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INCORPORATION OF L-VALINE BY MELANO-PROTEIN PARTICLES *IN VITRO*

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SUMMARY

A particle fraction has been isolated from mouse-melanoma tissue by differential centrifugation. This particle fraction differs in several biochemical respects from tumor mitochondria or tumor microsomes. The energy requirements for the incorporation of valine are met in an integrated manner by glycolytic enzymes associated with the particles, and the addition of exogenous ATP and an ATP-generating system does not stimulate the incorporation process. Agents that deplete mitochondria of ATP and uncouple phosphorylation (dinitrophenol, dicoumarol, cyanide or anaerobiosis) do not inhibit the incorporation of amino acids. Amino acid-activating enzymes and soluble RNA are present in the particles. The particles contain numerous melanin granules in various stages of synthesis, and this suggests that the particle fraction may participate in the formation of the protein matrix of the mature melanin granules.

INTRODUCTION

In recent years the formation of melanin granules in melanocytes has been studied in considerable detail by electron microscopy. Although melanin-containing granules have been detected throughout the endoplasmic reticulum, the smallest granules are constantly observed within the vesicles of the Golgi apparatus[1,2]. A procedure for the separation of melanin granules from mitochondria has been described[3], indicating that melanin granules and mitochondria are distinct cytoplasmic constituents.

The enzymes responsible for melanin formation have been studied in purified melanin granules[3], but the pathways for the biosynthesis of the protein moiety of the mature granules remain obscure. To determine the possible relationships between melanin granule formation and the synthesis of melano-protein, the amino acid incorporation activity of different cell constituents of Harding-Passey mouse melanoma tissue was studied *in vivo* and *in vitro*[4]. A procedure was developed for the isolation of a fraction of melano-protein particles containing RNA and lipids, and results obtained *in vivo* showed that these particles took up and retained amino acids. These results were further borne out *in vitro* by the ability of the isolated melano-protein particles to incorporate amino acids into protein[4]. When compared with other

Abbreviations: CP, carbamyl phosphate; HMP, *p*-hydroxymercuribenzoate.

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cell fractions *in vitro* the incorporation capacity of melano-protein particles was higher than that of microsomes or mitochondria in long-term experiments. This communication describes additional biochemical properties of the melano-protein particles, and identifies ATP generated during glycolysis as the main energy source for the incorporation reaction.

MATERIALS AND METHODS

Chemicals

Sucrose, KCl and $MgCl_2$ were analytical grade. CP, NAD, RNAase, and the amino acids used were obtained from the Nutritional Biochemical Corporation. Carbamyl phosphokinase was prepared from *Streptococcus faecalis* by the method of MOKRASCH *et al.*[5]. ATP, ADP, and Tris were the products of the Sigma Chemical Company. L-[^{14}C]Valine (1.54 μmoles/μC) and [^{32}P]phosphate were purchased from the Radiochemical Centre, Amersham (Great Britain).

Tumor

The transplantable solid Harding-Passey mouse melanoma was carried in four-week old commercially purchased mice. Semi-aseptic transplantation of tumor tissue was made subcutaneously using a trocar with tissue 3 mm × 5 mm. To obtain larger quantities of tumor tissue, two transplants were made on each side of the mouse. For fractionation studies tumors transplanted 40–60 days earlier and weighing 3–5 g were used.

Tissue fractionation

The tumor was dissected free from connective tissue, weighed, minced with scissors, and transferred to an all glass Potter–Elvehjem homogenizer with a loose-fitting glass pestle. The tissue was homogenized with 2.3 volumes of medium containing 0.035 M Tris buffer (pH 7.8), 0.01 M $MgCl_2$, 0.025 M sucrose, and 0.05 M KCl. Nuclei and cell debris were removed by centrifugation for 5 min at 600 × *g* (all centrifuge forces are calculated from bottom of the tube) in the International Centrifuge HR-1. The nuclei and cell debris were transferred with 1 volume of medium to the homogenizer and rehomogenized. This procedure was repeated two times. After the third resuspension and homogenization the supernatant obtained after centrifugation was nearly colorless. The supernatant fractions were combined and centrifuged for 10 min at 10000 × *g* in the International Centrifuge Model HR-1. An intense black pellet was obtained. The pellet was resuspended in an equal volume of medium, and placed as the top layer on a 5-ml Spinco centrifuge tube previously layered with 3.0 ml 1.5 M sucrose. The tubes were centrifuged 45 min at 173000 × *g* in the swinging bucket head SW 39 of the Spinco centrifuge. A black pellet was collected at the bottom of the tube. The tubes were drained of sucrose, frozen in an alcohol–dry ice bath, and lyophilized 6 h. The dark brown powder was stored over silica gel in a desiccator at −20°. To reconstitute the particles, the preparation was resuspended in the appropriate medium by gently homogenizing with a homogenizer fitted with a loose glass pestle, and centrifuged for 10 min at 10000 × *g*. The supernatant was discarded, and the particles resuspended in medium.

The particles were stable when stored lyophilized at −20° for periods of up to three weeks. A loss of 10–50% of the initial activity occurred during the lyophili-

zation process. A variation in the activity of particles isolated from individual tumors was encountered. In the experiments described, particles from a minimum of eight tumors have been collected, combined, and assayed. Under these conditions, uniform incorporating activities were obtained.

Rat-liver mitochondria

Mitochondria were prepared as described by ERNSTER AND LOW[6].

Analysis

Lactic acid was determined by the method of BARKER AND SUMMERSON[7], protein by the method of LOWRY *et al.*[8], RNA by the orcinol method of CERIOTTI[9], and amino acid activation by the procedure of VON DER DECKEN[10]. End group analysis was carried out by the method of SANGER[11].

The reaction mixture for studying the incorporation of amino acids into RNA and protein contained 0.5 μC L-[^{14}C]valine (1.54 μmoles/μC), 3.0 mg lyophilized melanoma particles, 1.0 μmole ADP, 5.0 μmoles $MgCl_2$, 10 μmoles K_2HPO_4, 10 μmoles glucose, 0.1 μmole NAD, 75 μmoles sucrose, 12.5 μmoles KCl, and 20 μmoles Tris buffer (pH 7.8), in a total volume of 1 ml.

After incubation at 35° with shaking, the reaction mixture was chilled to 0°, and proteins and nucleic acids precipitated with one volume of cold 10% trichloroacetic acid. The precipitates were transferred to glass centrifuge tubes fitted with a ground glass pestle, and ground twice with cold 5% trichloroacetic acid. When labeling of RNA was determined, the trichloroacetic acid precipitates were suspended in water and adjusted to pH 5.6 with acetate buffer (pH 5.6). The nucleic acids were extracted with an equal volume of water-saturated phenol, and precipitated at −20° overnight with 2 volumes of 95% ethanol. The precipitate was washed once with ethanol – ether (3:1), and with ether until dry. Proteins, after separation from RNA, were washed with the same series of organic solvents used for RNA. The dried proteins or RNA were pressed on aluminum planchets and counted to collect 1000 counts in three counting periods using a thin window Geiger–Müller counter. Samples were corrected for variations in the protein content from self-absorption curves prepared from labeled rat-liver proteins. The counts are given as counts/min/mg protein at infinite thinness.

Oxygen uptake of the particles and mitochondria was studied with a stationary platinum electrode following the procedure described by CHANCE AND WILLIAMS[12]. The reaction mixture consisted of 50 μmoles KCl, 33 μmoles Tris buffer (pH 7.6), 8 μmoles $MgCl_2$ and 50 μmoles sucrose in a final volume of 1.5 ml. 1.0 ml of the incubation medium was added to the cuvette before starting the experiment. Other substances were added during the incubations at the appropriate times; 0.2 ml of rat-mitochondrial suspension in 0.25 M sucrose or 0.2 ml of melanoma particles corresponding to a protein content of 8 mg was used in each experiment.

RESULTS

Time-dependent incorporation of L-[^{14}C]valine into protein and s-RNA

As shown in Fig. 1, the incorporation of L-[^{14}C]valine into protein by isolated melano-protein particles continued over a time interval of 2 h at an increasing rate. Incorporation into s-RNA reached a maximum and a saturated rate at 1 h.

Energy requirements

The incorporation at any time interval was independent of added ATP, cell sap, or exogenous amino acids (Table I). The particles metabolized glucose to lactic acid in an amount sufficient to account for the observed incorporation of valine into protein. Agents which inhibit lactic acid formation (fluoride, arsenate, iodoacetate)

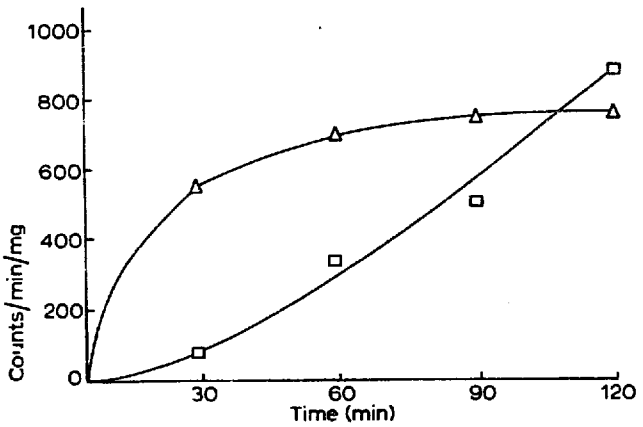

Fig. 1. Time dependent incorporation of L-[^{14}C]valine into protein □—□ and RNA (△—△) by melano-protein particles. The reaction components consisted of 0.5 μC L-[^{14}C]valine (1.54 μmoles/μC), 3.0 mg melano-protein particles, 1.0 μmole ADP, 5.0 μmoles $MgCl_2$, 10 μmoles K_2HPO_4, 10 μmoles glucose, and 20 μmoles Tris buffer (pH 7.8) in a final volume of 1.0 ml.

TABLE I

REQUIREMENTS FOR THE INCORPORATION OF L-[^{14}C]VALINE BY MELANOMA-PROTEIN PARTICLES

The complete system consisted of 0.5 μC L-[^{14}C]valine (1.54 μmoles/μC), 3.0 mg melanoma-protein particles, 1.0 μmole ADP, 5.0 μmoles $MgCl_2$, 10 μmoles K_2HPO_4, 10 μmoles glucose, and 20 μmoles Tris buffer (pH 7.8) in a final volume of 1.0 ml. Incubation time, 2 h at 35°.

Addition	*Counts/min/mg*
Complete system	150
Complete system plus 1.0 μmole ATP	151
Complete system plus 1.0 μmole ATP, 10.0 μmoles CP, 15 μg CP phosphokinase	147
Complete system plus 0.2 ml tumor cell sap	135
Complete system plus 0.01 μmole complete amino acid mixture	150

inhibited the incorporation of valine into protein by the particles (Table II). Other agents which affect aerobic energy production had no effect as seen from Table III. The addition of an energy source and an energy-generating system partially reversed the inhibition by arsenate (Table II). L-Valine was only incorporated to a minor extent at the N-terminal end, as evidenced by the limited reaction with FDNB (Table IV). Solubilization in 30% KOH followed by reprecipitation with trichloroacetic acid did not decrease the specific activity of the labeled proteins.

Optimum metal ion concentration

The particles were stimulated by potassium and magnesium ions. Optimum potassium ion concentration was $5 \cdot 10^{-2}$ M, and optimum magnesium concentration $1 \cdot 10^{-2}$ M.

Enzymic activity of particles

In addition to the enzymes of glycolysis, the particles contained amino acid-activating enzymes (Table V). An amino acid-dependent pyrophosphate exchange reaction with ATP was observed with twelve amino acids; no activation or an inhibition was seen with four others.

TABLE II

EFFECT OF GLYCOLYTIC INHIBITORS ON LACTIC ACID FORMATION AND INCORPORATION OF L-[^{14}C]VALINE BY MELANOMA-PROTEIN PARTICLES

The complete system and reaction conditions are as described under Table I.

Addition	*Concentration*	*Counts/min/mg protein*	*μmoles lact-ie/h*
Complete system	—	165	0.21
Complete system plus NaF	$2.5 \cdot 10^{-2}$ M	5	0.02
Complete system plus Na_2HAsO_4	$2.5 \cdot 10^{-2}$ M	77	0.04
Complete system plus Na_2HAsO_4	$2.5 \cdot 10^{-2}$ M	121	1.05
CP	$1.0 \cdot 10^{-2}$ M		
ATP	$1.0 \cdot 10^{-2}$ M		
CP-kinase	15 μg		

TABLE III

EFFECT OF RESPIRATORY INHIBITORS ON INCORPORATION OF L-[^{14}C]VALINE BY MELANO-PROTEIN PARTICLES

The complete system and reaction conditions are as described under Table I.

Addition	*Concentration*	*Counts/min/mg*
Complete system	—	165
Complete system plus dicoumarol	$1 \cdot 10^{-4}$ M	165
Complete system plus dinitrophenol	$2 \cdot 10^{-4}$ M	147
Complete system plus pentachlorophenol	$1 \cdot 10^{-4}$ M	60
Complete system plus NaCN	$1 \cdot 10^{-4}$ M	165
Complete system plus nitrogen*		165

* Flasks were flushed with 100% nitrogen for 10 min at 0° and sealed.

TABLE IV

PERCENT N-TERMINAL L-[^{14}C]VALINE INCORPORATED INTO PROTEIN BY MELANO-PROTEIN PARTICLES.

Expt.	*Total counts/min*	*Ether-soluble counts/min*	*Percent N-terminal*
1	1675	28	1.6
2	785	24	3.3
3	1677	27	1.6

Neither RNAase nor lecithinase inhibited the incorporation significantly. HMB completely inhibited the reaction. The inhibition could be reversed by GSH (Table VI). GSH, as also shown in Table VI, stimulated the incorporation.

Figs. 2 and 3 show the negligible respiratory activity of the melano-protein

particles and the rapid uptake of oxygen by liver mitochondria under the same experimental conditions. At a protein content equal to that of the mitochondria and in excess of that employed in the incorporation experiments, no oxygen uptake of the particles was observed under conditions where an active incorporation of L-valine

TABLE V

AMINO ACID-DEPENDENT PYROPHOSPHATE–ATP EXCHANGE BY MELANO-PROTEIN PARTICLES

The reaction components consisted of 0.6 μmole amino acids, 1.2 μmoles ATP, 1.2 μmoles $MgCl_2$, 12.0 μmoles KF, 30 μmoles Tris (pH 7.8), 1.0 mg enzyme protein and 0.8 μmole [^{32}P]PP_i (100000 counts/min/μmole). The reaction flasks were incubated 60 min at 35°. After the incubation 0.05 ml of chromatography solvent was added, the tubes centrifuged, and 10 μl of the supernatant applied to Whatman No. 1 paper. The papers were developed in isobutyric acid – ammonia – water (50:1:20) for 72 h at 18° by the descending technique. The radioactive ATP was located by radioautography, and quantitative values obtained by cutting out the ATP spot and counting in a thin-window counter.

Amino acid	*Total counts/min*	*Percent total exchange*
—	1 720	1.39
L-Cysteine	11 500	9.25
L-Asparagine	6 450	5.20
L-Glutamic	6 450	5.20
L-Aspartic	6 450	5.20
L-Glutamine	5 400	4.33
L-Histidine	4 900	3.97
L-Threonine	4 700	3.81
L-Lysine	4 500	3.63
L-Leucine	4 440	3.53
L-Serine	4 300	3.45
L-Arginine	3 700	2.92
L-Valine	3 550	2.86
L-Tryptophan	2 600	2.10
L-Methionine	2 500	2.01
L-Isoleucine	2 450	1.98
L-Proline	1 500	1.25
L-Tyrosine	1 117	0.86
L-Alanine	1 100	0.80
L-Phenylalanine	970	0.79
Glycine	625	0.52

TABLE VI

EFFECT OF RNAASE, LECITHINASE, PUROMYCIN, AND SULFHYDRYL REAGENTS ON INCORPORATION OF L-[^{14}C]VALINE BY MELANO-PROTEIN PARTICLES

Complete system and conditions are as described under Table I.

Addition	*Concentration*	*Counts/min/mg protein*
Complete system	—	211
Complete system plus RNAase	100 μg/ml	197
Complete system plus *Naja Naja* venom	200 μg/ml	163
Complete system plus GSH	10^{-2} M	330
Complete system plus HMB	10^{-3} M	2
Complete system plus GSH	10^{-2} M	
and HMB	10^{-3} M	344
Complete system plus puromycin	10^{-4} M	105

into the particles occurred. The particles showed no respiratory control as seen by the failure of ADP and P_i to stimulate oxygen uptake.

In the presence of succinate or other intermediates in the tricarboxylic acid cycle the respiration rate was not stimulated (Fig. 4). The particles did not contain any diffusible factor inhibiting oxygen uptake since rat-liver mitochondria continued to respire at a normal rate in the presence of the melano-protein particles (Fig. 2). Oxidative enzymes were, however, present in the particles. Added cytochrome *c*

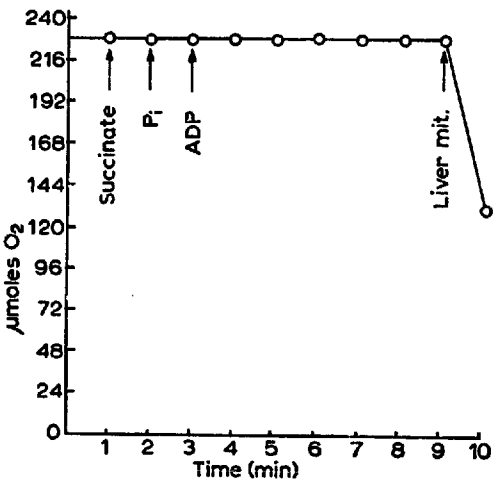

Fig. 2. Oxygen uptake by tumor-melanoma particles. The reaction components consisted of 50 μmoles KCl, 33 μmoles Tris buffer (pH 7.6), 8 μmoles $MgCl_2$, 50 μmoles sucrose, and 8 mg melanoma-protein particles in a final volume of 1.5 ml. 10 μmoles sodium succinate, 0.5 μmole P_i, 0.5 μmole ADP, and 8 mg rat-liver mitochondria (mit) were added at points indicated. Oxygen uptake was measured with a stationary platinum oxygen electrode at 23°.

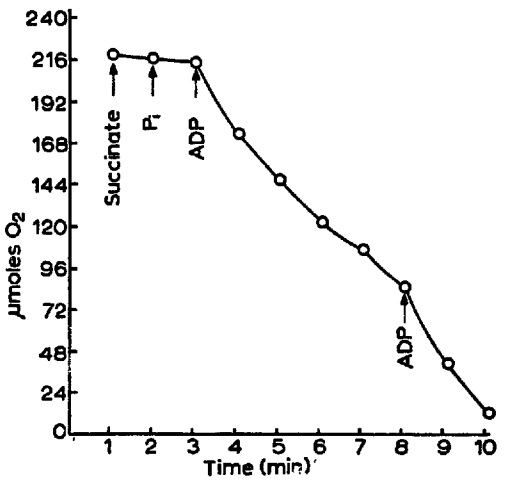

Fig. 3. Oxygen uptake by rat-liver mitochondria. The reaction components are as described in Fig. 2. 10 μmoles sodium succinate, 0.5 μmole P_i, and 0.5 μmole ADP were added at the points indicated.

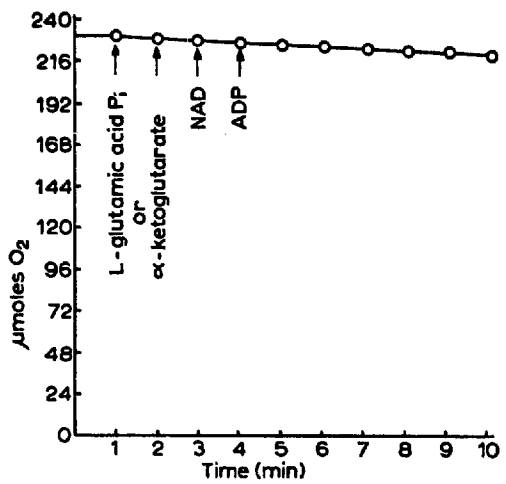

Fig. 4. Effect of tricarboxylic acid intermediates on oxygen uptake by tumor-melanoma particles. The reaction components are as described in Fig 2. 0.5 μmole P_i, 10 μmoles L-glutamic acid, or 10 μmoles α-ketoglutarate, 0.3 μmole NAD, and 0.5 μmole ADP were added at the points indicated.

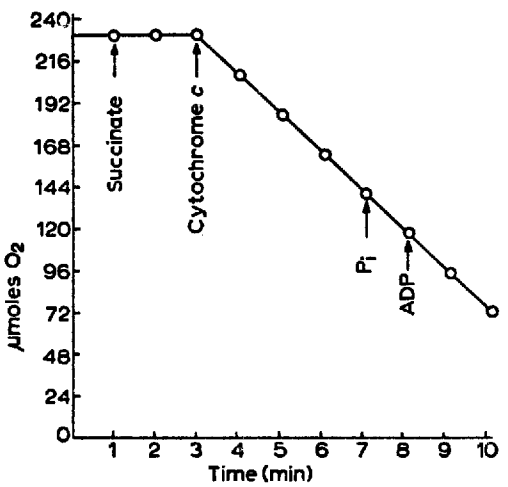

Fig. 5. Effect of cytochrome *c* on oxidation of succinate by tumor-melanoma particles. Reaction conditions are as described in Fig. 2. 10 μmoles succinate, 0.06 μmole cytochrome *c*, 0.5 μmole P_i, and 0.5 μmole ADP were added at the points indicated.

catalyzed an immediate uptake of oxygen in the presence of succinate. However, this oxidation was not linked to phosphorylation since added ADP and P_i did not stimulate the rate of succinate oxidation (Fig. 5).

DISCUSSION

The present studies show that for one type of particle preparation isolated from melanoma tissue, optimal incorporation of L-[^{14}C]valine is not dependent upon an active oxidative phosphorylation mechanism, but appears to be linked with energy generated during glycolysis. Under conditions where active incorporation of valine occurs, no respiration of the particles can be detected. The failure of the tumor to meet the energy requirements for incorporation of valine from oxidative phosphorylation is further established by the complete lack of an effect on the incorporation process by agents that effectively halt oxidative phosphorylation in mitochondria. With the exception of pentachlorophenol, no agent that affects oxidative phosphorylation significantly altered the incorporation of L-valine into protein.

The inhibition by pentachlorophenol is of interest. Parallel studies with liver microsomes have shown a similar effect and indicate that the inhibition by pentachlorophenol cannot be explained by the uncoupling of oxidative phosphorylation. The inhibition cannot be reversed by the addition of tyrosine[13] and it does not appear that pentachlorophenol competitively inhibits the incorporation of tyrosine or other amino acids. The mechanism for the inhibition of incorporation with pentachlorophenol is not understood, but the high lipid solubility of this reagent and the presence of lipid[4] in the particles may be related.

The failure to inhibit the system with RNAase may be due to an inability of the enzyme to attack the ribonucleic acid present within the particle. The fact that the labeling of soluble RNA precedes the incorporation into protein and the steady state of incorporation into this fraction suggests that RNA is involved in the incorporation reaction.

A variation in the incorporating activity of particles isolated from individual tumors has been encountered. Attempts to activate inactive particles or particles with a reduced incorporating activity by means of supplementation with energy, sulfhydryl reagents, liver and yeast extracts, nucleotides and metal ions have not been successful. Inactive particle preparations show little or a reduced ability to form lactic acid, and appear more highly melanized than corresponding active particles.

The energy requirement made evident by the addition of arsenate and the inhibition by puromycin are in accord with the view that valine is being incorporated into protein as a result of peptide bond formation. The final proof that melano-protein particles are able to synthesize proteins *de novo* will, however, depend upon the isolation of a uniformly labeled protein. The melano-protein complex is insoluble under physiological conditions, and attempts to solubilize the protein components with detergents have been only partially successful.

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