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ANTIMITOCHONDRIAL EFFECT OF SATURATED MEDIUM CHAIN LENGTH (C_8–C_{13}) DICARBOXYLIC ACIDS

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Abstract—In isolated rat liver mitochondria, respiration was competitively inhibited by medium chain length (C_8 to C_{13}) dicarboxylic acids to different extents: the higher the number of carbon atoms up to C_{12}, the greater the inhibition. In particular, experiments on submitochondrial particles showed that the competitive inhibition concerned the following enzymes: NADH dehydrogenase, succinic dehydrogenase and reduced ubiquinone: cytochrome *c* oxido-reductase. These results tend to confirm the suggestion that the melanocytotoxic effect of dicarboxylic acids, which are also competitive inhibitors of tyrosinase, may be primarily due to an antimitochondrial effect rather than being tyrosinase-dependent.

We have previously demonstrated that dicarboxylic acids from C_8 to C_{13} are competitive inhibitors of tyrosinase *in vitro* [1] and that *in vivo* they are effective in the treatment of pigmentary disorders due to hyperfunction of melanocytes such as melasma and toxic melanoderma [2]. A definite cytotoxic effect on malignant melanocytes of lentigo maligna [3] and primary cutaneous human malignant melanoma has also been shown [4, 5].

It was originally thought that the melanocytotoxicity of these diacids was due to an anti-tyrosinase activity, but during the past few years evidence has accumulated suggesting that their effect is not strictly specific for, or limited to, melanocytes. In fact, patients suffering from melasma and also affected with acne reported a beneficial effect on both types of lesions when treated with a cream containing 20% azelaic acid (C_9) dicarboxylic acid. The effect on acne, which was later further investigated [6], cannot be due to anti-tyrosinase activity.

In addition, studies on the metabolism of C_8–C_{13} dicarboxylic acids orally administered to rats and human individuals showed that they are partially metabolized via beta-oxidation [7], indicating involvement of the mitochondrial function. Furthermore, investigations by electromicroscopic autoradiography of cultured normal and malignant melanocytes exposed to [^{3}H]dodecandioic acid revealed a concentration of radioactivity in relation to mitochondria and nuclei. No radioactivity was associated with the sites of location of tyrosinase within the cell, such as the membranes of rough endoplasmic reticulum, GERL, Golgi membranes, or melanosomes [8].

All these data directed our attention towards the possibility that C_8–C_{13} dicarboxylic acids might affect, in addition to tyrosinase, enzymes of mitochondrial respiration. This paper reports the results of an investigation into their effect on isolated rat liver mitochondria.

MATERIALS AND METHODS

Chemicals. All the drugs were obtained from Sigma, with the exception of the dicarboxylic acids which were purchased from Fluka.

Mitochondria. Rat liver mitochondria were isolated according to the method of Johnson and Lardy [9] in 0.25 M sucrose–Tris and were used within 3–4 hr. Mitochondria with an acceptor control ratio lower than 3, by using succinate as substrate, were not used in the experiments. Succinate, L-glutamate and hydroxybutyrate were used as substrates.

Determination of respiratory activity. The O_2 consumption was recorded amperometrically in 2 ml of reaction medium at 25° by using a Rank oxygen electrode (Rank Bros., Bottisham, U.K.) in the absence or presence of various concentrations of dicarboxylic acids: pimelic acid (C_7); suberic acid (C_8); azelaic acid (C_9); sebacic acid (C_{10}); undecandioic acid (C_{11}); dodecandioic acid (C_{12}); and tridecandioic acid (C_{13}).

Determination of redox states of the cytochromes. The redox states of the cytochromes were estimated from the difference spectra between 350 and 650 nm at room temperature by using an AMINCO DW 2 spectrophotometer in the split-beam mode. The reaction medium was that detailed by Bustamante *et al.* [10] except for the omission of bovine serum albumin and the inclusion, in some cases, of 2% (v/v) ethanol.

Oxido-reductase activities of sub-mitochondrial particles (SMP). SMP were prepared by exposing a suspension of liver mitochondria to ultrasound for 2 min in a Branson sonifier model B 12, and then by removing unbroken mitochondria and dense particles by centrifugation at 5000 *g* for 20 min. The supernatant was centrifuged at 30,000 *g* for 20 min. The pellet was resuspended in sucrose–Tris and tested by an AMINCO DW 2 spectrophotometer for various oxido-reductase activities, at a final concen-

tration of 0.8–1 mg/ml, in the absence or presence of dicarboxylic acids at various concentrations.

Enzymes were assayed by following the rate of change in absorbance at different wavelengths, in the presence of different substrates and acceptors: NADH dehydrogenase at 340 nm, 0.2–0.4 mM $NADH_2$ and oxygen; succinic dehydrogenase at 420 nm, 7 mM succinate and 1 mM ferricyanide or 1 mg cytochrome *c*; reduced ubiquinone:cytochrome *c* oxido-reductase at 550 nm, 0.2–0.5 mg/ml ubiquinone and 1 mg/ml cytochrome *c*; cytochrome *c*:O_2 oxido-reductase at 550 nm, 1 mg/ml reduced cytochrome *c* and O_2 [11].

The assays were conducted at 25° in a 2.5 ml system containing sub-mitochondrial particle suspensions and the above-mentioned substances; 1 mM KCN was included, except when the oxygen was the acceptor. Dicarboxylic acids were used either as disodium salts or as free acids dissolved in ethanol. Solubility problems, in the form of disodium salts or free acids, arose with dicarboxylic acids higher than C_{12}.

ADP and ATP analysis. ATP generation from mitochondria was measured by high pressure liquid chromatography (HPLC). The mitochondrial suspension (buffer 2.5 ml, succinate 7 mM, and ADP 250 μM) was filtered on a 0.5 μm pore size Millipore filter, and 30 μl was injected into the liquid chromatograph (1084 B, Hewlett Packard) which was provided with a scanning spectrophotometer from 190 to 540 nm.

The chromatograph included an integrator that gave peak areas and times for each peak in the chromatogram. The separations were obtained on a ion exchange column (Partisil SXS 10/25 SAX, Whatman). The chromatographic column was operated at 40°. Mobile phase: 0.5 M KH_2PO_4, pH 3.5. Flow rate: 0.8 ml/min. Detection: UV 280 nm.

Swelling. The amount of mitochondrial swelling induced by the dicarboxylic acids was measured as the change in absorbance at 560 nm in the presence or absence of substrates of respiration.

Protein measurement. Proteins were determined by the biuret method, with bovine serum albumin as standard.

Statistical analysis. All results are presented as mean ± the standard error of five experiments.

Table 1. Effect of C_8–C_{13} dicarboxylic acids on mitochondrial respiration tested by an oxygen electrode

Dicarboxylic acid	Respiration inhibition (%)
C_8, 20 mM	50 ± 5
C_9, 10 mM	60 ± 7
C_{10}, 10 mM	65 ± 8
C_{11}, 10 mM	70 ± 5
C_{12}, 10 mM	80 ± 6
C_{13}, 10 mM	78 ± 7

Each result represents the mean ± S.E. of seven experiments. Mitochondrial protein concentration: 1 mg/ml. Substrates: succinate or glutamate, 7 mM. C_7 Dicarboxylic acid did not inhibit respiration at concentrations higher than 20 mM.

RESULTS

Similar results were obtained by using dicarboxylic acids either as disodium salts or as free acids dissolved in ethanol.

Respiration of intact mitochondria

From Table 1 it is evident that in the presence of C_8–C_{13} dicarboxylic acids respiration is inhibited, and that the degree of inhibition is correlated with the chain length of the diacids up to C_{12}. The inhibition was slightly reduced after 1 hr incubation. In the case of pimelic acid (C_7), which is not a competitive inhibitor of tyrosinase, no inhibitory effect on respiration was observed, even at 50 mM concentration.

In Figs. 1 and 2 the results obtained by using

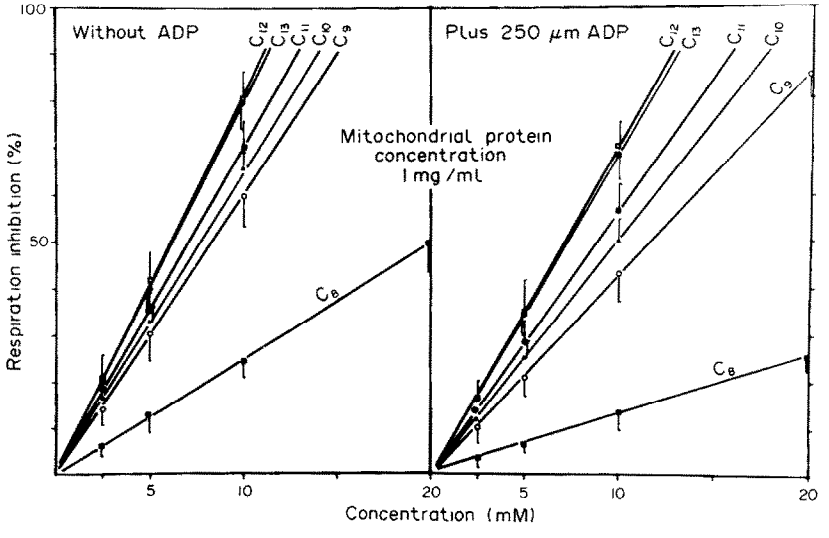

Fig. 1. Effect of varying amounts of C_8 to C_{13} dicarboxylic acids on mitochondrial respiration in the presence or absence of ADP, tested by an oxygen electrode. Mitochondrial protein concentration: 1 mg/ml. Clearly the degree of inhibition is related to the chain length of the diacids. Each value represents the average of five experiments ± S.D.

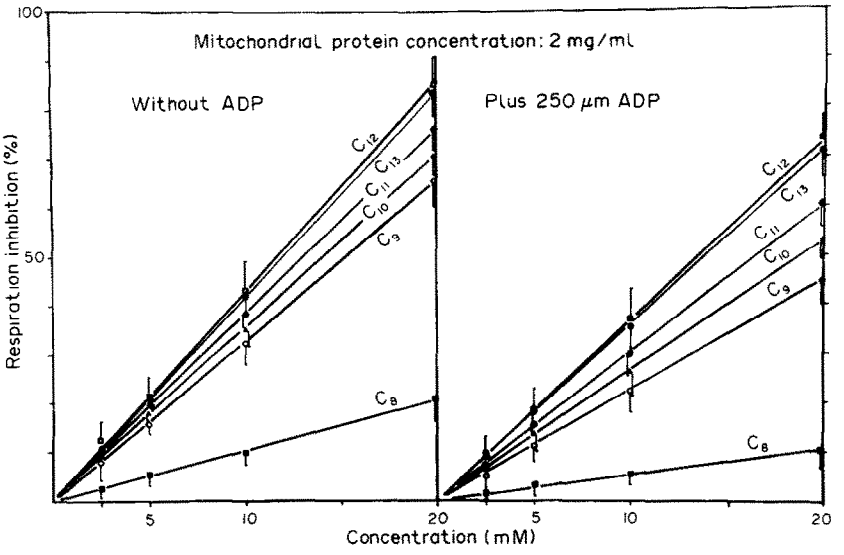

Fig. 2. Effect of varying amounts of C_8–C_{13} dicarboxylic acids on mitochondrial respiration in the presence or absence of ADP (250 μM), measured by an oxygen electrode. Mitochondrial protein concentration: 2 mg/ml. This figure shows that in addition to chain length (Fig. 1) the degree of inhibition is also related to mitochondrial protein concentration. Each value represents the average of five experiments ± S.D.

different concentrations of dicarboxylic acids from C_8 to C_{13} are presented. Two different concentrations of mitochondria were used in the presence or absence of 250 μM ADP (states 3 and 4, respectively). The results show that, also in the presence of ADP, the inhibitory effect on respiration was proportional to the concentration of each dicarboxylic acid and to the concentration of the mitochondrial protein, indicating a competitive mechanism of inhibition.

C_9 and C_{12} dicarboxylic acids at 20–30 mM concentration were capable of inhibiting 50–60% of the mitochondrial respiration also in the presence of 0.05 mM 2,4-dinitrophenol (state 3 u). The same effect was observed on freezing and thawing damaged mitochondria.

Figure 3 shows a typical spectrum from 350 to

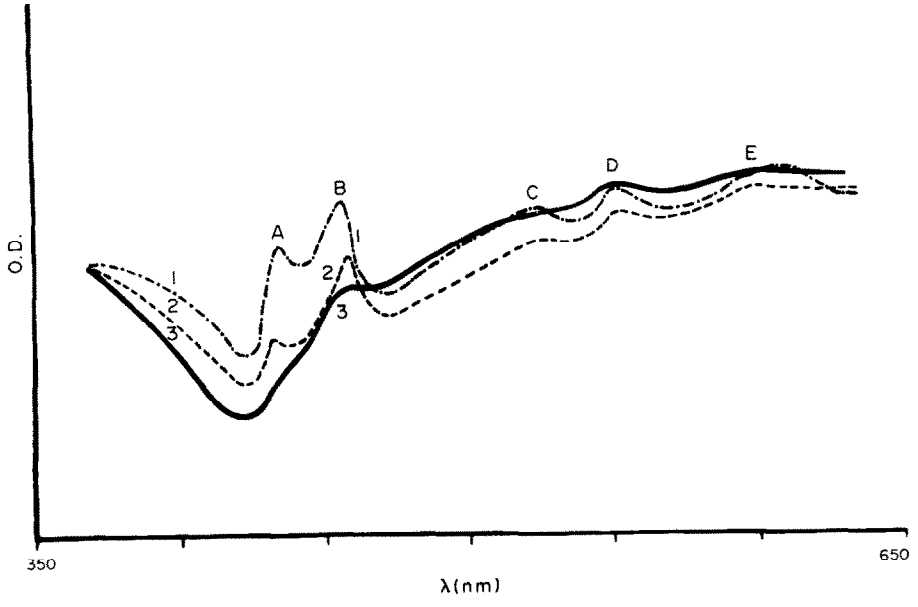

Fig. 3. Effect of C_9 dicarboxylic acid (10 and 5 mM) on state 4 mitochondrial respiration tested by using an AMINCO DW2 spectrophotometer in split-beam mode. Spectrum range, 350–650 nm; span, 0.5; mitochondrial protein concentration, 1 mg/ml. All the spectra were taken 15 min after the addition of 7 mM succinate. 1, Control (without C_9 dicarboxylic acid); 2, 10 mM C_9 dicarboxylic acid; 3, 5 mM C_9 dicarboxylic acid. Major absorption peaks for cytochromes: A, cytochrome *b*; B and C, cytochrome *a*; C and D, cytochrome *c*. It is evident that in the presence of C_9 dicarboxylic acid there is a slower rate of increase in the absorbance of all cytochromes. A similar effect was observed with the other dicarboxylic acids (C_{10}–C_{13}) tested.

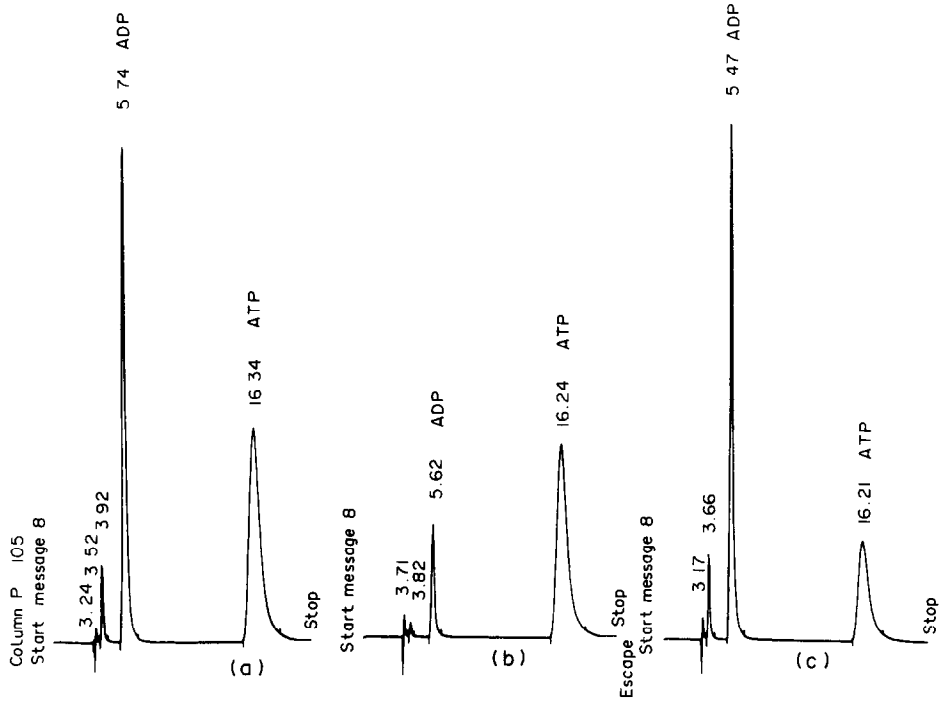

Fig. 4. Effect of C_9 dicarboxylic acid on ATP formation. The mitochondrial suspension (1 mg/ml protein concentration in reagent buffer) in the presence of 7 mM succinate and 250 μM ADP was filtered on 0.5 μm pore size Millipore and injected (50 μl) into the column. (a), ADP and ATP standard separation; (b), ATP generation by the mitochondrial suspension; (c), ATP generation by the same mitochondrial preparation in the presence of C_9 dicarboxylic acid. The inhibition of ATP formation is evident. Chromatographic conditions: column Partisil PXS 10/25 SAX (Whatman). Mobile phase: 0.5 M KH_2PO_4, pH 3.5; flow rate, 0.8 ml/min; slop sensitivity, 1; attenuation, 128 $\times$ 10^{-4} absorbance units/cm; detector, 280 nm.

650 nm of the reduced cytochromes in the presence and absence of azelaic acid; 7 mM succinate was used as substrate. In the presence of azelaic acid, also after 1 hr, there was a slower increase in the absorbance of whatever cytochrome, suggesting that the site, or sites, inhibited were located at a stage preceding the cytochrome chain.

ATP generation

Mitochondrial activity was also tested by measuring ATP formation by HPLC on an ion exchange column. Azelaic acid (10 mM) produced *ca* 40% inhibition in ATP formation from 1 mg/ml of mitochondrial protein preparation by using succinate or glutamate as substrate (Fig. 4), and the higher the amount of diacid, the lower the ATP formation and vice versa. The results obtained by this method confirm the previous results suggesting a competitive mechanism of inhibition.

Swelling test

Up to 20 mM concentration of dicarboxylic acids produced a slight but not significant change in the absorbance at 560 nm only in the presence of substrates.

Sub-mitochondrial particles

Dicarboxylic acids acted on different steps of the mitochondrial electron transport system: NADH dehydrogenase, succinic dehydrogenase, reduced ubiquinone:cytochrome *c* oxido-reductase, but did not inhibit cytochrome *c*:O_2:oxido-reductase (Table 2).

Figure 5 shows the kinetics, studied by using a double-beam spectrophotometer, of NADH dehydrogenase with O_2 as acceptor in the presence or absence of azelaic acid. Purified cytochrome *c* oxidase and NADH dehydrogenase enzymes gave similar results in terms of inhibition and competitive inhibition by the diacids.

DISCUSSION

The results of the present investigation tend to confirm the suggestion that the cytotoxic effect of C_8–C_{13} dicarboxylic acids may be primarily due to an antimitochondrial effect rather than being tyrosinase-dependent as previously thought on the basis of their effect upon melanocytes in hyperpigmentary disorders [2–5].

With isolated rat liver mitochondria and sub-mitochondrial particles, it is clear that dicarboxylic acids from C_8 to C_{13} inhibit cell respiration (Table 1), and this was confirmed by the measurement of ATP formation with HPLC on an ion exchange column (Fig. 4). They inhibit at least three electron transfer reactions of the respiratory chain, involving succinic dehydrogenase, NADH dehydrogenase and reduced ubiquinone:cytochrome *c* oxido-reductase (Table 2, Fig. 5).

Table 2. Effect of azelaic acid on submitochondrial particles

Substrate	Enzyme	Acceptor	Azelaic acid concentration (mM)	% Inhibition
$NADH_2$, 0.2 mM	NADH dehydrogenase	Oxygen	5.0	30 ± 4
			7.5	45 ± 5
			10.0	60 ± 4
Succinate, 7 mM	Succinic dehydrogenase	Ferricyanide	5.0	32 ± 4
		cytochrome *c*	7.5	50 ± 5
			10.0	68 ± 5
Reduced ubiquinone, 0.5 mg/ml	Reduced ubiquinone:cytochrome *c* oxido-reductase	Cytochrome *c*	5.0	28 ± 6
			7.5	40 ± 5
			10.0	54 ± 4

Each result represents the mean ± S.E. of five experiments. Sub-mitochondrial protein concentration: 0.8–1 mg/ml.

Analogous with tryosinase, which is an oxido-reductase, the mechanism of inhibition is of the competitive type, as shown by the fact that either ATP formation or the degree of inhibition was proportional to the concentration of mitochondrial proteins (Figs. 1 and 2). As with tyrosinase, the higher the number of C atoms, the greater the degree of inhibition of the mitochondrial enzymes; beyond C_{12}, the degree of inhibition does not increase, though, as described in Materials and Methods, technical problems related to solubility make measurement difficult at this level.

The concentration at which dicarboxylic acids exert their antimitochondrial action is high, in the order of 10 mM. It might be thought that this high concentration would be toxic *per se*, but the fact that C_7 dicarboxylic acid, at the same concentration, does not show any activity and that over the range C_8–C_{13} there is a gradient of respiratory inhibition suggests the contrary.

We have to emphasize that C_9 or C_{12} dicarboxylic acids, while active in the treatment of acne or pigmentary disorders due to hyperfunction or malignant proliferation of melanocytes, have no toxic effect on normal cells *in vivo* [3]. Furthermore, we have shown that C_9–C_{12} dicarboxylic acids are partially metabolized by beta-oxidation [7], and that azelaic acid when orally administered to rats and rabbits is not toxic or teratogenic [12]. It has been suggested [13] that the antitumoural effect of certain drugs may be due to their capability of interfering with mitochondrial respiration, and that tumoural cells are more sensitive than normal cells to antimitochondrial agents because mitochondrial deficiency might be one of their weak spots. The present results are in line with this view, and indicate that the cytotoxic effect of C_9 and C_{12} dicarboxylic acids on malignant melanocytes may be due to a similar mechanism.

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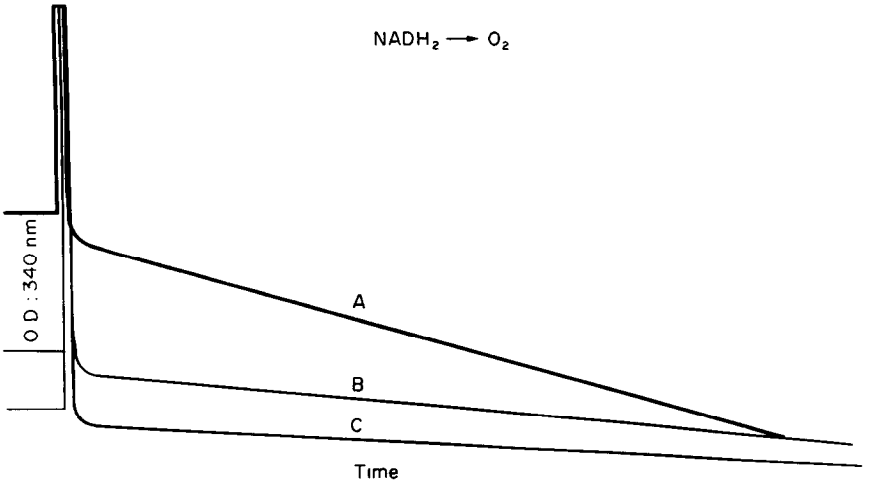

Fig. 5. Effect of C_9 dicarboxylic acid on NADH dehydrogenase. The kinetic study was carried out using a double-beam spectrophotometer in dual mode with 0.2 mM $NADH_2$ as substrate and O_2 as acceptor in the presence of 0.8 mg/ml submitochondrial particles protein concentration, following the rate change in absorbance at 340 nm. Mono 1, 340 nm; Mono 2, 290 nm; span, 0.5; time base, 10 sec/in. A, Control; B, effect of 5 mM C_9 dicarboxylic acid; C, effect of 10 mM C_9 dicarboxylic acid under the same conditions as the control.

REFERENCES

1. M. Nazzaro-Porro and S. Passi, *J. invest. Dermat.* **73**, 112 (1978).
2. M. Nazzaro-Porro and S. Passi, *Giorn. It. Dermat.* **113**, 401 (1978).
3. M. Nazzaro-Porro, S. Passi, L. Balus, A. S. Breathnach, B. Martin and G. Morpurgo, *J. invest. Dermat.* **73**, 112 (1979).
4. M. Nazzaro-Porro, S. Passi, G. Zina, A. Bernengo, A. S. Breathnach, S. M. Gallagher and G. Morpurgo, *Lancet* **ii**, 1109 (1980).
5. M. Nazzaro-Porro, S. Passi, G. Zina and A. S. Breathnach, in *Proceedings of the XVIth International Congress on Dermatology*. Kukita, A. and Seiji, M. (Eds). University of Tokyo Press, Tokyo, p. 229 (1983).
6. M. Nazzaro-Porro, S. Passi, M. Picardo, A. S. Breathnach, R. Clayton and G. Zina, *Br. J. Dermat.* **109**, 45 (1983).
7. S. Passi, M. Nazzaro-Porro, M. Picardo, G. Mingrone and P. Fasella, *J. Lipid Res.* **24**, 1140 (1983).
8. B. J. Ward, A. S. Breathnach, E. J. Robins, S. Passi and M. Nazzaro-Porro, *J. invest. Dermat.* **80**, 370 (1983).
9. D. Johnson and H. A. Lardy, in *Methods in Enzymology* (Eds. R. W. Eastbrook and M. E. Pullmann), Vol. 10, p. 94. Academic Press, New York (1967).
10. E. Bustamante, J. W. Sofer and P. H. Pederson, *Analyt. Biochem.* **80**, 41 (1977).
11. R. W. Estabrook and M. E. Pullmann (Eds.), *Methods in Enzymology*, Vol. 10. Academic Press, New York (1967).
12. G. Mingrone, V. Greco, M. Nazzaro-Porro and S. Passi, *Drug exp. clin. Res.* **9**, 447 (1983).
13. D. Wilkie, *J. Roy. Soc. Med.* **72**, 599 (1979).