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EFFECT OF ADENINE NUCLEOTIDES ON THE OXIDATION OF *N*-METHYL GROUPS IN LIVER MITOCHONDRIA

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SUMMARY

In phosphorylating mitochondria, isolated in 0.25 M sucrose and suspended in a glycylglycine–KCl medium at pH 7.4, the *N*-methyl group of sarcosine is oxidized to formaldehyde, formate, and CO_2. The initial rate of O_2 uptake in this system is only about half as great as with phosphate-washed mitochondria, in which the *N*-methyl carbon is oxidized only to the level of "active formaldehyde" and can be recovered as serine-β-carbon and/or formaldehyde. In the glycylglycine–KCl medium, the O_2 uptake with sarcosine occurs in a biphasic manner and the initial slower rate can be extended by the addition of Mg^{2+}, and ADP, AMP, or ATP. O_2 uptake is similarly restrained by ADP in mitochondria buffered with imidazole or pyrophosphate. The ADP effect is not observed in the presence of dinitrophenol. The patterns of O_2 uptake obtained with ADP in these various media are not altered when the oxidation of the formaldehyde, derived from the *N*-methyl group, is suppressed by the addition of either semicarbazide or rotenone. With dimethylglycine, another component of the "1-C cycle", the initial rate of oxidation in glycylglycine or imidazole is enhanced by ADP rather than being decreased. These results together with appropriate coenzyme analyses suggest that reactions of "one carbon compounds" can provide sensitive markers for assessing compartition of cofactors such as the pyridine nucleotides, flavins, and folates in the mitochondrial matrix.

INTRODUCTION

Earlier studies in our laboratory had demonstrated that both the rate and extent of oxidation of the *N*-methyl carbon of sarcosine in intact mitochondria are affected significantly by the composition of the reaction medium. O_2 uptake by mitochondrial suspensions in which sarcosine oxidation can be coupled to phosphorylation of ADP[1] is only about half as rapid as that observed when the mitochondria are swollen in isotonic phosphate buffers[2]. Similar oxidative patterns have been observed in the oxidation of choline by Williams[3], Kimura *et al.*[4], and more recently by Kagawa *et al.*[5,6]. The latter investigators concluded that in the presence of Mg^{2+} both adenosine di- and triphosphate decrease the permeability of intact mitochondria to choline[6]. Subsequent studies in our laboratory have demonstrated that the initial

rate of oxidation of sarcosine can likewise be lowered by the presence of adenine nucleotides and exogenous magnesium in the mitochondrial suspensions. The analyses to be described in the present paper have also assessed other variables in the composition of the reaction medium which can influence both the rate and extent of oxidation of the *N*-methyl carbon in intact mitochondria.

METHODS AND MATERIALS

Mitochondria of rat liver were isolated in 0.25 M sucrose or 0.15 M KCl as described previously[1,7].

Respiration was measured manometrically at 32 °C in a Gilson differential respirometer in a final volume of 2.6 ml, unless otherwise indicated. The compositions of the reaction mixtures are given in the legends of Table I and figures.

Mitochondrial swelling was monitored by measuring changes in nephelometric densities in a Klett photometer equipped with a No. 52 filter.

Loss of nucleotides and other components from the mitochondria was determined from spectrophotometric examination (210–700 nm) of supernatant solutions of preparations centrifuged at $15000 \times g$ for 10 min. Pyridine nucleotides were measured fluorometrically as described in Table I, and the folates were determined by the procedure of Herbert[8].

Formaldehyde was analyzed by the chromotropic acid method[9].

Sarcosine, free base, was obtained from Fluka AB and was recrystallized from ethanol–water. Dimethylglycine and the adenine nucleotides were purchased from the Sigma Chemical Company. All of the other reagents were commercial products of reagent quality.

RESULTS AND DISCUSSION

Effect of ADP and magnesium on rate of sarcosine oxidation in phosphorylating mitochondria

Under appropriate conditions O_2 uptake by mitochondria metabolizing sarcosine can occur in a biphasic manner and the initial slow rate can be extended by addition of ADP to the reaction medium, as illustrated by the data in Fig. 1. In the presence of exogenous ADP, the rate of oxidation may be only about half as great as when ADP is omitted from the reaction medium. Parallel experiments under the same conditions demonstrated that these patterns of oxidation are not observed for other substrates such as glutamate, succinate and α-glycerophosphate. The influence of ADP on sarcosine oxidation is Mg^{2+}-dependent. When exogenous Mg^{2+} is omitted from the reaction mixture, or when fluoride is added, the rate of O_2 uptake is no longer biphasic and is not altered by added ADP. Exogenous AMP and ATP influence sarcosine oxidation in a manner similar to ADP. GDP, IDP and UDP have no effect.

The restraining effect of ADP on sarcosine oxidation is also observed in mitochondria washed with KCl and buffered with either imidazole or pyrophosphate in place of the glycylglycine.

The action of ADP is sensitive to H^+ concentration, as demonstrated by experiments carried out in the imidazole buffer at pH 7.4 and 6.9. At pH 7.4 the rate

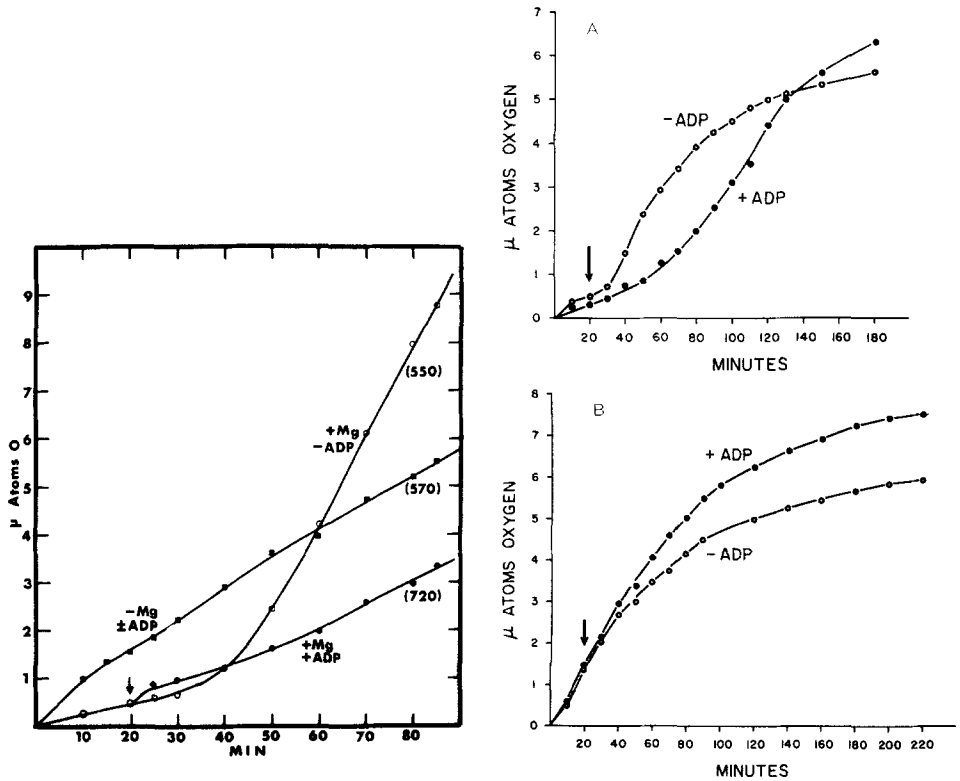

Fig. 1. Effect of ADP and Mg^{2+} on mitochondrial oxidation of sarcosine. Reaction mixture contained: mitochondria (14.5 mg protein); sucrose, 300 μmoles; glycylglycine, pH 7.4, 105 μmoles; KCl, 180 μmoles; potassium phosphate, pH 7.4, 10 μmoles; sarcosine, 20 μmoles; and where indicated, $MgCl_2$, 21 μmoles; ADP, 5μmoles; final volume 2.6 ml. The numbers in parentheses are the nephelometric densities (Klett photometer readings with the 520-nm filter) of the mitochondrial suspensions, measured at the end of the manometric analyses. The time of addition of ADP is indicated by the arrow. ○, −ADP; ●, +ADP; ■, $-Mg^{2+}$, ±ADP.

Fig. 2. Effect of pH on sarcosine oxidation in presence of ADP. (A) pH 7.4, (B) pH 6.9. Reaction components: mitochondria in 0.15 M KCl, 0.4 ml (12 mg protein); 0.15 M imidazole–0.03 M $MgCl_2$, pH 7.4 or 6.9, 0.7 ml; 0.3 M KCl, 0.6 ml; 0.15 M KCl, 0.8 ml; 0.1 M potassium phosphate, 0.1 ml; 0.1 M sarcosine, 0.2 ml; and 0.05 M ADP, 0.1 ml, added at 20 min as indicated. ○, −ADP; ●, +ADP.

of O_2 uptake is lowered in the presence of ADP (Fig. 2A), while at pH 6.9 sarcosine oxidation is stimulated by ADP and is sustained at a higher rate than in the absence of ADP (Fig. 2B).

ADP has no effect on the rate of sarcosine demethylation in a mitochondrial preparation ruptured by sonic irradiation or when the mitochondria are washed and suspended in a medium such as 0.075 M potassium phosphate at pH 7.4. As demonstrated by earlier studies from this laboratory[2,10] the mitochondria swell, but remain intact in this buffer and the initial rate of sarcosine oxidation is 2–3 times greater than in the glycylglycine–KCl system. Furthermore, there is no lag period in the O_2 uptake. The same effects of phosphate have been observed in the oxidation

of choline[5]. Like phosphate, formate is capable of penetrating the inner membrane of the mitochondrion[11]. It was of interest to find in the present experiments that the kinetics of oxidation of sarcosine in 0.075 M formate are similar to those observed in the phosphate medium.

Effects of ADP on dimethylglycine oxidation

Both of the *N*-methyl groups of dimethylglycine can be oxidized to active formaldehyde in intact mitochondria[12] by the sequential action of two enzymes, dimethylglycine dehydrogenase and sarcosine dehydrogenase[13]. It was observed in the current studies that, under the same conditions employed for sarcosine as substrate, the initial rate of oxidation of dimethylglycine is enhanced rather than being restrained in the presence of ADP. In further contrast to sarcosine oxidation, the rate of O_2 uptake with dimethylglycine is only about half as great in 0.075 M phosphate as in the imidazole or pyrophosphate systems. The same results are obtained when semicarbazide is present.

Factors affecting the extent of oxidation of the N-methyl carbon beyond the level of formaldehyde or the β-carbon of serine

When the mitochondria are swollen and uncoupled in 0.075 M phosphate, the oxidation of the *N*-methyl groups of sarcosine and dimethylglycine does not proceed beyond the level of "active formaldehyde"[12,14]. Similar results were obtained in the present experiments when formate was substituted for the phosphate. On the other hand, when the mitochondria are washed in 0.25 M sucrose or 0.15 M KCl and finally suspended in the KCl–glycylglycine medium (Fig. 1), the methyl carbon is oxidized to formate and CO_2 (ref. 1), even without the addition of ADP. When semicarbazide is added to this system as an aldehyde trapping agent[1,2], 75–90% of the oxidized methyl carbon can be isolated as formaldehyde, but the biphasic pattern of O_2 uptake is not affected. Similar results are obtained in the presence of rotenone.

The foregoing results suggested that the biphasic pattern of the rate of O_2 uptake with sarcosine and the oxidation levels attained by the *N*-methyl carbon are not determined solely by the degree of coupling of respiration and phosphorylation. In subsequent experiments, it was demonstrated that in mitochondria uncoupled by dinitrophenol, in either the glycylglycine or imidazole buffer systems, the O_2 uptake does not occur in the usual biphasic manner (Fig. 3A). The oxidation of the methyl group, however, still proceeds beyond the level of formaldehyde. The same results are obtained in the pyrophosphate buffer when the mitochondria are subjected to pentachlorothiophenol (Fig. 3B).

Earlier studies had demonstrated that FAD serves as a coenzyme in the oxidation of both "active formaldehyde" and formaldehyde. NAD^+, on the other hand, appears to be specific for the oxidation of formaldehyde[14]. Since swelling of mitochondria in phosphate results in "leakage" of intramitochondrial nucleotides[15], the metabolism of the *N*-methyl group of sarcosine in phosphate-treated mitochondria is limited to the formation of formaldehyde and/or the β-carbon of serine[14]. In the present experiments, the same results were obtained with mitochondria washed with formate. Further analyses (Table I) demonstrated that folates, like the pyridine nucleotides, are also lost from mitochondria exposed to phosphate. However, these

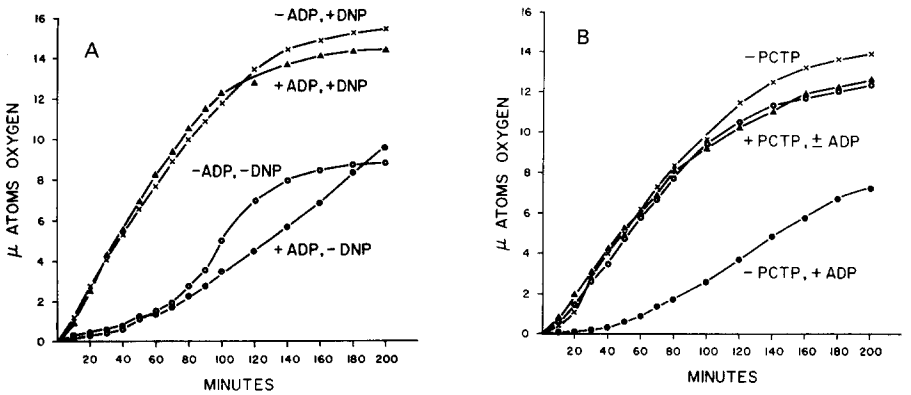

Fig. 3. Effects of dinitrophenol and pentachlorothiophenol on sarcosine oxidation in absence and presence of ADP. (A) Imidazole buffer and other components as described in Fig. 2A. ○, −ADP, −dinitrophenol; ●, +ADP, −dinitrophenol; ×, −ADP +dinitrophenol, ($5 \cdot 10^{-5}$ M); ▲, +ADP, +dinitrophenol. (B) 0.075 M potassium pyrophosphate, pH 7.5, 1.9 ml; mitochondria in 0.15 M KCl, 0.6 ml; sarcosine and ADP as described elsewhere. ×, −ADP, −pentachlorothiophenol; ●, +ADP, pentachlorothiophenol; ○, −ADP, +pentachlorothiophenol ($4 \cdot 10^{-5}$ M); ▲, +ADP, +*p*-chlorothiophenol.

TABLE I

FOLATE AND PYRIDINE NUCLEOTIDE CONTENT OF INTACT MITOCHONDRIA ISOLATED IN SUCROSE OR PHOSPHATE

The mitochondria were washed three times in 0.25 M sucrose or 0.075 M potassium phosphate, pH 7.5, as described previously. For the folate assays, the mitochondria were resuspended in 50 ml of 0.01 M sodium acetate, pH 7.3, containing 0.5 g ascorbic acid and assayed for protein. After heating 15 min, the precipitate was removed by centrifugation and the supernatant solution was filtered prior to microbiological assay. For the pyridine nucleotide determination the mitochondrial suspension was analyzed for protein and was then denatured with $(NH_4)_2SO_4$, at 80% of saturation. The NAD^+ (and $NADP^+$) in the supernatant solutions was reduced with a small quantity of dithionite and was measured fluorimetrically, using an appropriate internal standard of NAD^+. The analyses were made with a Farrand spectrofluorimeter (Mark I) by exciting at 354 nm and measuring the emission at 450 nm.

Medium for isolation of mitochondria	*Folates* (μg/g mitochondrial protein)*	*Pyridine nucleotides** (μmoles/g mitochondrial protein)*
0.25 M sucrose	89	7.5
75 mM phosphate, pH 7.5	32	2.6

* Calculated as folic acid.
** Calculated as NADH.

preparations are still capable of converting the oxidized *N*-methyl groups of sarcosine and dimethylglycine to serine-β-carbon at a high rate[14]. From the foregoing results it is apparent that reactions of the "one-carbon units" derived from the sarcosine methyl group can serve as sensitive enzymatic markers for assessing compartition of the pyridine nucleotides, flavins, and folates in the mitochondrial matrix[10].

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REFERENCES

1 Frisell, W. R. and Sorrell, N. C. (1967) *Biochim. Biophys. Acta* 131, 207–210
2 Frisell, W. R. and Mackenzie, C. G. (1955) *J. Biol. Chem.* 217, 275–285
3 Williams, G. R. (1955) *Fed. Proc.* 14, 304
4 Kimura, T., Singer, T. P. and Lusty, C. J. (1960) *Biochim. Biophys. Acta* 44, 284–297
5 Kagawa, T., Wilken, D. R. and Lardy, H. A. (1965) *J. Biol. Chem.* 240, 1836–1842
6 Wilken, D. R., Kagawa, T. and Lardy, H. A. (1965) *J. Biol. Chem.* 240, 1843–1846
7 Van Buskirk, J. J. and Frisell, W. R. (1967) *Biochim. Biophys. Acta* 143, 292–298
8 Herbert, V. (1966) *J. Clin. Pathol.* 19, 12–16
9 Frisell, W. R. and Mackenzie, C. G. (1958) in *Methods of Biochemical Analysis* (Glick, D., ed.) Vol. 6, pp. 63–77, Interscience Publishers, New York
10 Frisell, W. R., Patwardhan, M. and Mackenzie, C. G. (1965) *J. Biol. Chem.* 240, 1829–1835
11 Chapell, J. B. and Haarhoff, K. N. (1967) in *Biochemistry of Mitochondria* (Slater, E. C., Kaniuja, Z. and Wojtczak, L., eds), pp. 75–91, Academic Press, New York
12 Mackenzie, C. G. and Frisell, W. R. (1958) *J. Biol. Chem.* 232, 417–427
13 Frisell, W. R. and Mackenzie, C. G. (1962) *J. Biol. Chem.* 237, 94–98
14 Mackenzie, C. G. (1955) in *Amino Acid Metabolism* (McElroy, W. D. and Glass, B., eds), pp. 702–726, The Johns Hopkins Press, Baltimore, Md.
15 Kaufman, B. T. and Kaplan, N. O. (1960) *Biochim. Biophys. Acta* 39, 332–342