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OXIDATIVE PHOSPHORYLATION IN THE EPIDERMIS OF MAN AND THE HAIRLESS MOUSE*

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One of the most important intermediates in cellular biochemistry is adenosine triphosphate (ATP). This energy-rich phosphate ester is synthesized by use of energy released during metabolic reactions in the cell.

The main mode of ATP synthesis in most tissues is termed "oxidative phosphorylation" and is associated with the cytochrome system of electron transfer in mitochondria (Fig. 1). This process has been studied in detail in liver, kidney, and muscle preparations, but it has not been demonstrated in skin. Oxidative enzymes (1) are known to be present in the skin and uptake of radioactive phosphate (P^{32}) into various skin fractions, including ATP in tissue culture (2–6), has been observed.

This paper reports the demonstration of oxidative phosphorylation in the epidermis of man and that of the hairless mouse. A method is described for the measurement of ATP formation relative to the oxidation of succinate-1-C^{14} to $C^{14}O_2$ in epidermal specimens.

METHODS AND MATERIALS

Epidermis from hairless mice was obtained by scraping the whole stretched skin with a scalpel immediately after sacrifice of the animals; it was then weighed on a single-pan automatic balance. Human epidermis was obtained in sections 0.2 mm thick from volunteers under local anesthesia by means of a Castroviejo keratome (7). Tissue samples (10 to 30 mg) were placed in temperature-equilibrated vessels containing reaction media.

Evolution of $C^{14}O_2$ from the metabolism of succinate-1-C^{14} was taken as a measure of the respiratory activity of the tissue being examined. Succinate-1-C^{14}† was diluted with unlabeled succinate (to give a specific activity of approximately 10 μc per millimole). Reactions were carried out in stoppered, single-arm Warburg vessels; the center wells contained 0.1 ml of 15% sodium hydroxide and a filter-paper wick to trap the $C^{14}O_2$ that evolved. The reaction vessels contained 14 μmoles (60,000 cpm) of succinate-1-C^{14} and components as indicated (Fig. 2) and were run for 1 hour unless stated otherwise. Sulfuric acid was added from the side arm to stop the reactions and evolve the $C^{14}O_2$. Contents of the center well were then counted directly on a Packard Tricarb scintillation counter with diotol as the scintillating fluid (8).

Phosphate ester synthesis was determined by measuring incorporation of P^{32} into water-soluble organic phosphates. Carrier-free inorganic orthophosphate-32‡ was heated for 1 hour in dilute acid to destroy metaphosphates and pyrophosphates. The solution was then neutralized and diluted with 0.2 M potassium phosphate, pH 7.4, to give 2.5 to 4 $\times$ 10^7 counts per milliliter. Each reaction vessel contained 0.1 ml of this component. Other components were added as indicated in Figure 3 to give a final volume of 2.0 ml. Reactions were run for 30 minutes unless otherwise indicated; they were stopped by addition of 10 N sulfuric acid. Unreacted inorganic P^{32} was removed from the aqueous layer as the phosphomolybdate complex by extraction with butanol-benzene (9), and aliquots of the aqueous layer were counted on the scintillation counter in diotol.

The labeled organic phosphates were examined by two-dimensional paper chromatography in (1) isopropyl ether:formic acid (11:50) and (2) 0.1 M sodium phosphate, pH 7.0:ammonium sulfate:*n*-propanol (11:60:2) followed by radioautography.

When it was determined that optimal concentrations for succinate oxidation and phosphate ester synthesis were similar, $C^{14}O_2$ evolution and P^{32} incorporation were measured simultaneously on the same specimen. When optimal conditions differed, those best for phosphate uptake were selected. Though the reaction mixture contained both labeled succinate and phosphate, the P^{32} could be counted in a two-channeled scintillation counter with no interference from C^{14} when proper channel selection was made.

RESULTS

Succinate-1-C^{14} metabolism.—Both mouse and human epidermis readily metabolized succinate-C^{14} to $C^{14}O_2$. Figure 2 shows the conditions selected for assay after a series of preliminary tests. Triphosphopyridine nucleotide (TPN) and adenosine diphosphate (ADP), both of which are thought to enter mitochondria freely, stimulated formation of $C^{14}O_2$. Diphosphopyridine (DPN), cytochrome C and ATP

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‡ Obtained from Isoserve, Inc., Cambridge, Massachusetts.

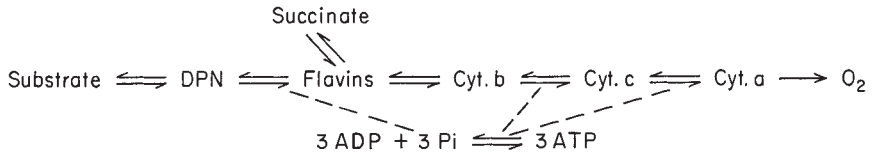

FIG. 1. Oxidative phosphorylation by the electron transport system of mitochondria.

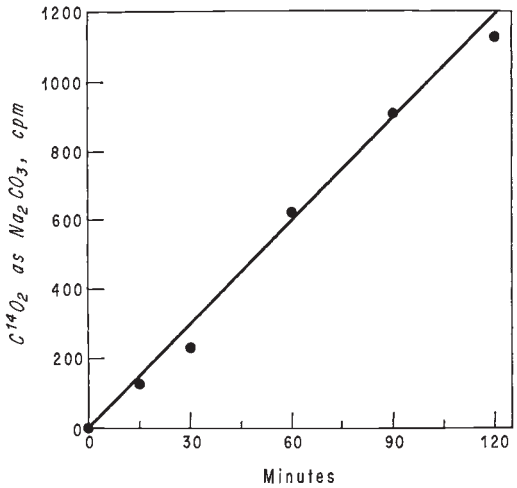

FIG. 2. Rate of $C^{14}O_2$ production. Each flask contained ADP, 1 μmole; inorganic phosphate, pH 7.4, 20 μmoles; Tris, pH 7.4, 10 μmoles; $MgCl_2$, 10 μmoles; TPN, 0.5 μmole; and succinate-1-C^{14}, 15 μmoles, diluted to volume with saline. Tissue specimens were added to start the reactions.

had no consistent stimulatory effect, presumably because they could not pass the cell membrane and enter the mitochondria. Higher concentrations of succinate (at the same specific activity) and of magnesium ion caused increased formation of $C^{14}O_2$ (Table I), but these conditions inhibit phosphorylation; consequently the lower concentrations were selected. The reaction rate was linear with time (Fig. 2). In 2 hours approximately 0.06 μmole of succinate-C^{14} was converted to $C^{14}O_2$. In subsequent studies, experiments were carried out for 1 hour.

The effect of other additives and of several respiratory inhibitors on the system is shown in Table 1. The Crabtree effect (10) is seen in this tissue; glucose produced a 13% inhibition of succinate oxidation.

Phosphate-32 uptake.—Initial experiments in which inorganic phosphate-32 was incubated with human epidermal slices showed that a considerable amount of the labeled component became attached to the tissue, and negligible organic phosphate radioactivity remained in the supernatant. The addition of ADP as a phosphate acceptor greatly increased the amount of organic phosphates in the supernatant, but more than 1 μmole of this cofactor was observed to be inhibitory, so that 10 μmoles of glucose was added to serve as an additional phosphate acceptor via the hexokinase reaction. Under optimal conditions (Fig. 3), as determined by other preliminary tests, only 6% of the organic phosphate remained bound to tissue, and this was neglected for expediency of assay. The rates of reaction with these additions are shown in Figure 3. Table II stresses the dependency of uptake of inorganic phosphate on the concentration of substrate, magnesium and phosphate acceptors. The effect of the various inhibitors further illustrates that this phosphorylation is related to respiration and electron transport.

The major product was tentatively identified as ATP by its Rf value during chromatography in the solvent systems described. However, several other radioactive spots appeared, the sum of which equaled more than 50% of the

TABLE I

*Effect of additives and inhibitors on succinate-1-C^{14} metabolism in mouse skin**

	Concentration, M	CPM/10 mg/hr
—	—	535
Succinate†	0.015	595
Glucose	0.05	465
Magnesium	0.1	710
Malonate	0.01	44
Methylene blue	0.1%	370
Fluoride	0.02	380
BAL	0.01	95
Cyanide	0.001	165
2,4-Dinitrophenol	0.0001	525

* Factors were added to the test system described in Figure 2.

† Specific activity of substrate was kept constant.

activity on the radioautograph. The only other component identified with certainty was inorganic orthophosphate, which probably resulted from chemical degradation of the products.

It was desirable to combine the experiments on the metabolism of succinate-1-C^{14} and on the uptake of phosphate-32 so that they might be performed simultaneously in a specimen of tissue. The data reported (Table III) for human tissue were obtained on specimens of uninvolved skin from volunteers who were patients who had various dermatoses. This skin may not have been normal and may account for the variability;* the data obtained with mouse skin were somewhat more consistent. The ratio of phosphate-32 taken up to $C^{14}O_2$ evolved was 2.3 for human skin and 2.1 for mouse skin.

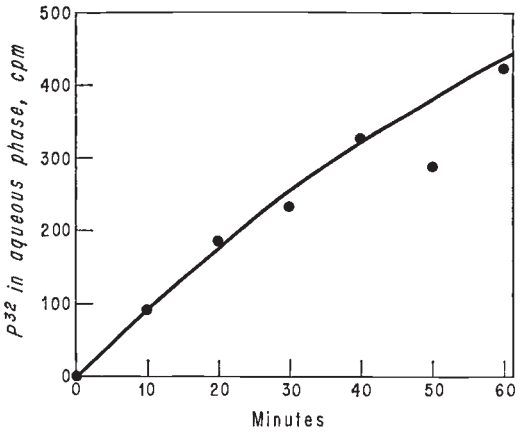

FIG. 3. Rate of phosphate-32 uptake. Each reaction contained ADP, 1 μmole; inorganic phosphate-32, pH 7.4, 20 μmoles; Tris, pH 7.4, 10 μmoles; glucose, 10 μmoles, $MgCl_2$, 5 μmoles; NaF, 12.5 μmoles; TPN, 0.5 μmole; and succinate, 15 μmoles, diluted to volume with saline. Tissue specimens were added to start the reactions.

TABLE II

*Effect of substrates, cofactors and inhibitors on P^{32} uptake into organic phosphates by mouse epidermis**

	Concentration, M	CPM/10 mg tissue per 30 min†
—	—	1010
Minus glucose	—	675
Minus succinate	—	337
Minus glucose and succinate	—	270
Succinate	0.015	805
Magnesium	0.1	215
Fluoride	0.03	565
Malonate	0.01	40
Cyanide	0.01	70
Azide	0.001	340
2,4-Dinitrophenol	0.0001	180

* Factors were added to or subtracted from the test system described in Figure 3. Correction was made for P^{32} decay (half life = 14.3 days).

† Counts presented are those of an aliquot approximately 1/15 of the total.

TABLE III

*Simultaneous measurement of succinate-1-C^{14} metabolism and phosphate-32 uptake in epidermal specimens**

Tissue source	CPM/10 mg tissue per 45 min†		
	Organic P^{32}	$C^{14}O_2$	$P^{32}/C^{14}O_2$
Human	268	141	1.9
	121	45	2.7
	261	89	2.9
	239	108	2.2
	290	89	3.3
	67	77	0.9
			(Av. 2.3)
Hairless mouse	519	266	1.95
	408	267	1.5
	484	207	2.3
	555	287	1.9
	784	347	2.25
	923	294	3.1
	603	295	2.0
			(Av. 2.1)

* Conditions were the same as those described in Figure 3 except that succinate-1-C^{14} replaced the unlabeled succinate. Reaction time was 45 minutes.

† Counts presented are those of a standard aliquot of the total.

COMMENT

It is becoming increasingly clear that the respiratory processes of the epidermis provide an important source of energy for the skin. Gilbert (10) and Cruickshank and associates

* Observations made since this manuscript was submitted indicate that such variability also exists in specimens taken from volunteers with normal skin.

(11) have presented data which indicate that lipid can provide the major energy substrate for epidermis. The results reported herein show that, under the conditions described, a major process for ATP synthesis in mouse skin involves the electron transport mechanism associated with Krebs cycle. The method described provides a means of study of this energy mechanism which has previously not been available for epidermis. More sophisticated studies on isolated mitochondria from this tissue need to be made, but the technic presented herein has the advantage of measuring the reactions of mitochondria *in situ* and of being sensitive enough that oxidative phosphorylation can easily be estimated in epidermal biopsy specimens. Such a method needs to be indirect, therefore, to avoid unnecessary manipulation of the tissue. That this nonspecific formation of organic phosphate-32 is a valid technic for measuring formation of ATP via oxidative phosphorylation is supported by two findings: (1) it is stimulated by succinate and by other factors which are known to stimulate oxidative phosphorylation in mitochondria, and (2) it is inhibited by respiratory inhibitors. While most of the inhibitors tested are not necessarily specific for this system, the action of malonate generally is identified with succinic dehydrogenase inhibition, and this compound abolished 96 per cent of the phosphorylation. Though glucose was necessary for optimal phosphorylation (Table II), it probably served as a phosphate acceptor via the hexokinase reaction rather than as a primary substrate for glycolytic phosphorylation. It accounted for only 9.1% of the total stimulation in phosphorylation (lines 3 and 4) observed when compared with the increase when succinate was present (lines 1 and 4).

The amount of phosphate consumed was small (0.2 to 0.3 μmole/hr/10 mg tissue) but was in a range comparable to that expected for the amount of succinate-C^{14} metabolized to $C^{14}O_2$(0.03 to 0.05 μmole). While phosphorylation was 10 times the actual amount of succinate oxidation, it should be noted that complete oxidation of 1 mole of succinate theoretically can produce 11 to 12 moles of ATP via the Krebs cycle. It thus appears that the exogenous succinate could have served as the major substrate for this phosphorylation. Any calculations must be considered speculative, however, since the following several factors are unknown or uncontrolled in an indirect assay of this nature. 1. The degree of phosphorylating potential of any tissue or preparation is dependent on the conditions of its measurement, and it is thus difficult to compare data obtained under different assay conditions. 2. It is not known whether the Krebs cycle is the only route of succinate metabolism that occurs in skin (12), nor does $C^{14}O_2$ measurement give any indication of the degree of partial degradation of succinate. 3. ATPase activity is easily demonstrated in epidermal preparations (13), but the relative amount of activity in unhomogenized tissue as used in the situation described herein is not known. Fluoride was added to inhibit this enzyme, but it also inhibits succinic dehydrogenase at higher levels, and its concentration must be limited.

No data were obtained on the amount of oxygen consumed in mouse skin under these conditions, but if the data of Fitzgerald and Klein (14) are within the range of this tissue, it can be estimated that more than five times as much oxygen is consumed as is necessary to generate the observed phosphorylation (assuming 3 moles of ATP per atom of oxygen).

We have used succinate metabolism as a measure of mitochondrial content of specimens because the succinic dehydrogenase system is found exclusively in the mitochondria. Since oxidative phosphorylation also occurs in mitochondria, the amount of $C^{14}O_2$ from the metabolism of succinate-C^{14} in a given reaction should serve as a useful reference with which to compare phosphate-32 uptake. We believe that this provides a more dependable measure of the phosphorylating potential of a specimen than weight or area, and in our hands it is more easily measured than deoxyribonucleic acid (DNA) content.

Most of these data were obtained using hairless mouse skin. Table III is not intended to imply that there are no qualitative or quantitative differences in skin from mice and humans, though it does show that the system established for mouse tissue can be applied to small specimens of human epidermis for the demonstration of oxidative phosphorylation.

SUMMARY

An indirect method for demonstrating oxidative phosphorylation in small specimens (10 to

30 mg) of epidermis from man and from the hairless mouse is presented. The amount of phosphate-32 incorporated into organic phosphate is measured and compared with the amount of $C^{14}O_2$ that is evolved from the metabolism of succinate-1-C^{14} during the reaction. The succinate-C^{14} metabolism serves as a reference to the mitochondrial content of the specimen.

That this method measures oxidative phosphorylation is supported by the following observations: (1) the major substrate under the conditions described is exogenous succinate, (2) when glucose is the sole substrate it accounts for only 9.1% of the observed phosphate uptake in mouse skin, and (3) the process of incorporation of phosphate and metabolism of succinate is inhibited completely or in part by malonate and by other respiratory inhibitors. From these findings and other data that are discussed, it seems likely that oxidative phosphorylation of mitochondria provides a major source of ATP in the epidermis.

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