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REGULATION OF METABOLIC PROCESSES IN MITOCHONDRIA

edited by

J. M. TAGER

Laboratory of Biochemistry, University of Amsterdam (The Netherlands)

S. PAPA

Department of Biochemistry, University of Bari (Italy)

E. QUAGLIARIELLO

Department of Biochemistry, University of Bari (Italy)

and

E. C. SLATER

Laboratory of Biochemistry, University of Amsterdam (The Netherlands)



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PROTEIN SYNTHESIS IN LOCUST-FLIGHT-MUSCLE SARCOSOMES

U. BRONZERT AND W. NEUPERT

Institute for Physiological Chemistry, University of Munich (Germany)

For most investigations on the incorporation of labelled amino acids into isolated mitochondria mammalian liver and heart have been used¹⁻²³. These tissues show very little or no growth. On the contrary, the object of our investigations, mitochondria from the flight muscle of *Locusta migratoria*, the African migratory locust, offers particular advantages. With the fifth and last moulting, the small wingless larvae develop into the adult locusts. During this time of imaginal moulting, the flight muscle and its mitochondria show a very marked increase in growth. Figs. 1 and 2, which are taken from a publication of Brosemer, Vogell and Bücher²⁴ may give an impression of this rapid growth. Mitochondria of the flight-muscle precursor from locusts 9-8, 6-5, 3-2 and 1 day before the imaginal moulting make up 6, 8, 15

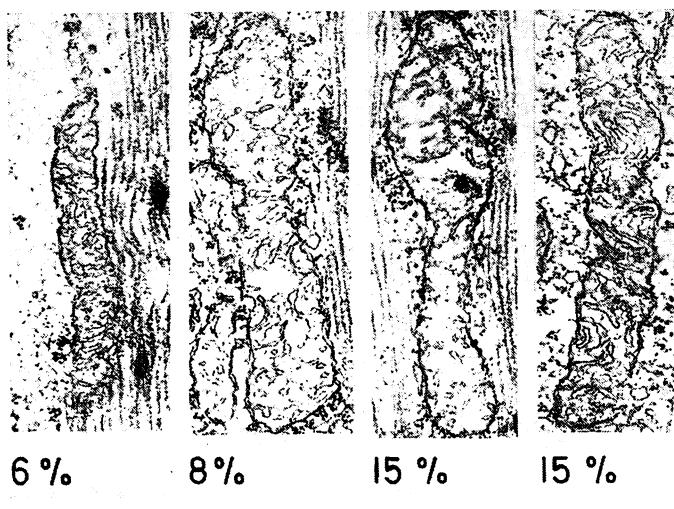


Fig. 1. The shape of mitochondria during development of the flight-muscle precursor. $\times 10\,000$. (a) 9-8 days; (b) 6-5 days; (c) 3-2 days; and (d) 1 day before imaginal moulting. The values give the mitochondrial volume as % of the total volume of muscle tissue.

and 15% of the total volume of the muscle tissue (Fig. 1). Fig. 2 demonstrates the conditions after the imaginal moulting. It presents mitochondria in the same electron microscopic magnification 2 h, and 3 and 8 days after imaginal moulting. On the 8th day the mitochondria make up 30% of the total volume of the flight muscle. At the same time, the total muscle volume increases by a factor of 10. Thus the total mitochondrial mass is increased by a factor of about 50 within the period described. Do these different mitochondria differ in their biochemical qualities, that is in their enzymatic equipment? In Fig. 3 enzymatic activity patterns of the flight muscle in different phases of development are shown. All activities of the enzymes presented, in part easily extractable, in part fixed to the structure and non-

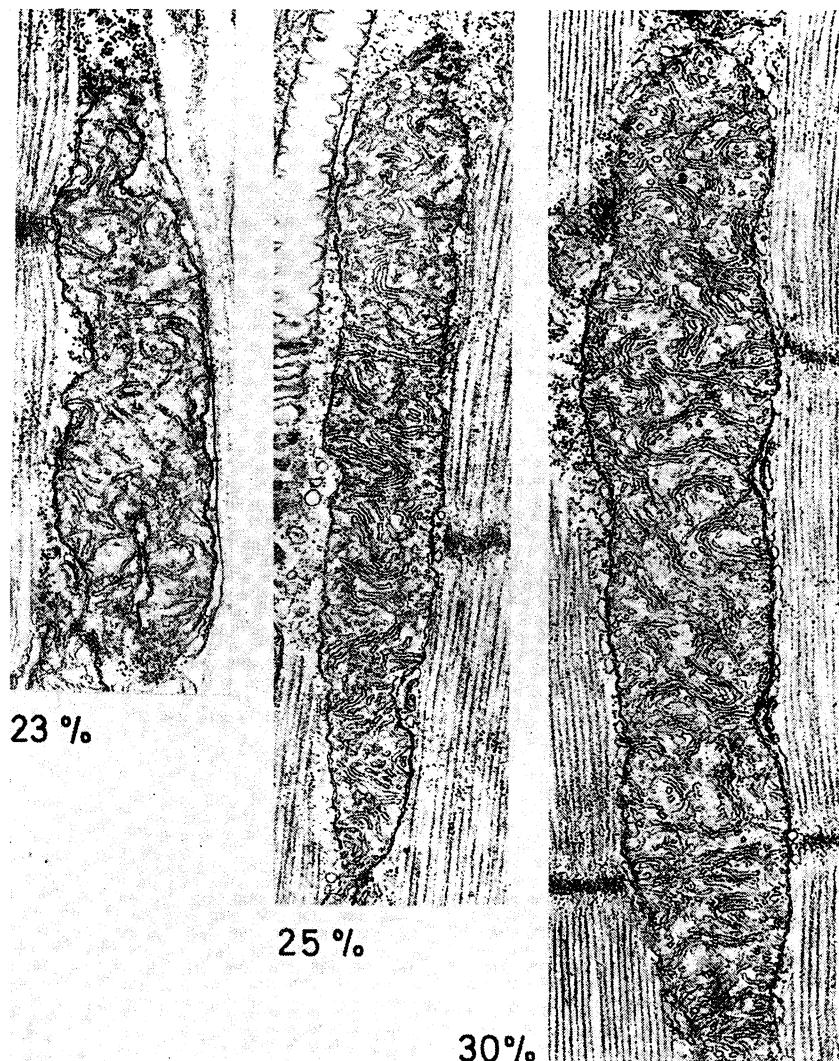


Fig. 2. The shape of mitochondria during development of the flight muscle. $\times 30000$. (a) 2 h; (b) 3 days; and (c) 8 days after imaginal moulting. The values give the mitochondrial volume as % of the total volume of muscle tissue.

extractable, are strongly proportional to that of α -glycerophosphate oxidase: malic dehydrogenase (EC 1.1.1.38), condensing enzyme (EC 4.1.3.7), succinate dehydrogenase (EC 1.3.99.1), isocitrate dehydrogenase (EC 1.1.1.4) and cytochrome *c*. There could also be mentioned: glutamic-oxaloacetic transaminase (EC 2.6.1.1), 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35), reduced NAD dehydrogenase (EC 1.6.99.3) and others. The mitochondria in all phases of the development already described appear to be enzymatically completely equipped and functioning. Fig. 4 is also taken from the publication of Brosemer, Vogell and Bücher²⁴. If the structurally bound α -glycerophosphate oxidase is taken as a measure of the amount of non-extractable protein, an increase of the mitochondrial mass by 1%/h is indicated during

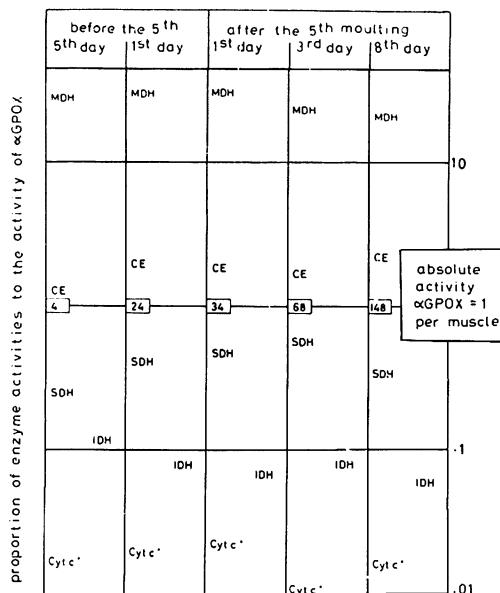


Fig. 3. Patterns of mitochondrial enzyme activity during the development of the dorsal longitudinal flight muscle. The ratio of the measured enzyme activities to that of α -glycerophosphate oxidase is given. The total activity of the latter enzyme is given in the boxes (μ moles/h/animal; 25°). Cyt. c^* means the reciprocal value of cytochrome c turnover of α -glycerophosphate oxidase.

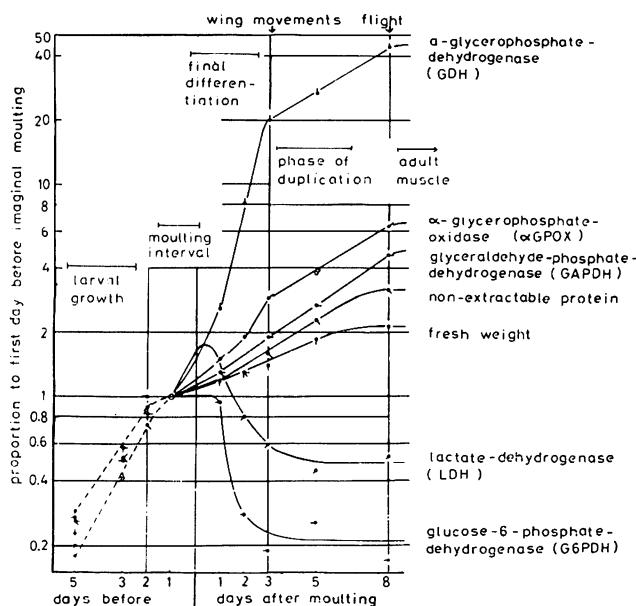


Fig. 4. Phases of development of biochemical patterns. Ordinate: Fresh weight, non-extractable protein and total enzyme activity of the dorsal longitudinal musculature in relation to the first day before moulting. Abscissa: Days before or after the imaginal moulting.

the doubling phase, which follows the phase of final differentiation after imaginal moulting.

In spite of numerous corresponding results of many authors in the field of protein biosynthesis in isolated mitochondria, there is still some doubt with regard to the biological significance of these investigations. The system which we have described reveals a rather distinct and quick regulation and should be especially suitable to give some information with respect to this problem. Its synthetic activity should exceed that of mitochondria from slowly growing tissues like liver and heart. Moreover, the regulation outlined by some morphological and enzymatic features should be detectable in the isolated mitochondria.

Accordingly, we developed a medium for incubation of isolated locust-flight-muscle mitochondria with a high and reproducible rate of incorporation. According to experiments, which will be described later on, we obtained the following convenient incubation medium: 0.1 M KCl; 10 mM triethanolamine buffer; 2 mM EDTA; 3 mM MgCl₂; 4 mM K₃PO₄; 2 mM ATP; and 1.6 mg/ml amino acid mixture. The final pH was 6.8. As tracers we used: [¹⁴C]algal protein hydrolysate, [¹⁴C]glycine, [¹⁴C]leucine; but mainly [¹⁴C]isoleucine with a specific activity of 9.1 mC/mmol which was diluted in the incubation medium by [¹²C]-isoleucine. Incubations were carried out at 23° under air in centrifuge tubes with a volume of 1.6 ml with vigorous shaking. The incubation mixture had a volume of 1.1 ml and contained 2–4 mg mitochondrial protein per ml. At the end of the incubation, mitochondria were precipitated with trichloroacetic acid and washed successively with trichloroacetic acid, ethanol-ether and ether. After 1 h of incubation with 0.4 μ C/ml undiluted [¹⁴C]isoleucine we obtained a specific activity of the mitochondria of 2.5 μ C/g. According

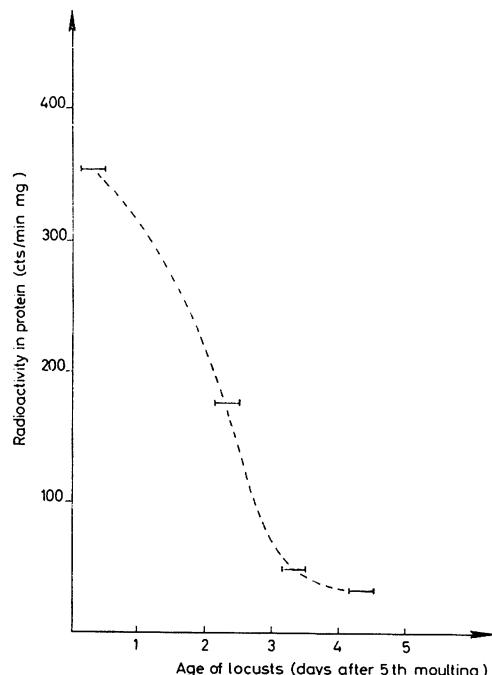


Fig. 5. Effect of the age of locusts on incorporation of amino acids into protein of isolated mitochondria. Incubation medium: 0.1 M KCl, 10 mM triethanolamine buffer, 2 mM EDTA, 3 mM MgCl₂, 4 mM K₃PO₄, 2 mM ATP, 1.6 mg/ml amino acid mixture, 0.2 μ C/ml [¹⁴C]isoleucine, and 7.5 μ g/ml [¹²C]isoleucine; pH 6.8. Incubation time, 1 h at 23°. (0.2 μ C gave 240 000 counts/min)

to a rough estimation 10% of the mitochondrial synthetic activity determined *in vivo*²⁴ could be established.

In order to prove that the alterations in growth intensity ascertained *in vivo* are reflected in the isolated mitochondria, we prepared mitochondria on 5 successive days under exactly identical conditions from a bulk of locusts which had moulted within 8 h. We incubated them under the conditions outlined. Fig. 5 shows the results of one experimental series. Concerning the first 4 days after moulting the incorporation activity decreases by about one order of magnitude.

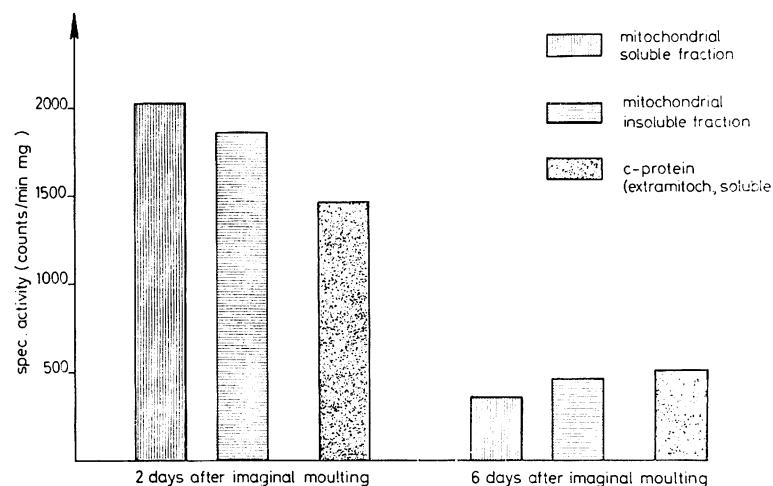


Fig. 6. Specific activity of flight-muscle fractions after injection of $0.3 \mu\text{C} [{}^{14}\text{C}]$ isoleucine (9.1 mC/mmol) per animal. Mitochondria were isolated 24 h after injection and, after mechanical disruption, extracted with 0.1 M phosphate buffer. c-Protein: Extramitochondrial protein fraction not sedimented after centrifugation at $100\,000 \times g$, but precipitable with 6% HClO_4 .

A strikingly similar result could be obtained by experiments *in vivo* (Fig. 6). Locusts of different age were injected with $[{}^{14}\text{C}]$ isoleucine. The specific activities of the mitochondrial protein fractions observed 24 h after injection may be taken as a measure for the synthetic activity of the intact animals. Almost the same decline of this synthetic activity can be seen. The synthetic activity measured *in vitro* appears to be dependent firstly on the state of the isolated mitochondria, that is, on some internal factors, and secondly on some external factors which are given by the composition of the incubation medium. In order to produce an isotonic medium, sucrose or KCl was used. With KCl, higher rates of incorporation could be obtained. A distinct dependence of the rate of incorporation on the concentration of KCl was observed (Fig. 7). The curve shows a maximum at a concentration of about 0.1–0.12 M. Increase of the concentration above this optimal one causes a marked decline of the rate of incorporation, more distinct than in the case of using sucrose.

Fig. 8 demonstrates the necessity for addition of Mg^{2+} for the incorporation. Compared with the incubation without added Mg^{2+} , a concentration of 3 mM brings about an increase of the specific activity of mitochondrial protein by a factor of about 6.

The effect of adding P_i is not so striking but yet clearly to be seen. Fig. 9 reveals a distinct maximum at a concentration of 4 mM. Higher concentrations lower the rate of incorporation to the value obtained without adding P_i .

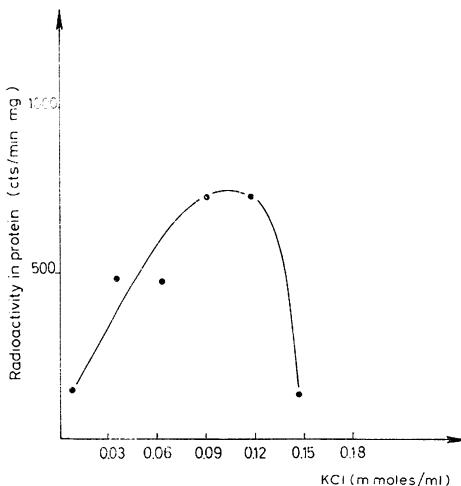


Fig. 7. Effect of concentration of KCl on incorporation of amino acids into protein of isolated mitochondria. Incubation medium: 10 mM triethanolamine buffer, 3 mM MgCl₂, 4 mM K₃PO₄, 2 mM ATP, 0.4 μ C/ml [¹⁴C]isoleucine, 7.5 μ g/ml [¹²C]isoleucine, and 1.6 mg/ml amino acid mixture; pH 6.8. Incubation time, 1 h at 23°.

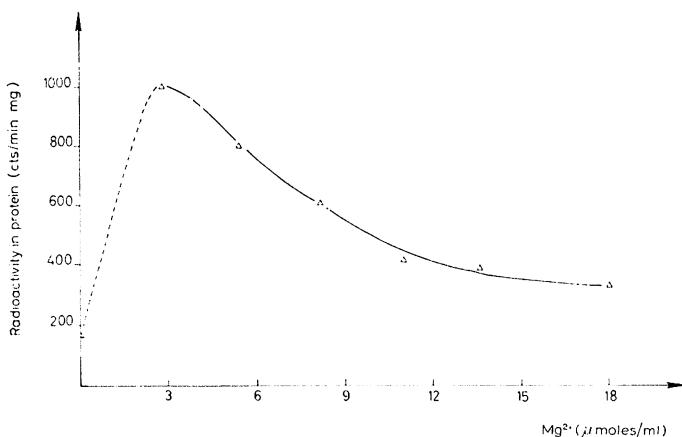


Fig. 8. Effect of added Mg²⁺ on incorporation of amino acids into protein of isolated mitochondria. Incubation medium: 0.1 M sucrose, 10 mM triethanolamine buffer, 2 mM EDTA, 8 mM KCl, 5 mM K₃PO₄, 3.5 mM ATP, 0.4 μ C/ml [¹⁴C]isoleucine, 7.5 μ g/ml [¹²C]isoleucine, and 1.6 mg/ml amino acid mixture; pH 7.0; Incubation time: 1 h at 23°.

The strong dependence of the rate of incorporation on oxidative phosphorylation is a phenomenon often described^{3,6,12,16,20-23}, which could also be shown in our experiments. In the absence of ATP and O₂ and omitting the shaking an almost complete depression of the incorporation is observed. Addition of KCN or CaCl₂ gives the same result.

In agreement with this, addition of ATP brings about a very obvious effect. As is to be recognized in Fig. 10, ATP at a concentration of 2 mM increases the rate of incorporation by a factor of 5-6. This finding is in contrast to experimental results obtained with mitochondria from liver and heart^{3,6,12,16,21}, where only little or no stimulation of the incorporation activity

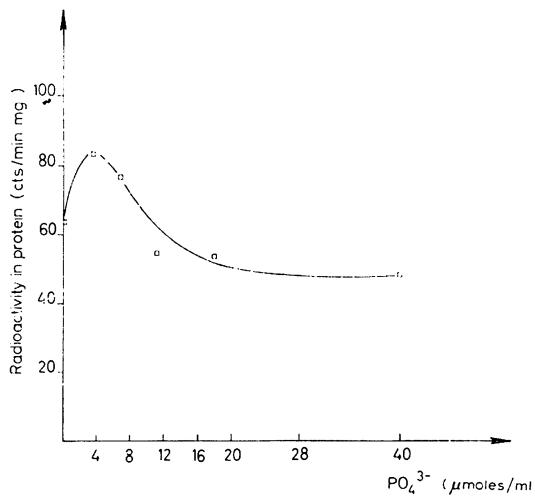


Fig. 9. Effect of concentration of added inorganic phosphate on incorporation of amino acids into protein of isolated mitochondria. Incubation medium: 0.1 M sucrose, 10 mM triethanolamine buffer, 2 mM EDTA, 8 mM KCl, 5 mM MgCl₂, 4.3 mM ATP, 0.4 μC/ml [¹⁴C]isoleucine, 15 μg/ml [¹²C]-isoleucine, and 1.6 mg/ml amino acid mixture; pH 6.8. Incubation time, 1 h at 23°.

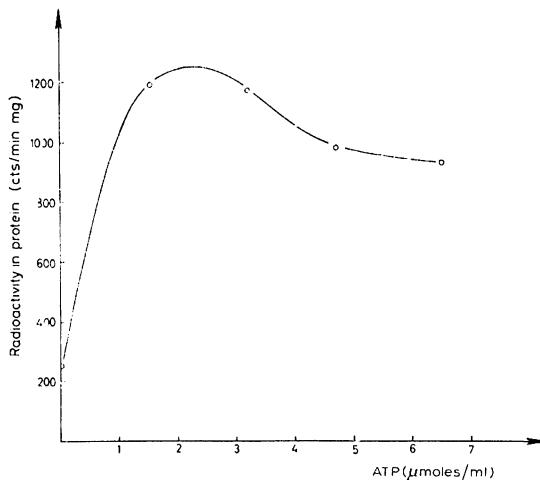


Fig. 10. Effect of concentration of added ATP on incorporation of amino acids into protein of isolated mitochondria. Incubation medium: 0.1 M KCl, 10 mM triethanolamine buffer, 2 mM EDTA, 9 mM MgCl₂, 5 mM K₃PO₄, 0.4 μC/ml [¹⁴C]isoleucine, 7.5 μg/ml [¹²C]isoleucine, and 1.6 mg/ml amino acid mixture; pH 7.0. Incubation time, 1 h at 23°.

could be demonstrated. The effect of addition of a non-radioactive amino acid mixture can be seen in Fig. 11. The optimal rate of incorporation is only reached at far higher concentrations of amino acids than those used in mitochondrial systems from liver and heart reported in the literature up to now^{3,6,12,16,23}. According to determinations by Kirsten²⁵, the flight muscle of *Locusta migratoria* shows a very high level of free amino acids. It amounts to concentrations of 0.3–1.5 μmoles/g fresh weight with respect to each amino acid. However, some amino acids

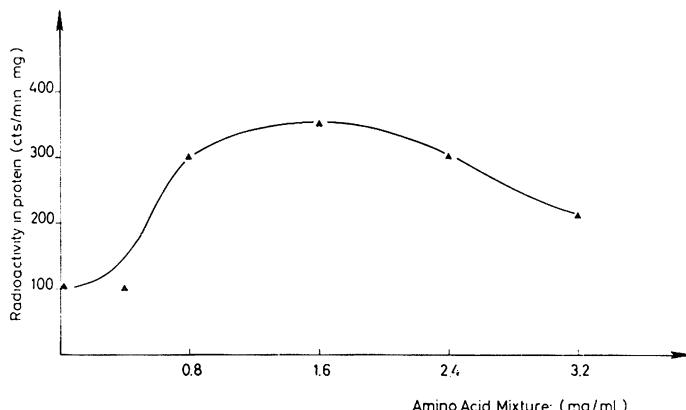


Fig. 11. Effect of addition of amino acid mixture on incorporation of $[^{14}\text{C}]$ isoleucine into protein of isolated mitochondria. Incubation medium: 0.1 M KCl, 10 mM triethanolamine buffer, 2 mM EDTA, 3 mM MgCl_2 , 4 mM K_3PO_4 , 2 mM ATP, 0.2 $\mu\text{C}/\text{ml}$ $[^{14}\text{C}]$ isoleucine, and 7.5 $\mu\text{g}/\text{ml}$ $[^{12}\text{C}]$ isoleucine; pH 6.8. Incubation time, 1 h at 23°.

with high substrate effect appear to have particularly high concentrations. The optimal concentration of the amino acid mixture in the incubation medium corresponds to a molarity on an average of about 0.5–1.3 μmoles with respect to each amino acid. This finding provides evidence for a correlation between the reaction *in vivo* and *in vitro*. In agreement with this, omitting tyrosine and cystine brought about a decrease of the incorporation rate of 38%. Protein biosynthesis may not proceed if mitochondria are not sufficiently provided with amino acids.

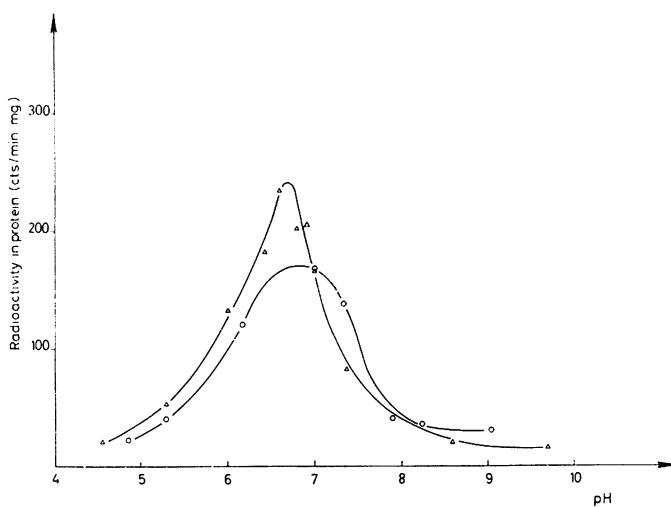


Fig. 12. Effect of pH on incorporation of amino acids into protein of isolated mitochondria. —○—○—, incubation without ATP. Incubation medium: 0.15 M sucrose, 5 mM triethanolamine buffer, 1 mM EDTA, 2.5 mM MgCl_2 , 5 mM KCl, 5 mM K_3PO_4 , 0.4 $\mu\text{C}/\text{ml}$ $[^{14}\text{C}]$ isoleucine, 7.5 $\mu\text{g}/\text{ml}$ $[^{12}\text{C}]$ isoleucine, and 1.6 mg/ml amino acid mixture. Incubation time, 1 h at 35°. —△—△—, incubation with ATP. Incubation medium: 0.1 M KCl, 10 mM triethanolamine buffer, 2 mM EDTA, 3 mM MgCl_2 , 4 mM K_3PO_4 , 2 mM ATP, 0.2 $\mu\text{C}/\text{ml}$ $[^{14}\text{C}]$ isoleucine, 7.5 $\mu\text{g}/\text{ml}$ $[^{12}\text{C}]$ isoleucine and 1.6 mg/ml amino acid mixture. Incubation time, 1 h at 23°.

A strong relationship exists between the synthetic activity of isolated mitochondria and the pH of the incubation medium. Maximal incorporation can be seen at pH 6.7–6.8 (Fig. 12). This is a relatively low value, lying about 0.5 units below the values for ribosomal protein biosynthesis reported in the literature.

These results lead to the suggestion that even in the case of ribosomes being present in the incubation medium there should be no or only little protein synthesis at the ribosomes. In order to test this, isolated microsomes were added to the complete incubation medium including mitochondria. After incubation, the activity incorporated into microsomal fraction and into the protein fraction released into the medium during incubation was determined. It appeared that the isolated ribosomes and the protein released into the medium within the limit of error contained no radioactivity. Consequently, contribution of ribosomes at least under the reaction conditions applied may be excluded. Moreover, the often reported inefficacy of ribonuclease on the incorporating system^{3,6,12,16,23} could be confirmed. Finally, electron microscopic pictures show the absence of ribosomes in preparations of isolated locust-flight-muscle mitochondria²⁶.

The possibility of bacterial contribution to incorporation is a serious problem when investigating mitochondrial protein synthesis. Our experiments furnish the following arguments against the existence of bacterial contamination:

- (1) The marked dependence of incorporation on KCl- and ATP-concentration as well as the strong dependence on the pH and
- (2) the inhibition of incorporation by low concentrations of Ca^{2+} , are not consistent with bacterial growth.
- (3) Heating of the mitochondria to 100° for a short time before adding to the incubation medium resulted in a decrease of incorporation to 6% of the value obtained with untreated mitochondria.
- (4) The time course of incorporation is a linear one, and in some experiments the

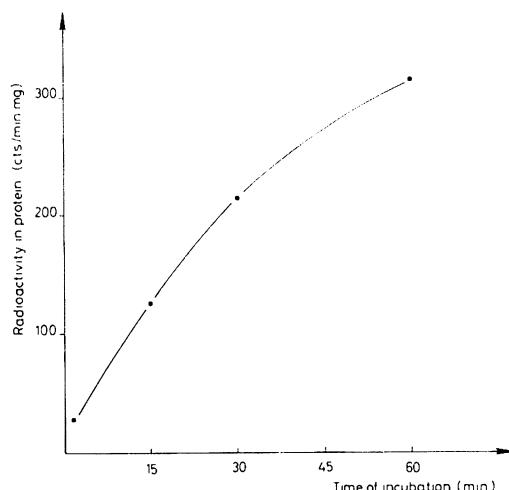


Fig. 13. Incorporation of amino acids into protein of isolated mitochondria as a function of incubation time. Incubation medium: 0.15 M sucrose, 5 mM triethanolamine buffer, 2 mM EDTA, 2.5 mM MgCl_2 , 5 mM KCl, 5 mM K_3PO_4 , 1 $\mu\text{C}/\text{ml}$ [^{14}C]isoleucine, 7.5 $\mu\text{g}/\text{ml}$ [^{12}C]isoleucine, 1.6 mg/ml amino acid mixture. The pH was 7.0. Temperature, 35°.

synthetic activity even decreases within the first hour (Fig. 13). Not before 3 h does the linear course change into an exponential one.

(5) The rate of incorporation is strongly dependent on the age of the locusts from which the mitochondria were isolated. This finding is not to be reconciled with bacterial contamination.

(6) If bacteria incorporate amino acids, soluble and insoluble protein fractions are expected to become labelled to the same extent. The experiments described below, which show clearly different labelling of soluble and insoluble fractions, give strong additional support that the contribution of bacteria to incorporation is negligible.

Now, having supplied some arguments for the biological significance of the incorporation *in vitro* the question may be asked: What is the relationship between incorporation *in vitro* and incorporation *in vivo*? In order to study this problem, locusts were injected with [¹⁴C]isoleucine shortly after the imaginal moulting. Mitochondria were isolated 24 h after injection. Subsequently the mitochondria were mechanically disrupted and extracted with 0.1 M phosphate buffer (pH 7.3). We obtained a soluble and an insoluble fraction in a ratio by weight of 35% and 65%. Labelling of both fractions occurs almost to the same extent (Fig. 6). In the intact organ, growth brings about a synchronous increase of soluble and insoluble mitochondrial proteins.

For investigation of the behaviour *in vitro*, mitochondria were extracted after incubation with [¹⁴C]isoleucine. The 3 following fractions were prepared: (1) Protein released into medium during incubation, (2) Protein extractable by subsequent treatment with Ultraturrax in 0.1 M phosphate buffer (pH 7.3) followed by vigorous stirring, (3) The insoluble remaining fraction.

Fraction 1 contains 19%, fraction 2, 16% and fraction 3, 65% of the total mitochondrial mass. Fig. 14 illustrates the results of these experiments. In the insoluble fraction (fraction 3) more than 97% of the total activity incorporated is found. Both soluble fractions together (fraction 1 + 2) contain less than 3% of the total activity incorporated. This value

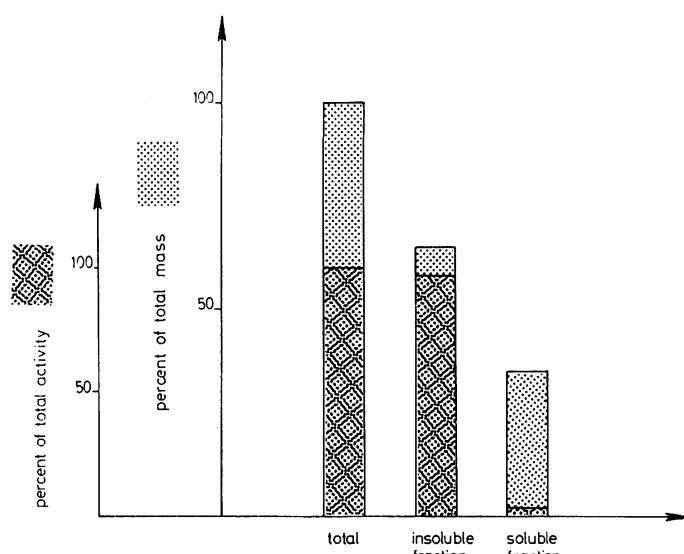


Fig. 14. Fractionation of mitochondria after incubation with [¹⁴C]isoleucine.

lies at the limit of error of the value of zero-time experiments. The specific activity of the protein released into the medium (fraction 1) amounts to 5% of that of the insoluble fraction.

These experimental results indicate that incubation of isolated mitochondria does not cause synthesis of soluble and extractable mitochondrial enzymes including most enzymes of the Krebs cycle, of the respiratory chain and of fatty acid oxidation.

In summary, the main results of our investigations on the incorporation of amino acids into isolated mitochondria from locust-flight muscle are as follows:

(1) The experimental results on the incorporation of amino acids by isolated mitochondria from other tissues could be generally confirmed. The composition of the incubation medium resembles with some exceptions those already described by other authors^{6,12,16,22}. However, we see the following differences: A strong effectiveness of ATP and of amino acid mixture, as well as a marked dependence on the pH.

(2) The rate of incorporation of amino acids into isolated mitochondria from locust-flight muscles is distinctly higher than that reported for mitochondria from non-growing tissues. During the phase of maximal synthetic activity, the rate of incorporation into isolated beef-heart mitochondria¹⁹ under comparable conditions is exceeded by a factor of about 10, and the rate of incorporation reported for isolated rat-liver mitochondria^{6,12} by a factor of almost 100. The values used for this comparison are obtained from mitochondria from locusts one day after imaginal moulting. 5–6 days after imaginal moulting, when growth slows down, the rate of incorporation decreases to the level in mitochondria from non-growing beef heart.

(3) Furthermore, the biological significance of the incorporation of amino acids *in vitro* has been investigated. Strong dependence of the synthetic activity of the isolated mitochondria on the synthetic activity of the animals, from which the mitochondria were isolated, could be established. Mitochondrial protein biosynthesis is regulated in such a way that there is a synchronous course in the activity with respect to microsomal protein biosynthesis (Fig. 6) The occurrence of mitochondrial DNA has been shown by several authors in various tissues recently^{27–29,35}. It may be possible that the distinct regulation outlined occurs *via* this DNA.

(4) According to investigations on various subjects, automultiplication of mitochondria or mitochondrial precursors has been supposed by several authors^{30–33}. The fractionation experiments show that this automultiplication must be regarded as partial. The findings described strongly resemble those of Roodyn^{7,10} and Truman³¹. They proposed on the basis of their fractionation experiments that mitochondrial protein biosynthesis is limited to the insoluble structural protein. Our experiments support this hypothesis. Further experiments are necessary to obtain further enlightenment on this problem.

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