levels of piericidin A equivalent to levels required to inhibit NADH oxidase. This low sensitivity of the NADH oxidase in *Azotobacter* particles may indicate that this system is different from the NADH system in mitochondria.

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References

Asano, A., and Brodie, A. F. (1964), *J. Biol. Chem. 239*, 4280.

Breidenbach, R. W., and Beevers, H. (1967), *Biochem. Biophys. Res. Commun. 27*, 462.

Chance, B. (1952), *Nature 169*, 215.

Crane, F. L., and Ehrlich, B. (1961), *Arch. Biochem. Biophys. 89*, 134.

Ernster, L., Dallner, G., and Azzone, G. F. (1963), *J. Biol. Chem. 238*, 1124.

Gornall, A. G., Bardawill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177*, 751.

Hall, C., Wu. M., Crane, F. L., Takahashi, N., Tamura, S., and Folkers, K. (1966), *Biochem. Biophys. Res. Commun. 25*, 373.

Hatefi, Y., Haavik, A., and Griffiths, D. E. (1962), *J. Biol. Chem. 237*, 1676.

Hatefi, Y., Haavik, A., and Jurtshuk, P. (1960), *Biochem. Biophys. Res. Commun. 3*, 281.

Jacobs, E. E., Andrews, E. C., and Crane, F. L. (1964), *in* Oxidases and Related Redox Systems, Vol. II, King, T. E., Mason, H. S., and Morrison, M., Ed., New York, N. Y., Wiley, p 784.

Jurtshuk, P., Aston, P., and Old, L. (1967), *J. Bacteriol. 93*, 1069.

King, T. E., Nickel, K. S., and Jensen, D. R. (1964), *J. Biol. Chem. 239*, 1989.

Löw, H., and Vallin, I. (1963), *Biochim. Biophys. Acta 69*, 36.

Pollard, C. J., and Bieri, J. G. (1958), *Biochim. Biophys. Acta 30*, 658.

Slater, E. C. (1950), *Biochem. J. 46*, 484.

Takahashi, N., Auzuki, A., and Tamura, S. (1965), *J. Am. Chem. Soc. 87*, 2066.

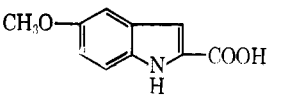
Inhibition of Gluconeogenesis and α-Keto Oxidation by 5-Methoxyindole-2-carboxylic Acid*

Norman Bauman and C. J. Hill

ABSTRACT: 5-Methoxyindole-2-carboxylic acid (MICA) is known to produce hypoglycemia *in vivo* by inhibition of hepatic gluconeogenesis. Its effects in liver slices and subcellular systems have been examined. MICA blocks gluconeogenesis at the level of mitochondrial CO_2 fixation, but does not affect solubilized pyruvate carboxylase.

MICA also specifically blocks mitochondrial oxidation of pyruvate and α-ketoglutarate, an effect reversible *in vitro* by lipoic acid.

5-Methoxyindole-2-carboxylic acid (MICA)[1] produces hypoglycemia in animals through inhibition of hepatic gluconeogenesis (Bauman *et al.*, 1967; Hanson *et al.*, 1967). In particular, it has been shown that it inhibits glucose production by the isolated perfused rat liver and at the same time decreases oxidation of lactate (N. Bauman and B. Pease, unpublished data). The present work extends these studies to liver slices and subcellular systems, demonstrating that MICA blocks mitochondrial oxidation of α-keto acids, an effect reversible *in vitro* by lipoic acid.



MICA

Experimental Section

Materials and Methods

MICA was purchased from the Aldrich Chemical Co. and recrystallized from water. It was dissolved in 0.15 M sodium bicarbonate or, in experiments where bicarbonate concentration might be critical, was suspended in water, to which 1 N KOH was added dropwise until the acid was dissolved and the pH was 7.5

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[1] Abbreviations used that are not listed in *Biochemistry* 5, 1445 (1966), are: MICA, 5-methoxyindole-2-carboxylic acid; PEP, phosphoenolpyruvate.

TABLE I: Effect of MICA on Glucose Production and Oxidation of Lactate and Pyruvate by Rat Liver Slices.[a]

	Carbohydrate Content (μmoles of glucose/50 mg of liver)	Substrate Converted into CO_2 during 2nd Incubn (%/50 mg of liver)
Expt 4466C115 DL-lactate-U-^{14}C		
Control incubated 1 hr only	0.13 ± 0.01[b]	(0.12 ± 0.005)[c]
Control incubated 1 hr + 2 hr	0.76 ± 0.09	1.96 ± 0.14
MICA in first incubation only	0.14 ± 0.04	0.73 ± 0.06
MICA in second incubation only	0.08 ± 0.02	0.43 ± 0.01
Expt 4466C151 pyruvate-1-^{14}C		
Control incubated 1 hr only	0.02 ± 0.005	(0.1 ± 0)[c]
Control incubated 1 hr + 2 hr	0.66 ± 0.02	13.7 ± 0.4
MICA in first incubation only	0.08 ± 0.03	1.8 ± 0.3
Expt 4466C152 pyruvate-2-^{14}C		
Control incubated 1 hr only	0.02 ± 0.005	(0.5 ± 0)[c]
Control incubated 1 hr + 2 hr	0.88 ± 0.08	6.8 ± 0.3
MICA in first incubation only	0.16 ± 0.02	2.2 ± 0.1

[a] Liver slices weighing 40–80 mg were prepared from a fasted rat. They were incubated 1 hr at 37° with shaking in sealed vials containing 2.8 ml of Krebs–Ringer bicarbonate buffer and 0.2 ml of 0.15 M DL-lithium lactate or sodium pyruvate, as indicated, under 95% O_2–5% CO_2 (first incubation). The slices were then transferred to fresh medium of the same composition, containing in addition about 0.1 μCi of the indicated radioactive substrate. They were then, unless otherwise indicated, incubated for 2 hr under the same conditions (second incubation). When indicated, vials also contained 300 μg of MICA. At the end of the second incubation the vials were chilled, the papers were moistened with 10 N NaOH, and CO_2 was released by addition of H_2SO_4. After another 30-min shaking the papers were removed and counted. The slices and media were hydrolyzed at 100° for 2 hr, acidity was 0.6 N. The mixtures were cooled, carefully neutralized with solid $BaCO_3$, and an aliquot of the supernatant was taken for glucose analysis. Each value is an average of three to six duplicate vials, corrected to a tissue weight of 50 mg. [b] Mean plus and minus standard error of the mean. [c] There was no second incubation; this value is a blank, presumably representing the $^{14}CO_2$ contamination of the radioactive substrate.

Organic chemicals were obtained from the following suppliers. PEP, potassium pyruvate, ITP, glutathione, sodium DL-β-hydroxybutyrate, thiamine pyrophosphate, α-ketoglutaric acid, Li_3CoA, and trilithium acetyl-CoA from Calbiochem; K-ATP from Schwarz BioResearch, Inc.; fumaric acid, oxalacetic acid, and sodium pyruvate from Nutritional Biochemicals Corp.; Tris and octanoic acid from Sigma Chemical Co.; sucrose (Enzyme Grade) from Mann Research Laboratories, Inc.; succinic acid from Eastman Organic Chemical Co.; anion-exchanger AG1-X8 (Cl) from Bio-Rad Laboratories. All compounds were used in aqueous solution; acidic compounds were adjusted to neutrality with NaOH or KOH, as indicated in the tables.

Inorganic chemicals (reagent grade) were from Fisher Scientific Co., Merck & Co., or Mallinckrodt Chemical Works.

Na_2CO_3-^{14}C (25 mCi/mmole) was purchased from the Chemical Tracer Corp. Sodium lactate-U-^{14}C (60 mCi/mmole) was purchased from the Volk Co.; radiopurity was certified by the manufacturer to be over 99%. Sodium pyruvate-1-^{14}C (4.7 mCi/mmole) and sodium pyruvate-2-^{14}C (3–4 mCi/mmole) were from the New England Nuclear Co. Because of its rapid deterioration (Silverstein and Boyer, 1964), radiopyruvate was frequently assayed for radiopurity by preparing the crystalline 2,4-dinitrophenylhydrazone of an isotopically diluted sample and comparing the specific activity of the derivative with that of the original. When necessary, radiopyruvate was repurified by chromatography on AG1-X8 (Cl) (Marcus and Shannon, 1962). All samples used had radiopurity of over 90% as estimated by the above method. ^{14}C was counted in a liquid scintillation counter (Packard Tri-Carb) in "DAM 611" (Davidson and Feigelson, 1957) or 0.4% 2,5-diphenyloxazole, 0.005% 1,4-bis[2-(5-phenyloxazolyl)]benzene in toluene mixed with methyl Cellosolve (ethylene glycol monomethyl ether). Quenching was compensated when necessary by use of internal standards.

Adult male rats of the CFE strain (Carworth Farms), weighing 150–200 g, were used throughout. They were fed Purina Chow *ad libitum* except when fasted for 18–24 hr.

Liver slices were cut from freshly excised livers by use of a mechanical chopper (McIlwain and Buddle, 1953). They were incubated in sealed vials under 95% oxygen, 5% CO_2 on a Dubnoff shaker at 37°. When

TABLE II: Lack of Effect of MICA on Rat Liver Phosphoenolpyruvate Carboxykinase.[a]

Experiment 8952B92	PEP Produced (μmole/min)
Complete system	0.07
Add MICA (333 μg/ml)	0.07
Omit oxalacetate	0.01
Zero time	0

[a] Incubations contained (in micromoles): glutathione (2.5), Na-ITP (15), NaF (30), Tris-HCl (pH 8.0) (67), $MgSO_4$ (22), sodium oxalacetate (10), and 0.2 ml of 105,000*g* supernatant of rat liver in a total volume of 1.5 ml. After 6-min incubation at 30° reactions were stopped by addition of 0.25 ml of 35% (w/v) trichloroacetic acid. The supernatants were analyzed for PEP as indicated in Methods.

TABLE III: Effect of MICA on CO_2 Fixation in Rat Liver Slices (expt 4466C-183).[a]

MICA (μg/ml)	cpm of CO_2 Fixed/ 100 mg of Liver
0	2454
9	1679
89	584
0 (unincubated)	374

[a] Mixtures contained (in micromoles): $CaCl_2$, 6; KCl, 81; $KHCO_3$, 39; potassium pyruvate, 69; Na_2CO_3-^{14}C (199,000 cpm) and 100 mg of liver slices from a fasted rat; and MICA or isotonic KCl as indicated, in a volume of 1.1 ml. The vials were sealed with an atmosphere of 95% O_2–5% CO_2 and shaken at 37° for 2 hr. Incubations were stopped by addition of 0.3 ml of 1.2 M $HClO_4$, transferred quantitatively with water wash to homogenizer tubes, and homogenized. After CO_2 was bubbled through to drive out $^{14}CO_2$, an aliquot of the whole mixture was counted.

radioactive CO_2 was to be collected, a filter paper was suspended from the stopper and alkalinized by injection just before ending the incubation by injection of dilute H_2SO_4. Krebs–Ringer bicarbonate buffer (Umbreit *et al.*, 1957) was used except in CO_2 fixation experiments, when the conditions were essentially those of Buchanan *et al.* (1942). Rat liver mitochondrial and supernatant fractions were prepared by the method of Hogeboom (1955).

Mitochondrial pyruvate carboxylase was extracted by the method of Scrutton and Utter (1965), modified by addition of 2 mM 1,4-dithiothreitol to the Tris-acetate buffer (W. McClure and H. A. Lardy, personal communication).

PEP was assayed as mercury-labile organic phosphorus (Nordlie and Lardy, 1963). PEP carboxykinase was assayed by production of PEP from oxalacetate and ITP; the conditions were essentially those of Foster *et al.* (1966). Glucose was assayed by the use of a glucose oxidase–peroxidase reagent (Worthington Biochemicals Corp.). Mitochondrial oxygen consumption was measured at 30° in the Warburg apparatus by standard techniques (Umbreit *et al.*, 1957). Protein was measured by the biuret method.

Results

Carbohydrate Production by Liver Slices. Rat liver slices were incubated in media containing lactate or pyruvate as substrate. Their carbohydrate contents were estimated by measuring the total glucose obtained on acid hydrolysis. Net carbohydrate production could then be estimated by subtracting the mean carbohydrate content of slices incubated for 1 hr from that of similar slices transferred at the end of the first hour of incubation to fresh medium and incubated an additional 2 hr. During this second incubation the substrate was radioactively labeled, so that oxidation of the substrate to CO_2 could be measured as well. Some of the slices were treated with MICA, either during the first incubation or during the second incubation; their carbohydrate contents were measured at the end of the second incubation. The data so obtained are given in Table I.

It can be seen that in each experiment there was net carbohydrate production in the controls, *i.e.*, there was more carbohydrate at the end of 3 hr than at the end of 1 hr. In each experiment treatment with MICA resulted in a carbohydrate content at 3 hr which was much less than the net synthesis in the controls, so we may say *a fortiori* that if there was any net synthesis of carbohydrate in the MICA-treated slices, it was much less than in the controls.

Similarly we note that oxidation of substrate is impaired by concurrent or prior treatment with MICA. That prior treatment with MICA (*i.e.*, during the first incubation) diminishes carbohydrate synthesis and substrate oxidation during the second incubation suggests that MICA is not easily washed out of the tissue.

PEP Carboxykinase. A major enzyme in the production of glucose is PEP carboxykinase. Therefore, the 105,000*g* supernatant of rat liver was studied for possible effects of MICA on PEP carboxykinase activity. The results of these studies are shown in Table II. It is evident that MICA does not affect this enzyme.

CO_2 Fixation in Liver Slices. Another measurement of gluconeogenesis in liver slices is the fixation of CO_2. Table III shows the results of an experiment in which the fixation of $NaHCO_3$-^{14}C into material not volatile in acid was inhibited by MICA.

CO_2 Fixation in Mitochondria. A similar experiment was performed with isolated mitochondria; fixation of $NaHCO_3$-^{14}C into material not volatile in acid was determined. Table IV shows that this reaction was dependent on pyruvate and was also inhibited by

TABLE IV: Effect of MICA on CO_2 Fixation and Pyruvate Oxidation by Rat Liver Mitochondria.[a]

	CO_2 Fixed (mμmoles)	Pyruvate-1-^{14}C Converted into CO_2 (mμmoles)
Expt 8952B151[a]		
Complete system	29	
Add MICA (8.9 μg/ml)	9	
Add MICA (89 μg/ml)	1	
Omit pyruvate	0	
Expt 8952B185[b]		
Complete system	69	24
Add MICA (30 μg/ml)	36	21
Add MICA (100 μg/ml)	18	11

[a] The complete system contained the following (in micromoles): Tris-HCl buffer, pH 7.5 (51); $MgCl_2$ (10); sodium phosphate buffer, pH 7.4 (4); K-ATP (3); NaF (7); $KHCO_3$ (10); sodium pyruvate (7); $NaHCO_3$-^{14}C (0.1 μCi); 3 mg of mitochondrial protein; and sufficient 0.25 M sucrose to bring the volume to 1.2 ml. Incubation was for 5 min at 30° after which 0.3 ml of 1 N $HClO_4$ was added, the samples were agitated to allow CO_2 to escape, and an aliquot of the residue was counted. [b] Conducted similarly, with the following exceptions. (1) The amounts of the above constituents, in micromoles, were: 50, 8, 4, 3, 5, 10, and 10, respectively. (2) Some of the vials contained 0.1 μCi of pyruvic-1-^{14}C acid instead of $NaHCO_3$-^{14}C. (3) The CO_2 liberated at the end of the incubations with radiopyruvate was collected on papers and counted. All figures are averages of two duplicate determinations and have been corrected for blanks determined on unincubated mixtures. Blanks were approximately 50% of the complete system in expt 8952B151 and 10% in expt 8952B185.

TABLE V: Effect of MICA on Pyruvate Carboxylase.[a]

Experiment 8952B48	CO_2 Fixed (μmole/min)
Complete System	0.12
Add MICA (100 μg/ml)	0.11
Omit acetyl-CoA	0.003
Substitute CoA for acetyl-CoA	0.007
Omit pyruvate	0

[a] Complete system contained (in micromoles): trithanolamine-HCl–NaOH buffer, pH 7.0 (34); $MgSO_4$ (6); K-ATP (1.3); $KHCO_3$ (13); 1,4-dithiothreitol (1.0); potassium pyruvate (3.8); trilithium acetyl-CoA (0.6); 0.2 ml of mitochondrial extract (see Methods); and Na_2-CO_3-^{14}C (0.06 μCi) in a final volume of 0.7 ml. It was incubated 5 min at 30°. The incubation was stopped and the CO_2 was released with 0.6 ml of 10% trichloroacetic acid. An aliquot of the supernatant was counted. Each value is the mean of duplicates and has been corrected for a blank of 0.022 μmole.

MICA. There is concurrent inhibition of pyruvate decarboxylation.

Mitochondrial Pyruvate Carboxylase. The fixation of CO_2 into oxalacetate is catalyzed by pyruvate carboxylase. This enzyme was extracted from mitochondria and assayed in the presence and absence of MICA. Table V shows that the reaction was dependent on acetyl-CoA (Utter and Keech, 1963) and that it was unaffected by MICA. As no direct effect of MICA on pyruvate carboxylase could be demonstrated, MICA might act by depriving pyruvate carboxylase of its other substrate, ATP, or of its cofactor, acetyl-CoA. Therefore, the effects of MICA on mitochondrial oxidative metabolism were examined.

Mitochondrial Oxidation and Phosphorylation. Mitochondrial suspensions were tested in the Warburg respirometer for their ability to oxidize various substrates in the presence of MICA. Table VI shows that oxidation of β-hydroxybutyrate and succinate was only minimally inhibited by MICA. (In separate experiments the P:O ratio was determined with succinate as substrate; it was unaffected by the presence or absence of MICA.) The oxidation of pyruvate, however, was depressed 73% by MICA. Moreover, when fumarate was provided as an acetyl acceptor, pyruvate oxidation was increased threefold but was still inhibitable to the same extent by MICA, suggesting that MICA blocks oxidation of pyruvate to acetyl-CoA. This was confirmed by generating acetyl-CoA from octanoate, thus bypassing oxidation of pyruvate. There was again a marked stimulation of oxidation when fumarate was provided as an acceptor, but now there was no inhibition by MICA. Further confirmation of this hypothesis was found in the complete suppression of the oxidation of α-ketoglutarate, a reaction known to parallel the oxidation of pyruvate.

Reversal of MICA Inhibition of α-Keto Oxidation. The known cofactors of the pyruvate oxidation system, thiamine, thiamine pyrophosphate, and lipoic acid, were tested for their ability to reverse MICA inhibition of α-ketoglutarate oxidation. Of these substances, only lipoic acid was effective. That this was not simply a nonspecific effect of a sulfhydryl compound was shown by the inability of 1,4-dithiothreitol to substitute for lipoic acid (Table VII).

Discussion

MICA has been found to inhibit hepatic gluconeogenesis both *in vivo* and *in vitro*. We have found a block in the conversion of three-carbon compounds into carbohydrates, agreeing well with the work of Hanson *et al.* (1967) demonstrating a block *in vivo*

TABLE VI: Effect on MICA on Oxidation by Rat Liver Mitochondria.[a]

Substrate	Oxygen Consumed (μatoms/20 min) Control	MICA (100 μg/ml)	% Inhibn
Expt 9125B42			
Pyruvate	6.2	1.7	73
Fumarate	3.4	2.3	32
Pyruvate + fumarate	19.8	4.9	75
Expt 9125B51			
Pyruvate + fumarate	14.3	3.7	74
Expt 9125B75			
Octanoate	2.9	5.1	Negative
Octanoate + fumarate	19.3	16.9	12
Expt 9125B67			
DL-β-Hydroxybutyrate	6.1	5.0	18
Succinate	13.8	12.0	13
α-Ketoglutarate	6.5	0	100

[a] Warburg flasks were equilibrated for 5 min, after which incubation was started by tipping 15 μmoles of each substrate and, when indicated, 300 μg of MICA from the side arm into the main compartment, which contained 40 μmoles of potassium phosphate buffer (pH 7.4), 20 μmoles of $MgSO_4$, 6 μmoles of Na-ATP, mitochondrial suspension containing about 15 mg of mitochondrial protein, and sufficient 0.25 M sucrose to make the final incubation volume 3 ml. Incubation was for 20 min at 30°. Each experiment was run with a single preparation of mitochondria from one fasted rat. Each value is an average of two to five flasks.

TABLE VII: Reversal of MICA Inhibition of α-Ketoglutarate Oxidation.[a]

Addition	Oxygen Consumed (μatoms/20 min) MICA (100 μg/ml)	No MICA
Expt 9125B79		
None	5.1	14.5
Thiamine hydrochloride	4.3	
Thiamine pyrophosphate	3.0	
DL-Lipoic acid	10.6	
10 μmoles of additional α-ketoglutarate	2.6	
Expt 9125B83		
None	3.2	8.8
DL-Lipoic acid	7.9	10.1
Dithiothreitol	1.7[b]	10.9

[a] Incubation conditions were as in Table VI, except that 3 μmoles of the indicated addition and 5 μmoles of the substrate (α-ketoglutarate) were in the main compartment and MICA (300 μg) or an equivalent volume of sucrose was tipped in at the end of the equilibration period. Each value is the mean of two flasks. [b] MICA concentration was 30 μg/ml.

at the level of pyruvate carboxylase. Our finding that mitochondria fail to fix radioactive CO_2 in the presence of MICA also suggests that the block might be at the level of pyruvate carboxylase. However, our data do not exclude as an alternative explanation a block in the series of reactions: oxalacetate → aspartate → arginosuccinate → fumarate → malate → oxalacetate, which randomize C-1 and C-4 of oxalacetate through the symmetric intermediate fumarate and are necessary to gluconeogenesis in liver (Hiatt *et al.*, 1958; Lardy *et al.*, 1965). Should such a block exist, radioactive CO_2, fixed into C-4 of oxalacetate, might again be lost, resulting in an apparent inhibition of pyruvate carboxylase, consistent with our data. This would also be consistent with the demonstrated lack of effect on the solubilized enzymes pyruvate carboxylase and PEP carboxykinase.

We have also shown that MICA, in concentrations less than or equal to those employed *in vivo*, inhibits mitochondrial oxidation of α-keto acids, presumably by interfering with the lipoate-dependent step, as the effect is reversible by lipoic acid. Although this finding need not be related to the block in gluconeogenesis, it is tempting to speculate that a relationship exists, *i.e.*, that the decrease in oxidation of pyruvate causes a relative lack of ATP, a substrate of pyruvate carboxylase, or of acetyl-CoA, a necessary cofactor of pyruvate carboxylase. This hypothesis is subject to experimental test, *e.g.*, by determining the effect of other generators of acetyl-CoA, such as octanoate, or of ATP, such as β-hydroxybutyrate, on the fixation of CO_2 in mitochondria.

The hypoglycemic activity of MICA, of course, suggested it as an agent in the treatment of diabetes mellitus. The present and previous studies (Bauman *et al.*, 1967; Hanson *et al.*, 1967; N. Bauman and B. Pease, unpublished data) on the mode of action of MICA show that it causes hypoglycemia by interfering with oxidative metabolism and gluconeogenesis. This is antithetic to all schemes of antidiabetic therapy.

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References

Bauman, N., Pease, B., and Hill, C. (1967), *Federation Proc. 26*, 507.

Buchanan, J. M., Hastings, A. B., and Nesbett, F. B. (1942), *J. Biol. Chem. 145*, 715.

Davidson, J. D., and Feigelson, P. (1957), *Intern. J. Appl. Radiation Isotopes 2*, 1.

Foster, D. O., Ray, P. D., and Lardy, H. A. (1966), *Biochemistry 5*, 563.

Hanson, P. L., Ray, P. D., and Lardy, H. A. (1967), 154th National Meeting of the American Chemical Society, Chicago, Ill., Sept, Abstract 238C.

Hiatt, H. H., Goldstein, M., Lareau, J., and Horecker, B. L. (1958), *J. Biol. Chem. 231*, 303.

Hogeboom, G. H. (1955), *Methods Enzymol. 1*, 16.

Lardy, H. A., Paetkau, V., and Walter, P. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 1410.

Marcus, A., and Shannon, L. M. (1962), *J. Biol. Chem. 237*, 3348.

McIlwain, H., and Buddle, H. L. (1953), *Biochem. J. 53*, 412.

Nordlie, R. C., and Lardy, H. A. (1963), *J. Biol. Chem. 237*, 2259.

Scrutton, M. C., and Utter, M. F. (1965), *J. Biol. Chem. 240*, 1.

Silverstein, E., and Boyer, P. D. (1964), *Anal. Biochem. 8*, 470.

Umbreit, W. W., Burris, R. H., and Stauffer, J. F. (1957), Manometric Techniques, Minneapolis, Minn., Burgess.

Utter, M. F., and Keech, D. B. (1963), *J. Biol. Chem. 238*, 2603.

Studies on Energy Transfer in Mitochondrial Oxidative Phosphorylation. III. On the Interaction of Adenosine Diphosphate with High-Energy Intermediates*

Rudolf H. Eisenhardt and Otto Rosenthal

ABSTRACT: The initial rapid phase of adenosine triphosphate formation (ATP jump) that is observed when adenosine diphosphate (ADP) is added to mitochondrial suspensions incubated in presence of inorganic phosphate (P_i) and substrate has been further investigated. Direct determinations of the rate and magnitude of the ATP jump are reported in the presence of β-hydroxybutyrate and of succinate, respectively. When succinate rather than β-hydroxybutrate is the substrate, the rate of the ATP jump is considerably enhanced. With either substrate, uncoupling concentrations of 2,4-dinitrophenol are without effect on the ATP jump, though the slower subsequent steady-state phosphorylation is inhibited. The results are interpreted to signify that the ATP jump is due to the rapid interaction of ADP and P_i with preformed high-energy intermediates and that it represents the actual phosphorylation reaction. Steady-state phosphorylation, which is that normally observed, is limited by the rate at which these intermediates are resynthesized. This step is considered to be rate limiting, which suggests that the intermediates are isolated from and independent of the members of the respiratory chain. It is concluded that uncouplers act on the respiratory chain side of the rate-limiting step.

The principal function of the mitochondrial electron transport system is to provide the energy required to combine ADP[1] with P_i to produce ATP. Although Boyer (1965) and Mitchell (1966) have advanced alternate hypotheses, most current theories of oxidative phosphorylation invoke the existence of some intermediary energy carriers, the so-called high-energy intermediates, to explain the transfer and subdivision of free energy from the electron transport system to the phosphorylation site.

Notwithstanding a considerable search for such intermediates, no convincing *chemical*, as contrasted to

* From the Harrison Department of Surgical Research, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104. *Received May 1, 1967.* For papers I and II of this series, see Schachinger *et al.* (1960) and Eisenhardt and Rosenthal (1964), respectively. This work was supported in part by Grant CA-06266 from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966) are: dimethyl-POPOP, 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene; POP, 2,5-diphenyloxazole.