ammonium sulphate–0.1 M-sodium phosphate buffer (pH 6.8)–propan-1-ol (30:50:1, w/v/v; R_F 0.52) and in butan-1-ol–acetic acid–water (2:1:1, by vol.; R_F 0.40). The spectrum at pH 7 was the same as that described by Carter & Cohen (1956); the nucleotide:phosphate ratio, based on the molar extinction coefficient reported by these authors (ϵ 1.96×10^4 at 268 m μ), was 0.92:1.00.

* Deceased 27 September 1963.

† Abbreviation: thio-IMP, 6-thioinosine 5'-phosphate.