Biochemical Pharmacology, Vol. 23, pp. 203–213. Pergamon Press, 1974. Printed in Great Britain.

EFFECTS OF PYRAZOLE, 4-BROMOPYRAZOLE AND 4-METHYLPYRAZOLE ON MITOCHONDRIAL FUNCTION*

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(*Received* 3 *March* 1973; *accepted* 15 *June* 1973)

Abstract—Pyrazole, an inhibitor of alcohol dehydrogenase, has been widely used in studies of ethanol metabolism. Since its specificity has recently been questioned, we studied the effects of pyrazole, methylpyrazole and bromopyrazole on mitochondrial function. These compounds inhibited oxidative phosphorylation, the $ATP^{-32}P$ exchange reaction, and energy dependent and independent calcium uptake. With α-ketoglutarate as substrate, state 3 (coupled) respiration was inhibited, whereas state 4 (resting) respiration was not affected. By contrast, state 4 respiration was stimulated when succinate or ascorbate served as the substrate, while state 3 respiration was slightly inhibited. Regardless of the substrate, the respiratory control ratio was depressed. The activities of succinic dehydrogenase and cytochrome oxidase were stimulated by pyrazole and its derivatives, which may explain the stimulation of succinate and ascorbate oxidation. The inhibitory effects of these compounds were reversed by washing the mitochondria, indicating that no permanent damage to mitochondria had occurred. This is supported by the lack of stimulation of latent ATPase activity and the unchanged barrier to the penetration of NADH. Pyrazole and its derivatives decreased the uptake of citrate and glutamate, but stimulated that of phosphate and malate. Methylpyrazole and bromopyrazole inhibited the transport of reducing equivalents into the mitochondria, as catalyzed by the malate-aspartate, fatty acid and α-glycerophosphate shuttles. The data mandate caution in advocating the therapeutic use of pyrazole or its derivatives in man, and suggest that the use of pyrazole to assess ethanol metabolism and its sequelae *in vivo* may have limitations.

PYRAZOLE, an inhibitor of alcohol dehydrogenase,[1,2] has been extensively used to assess the role of this enzyme in ethanol metabolism and to study the consequences of ethanol metabolism.[3–5] Moreover, it was suggested that pyrazole may be useful in the treatment of methanol poisoning,[6] and 4-methylpyrazole has been used to inhibit ethanol oxidation in man.[7] However, pyrazole has been shown to have effects on the liver other than inhibition of alcohol dehydrogenase, particularly on microsomal functions. *In vitro*, 2–4 mM pyrazole inhibited microsomal ethanol oxidation and the activities of aniline, pentobarbital and benzpyrene hydroxylases.[8] Chronic administration of this compound produced ultrastructural changes in the liver,[8] retarded growth[8] and reduced hepatic triglyceride content.[9] Conspicuous hepatic necrosis was produced by the combined administration of ethanol and pyrazole.[10] Since mitochondria are important in ethanol metabolism, because of their role in the reoxidation of NADH produced by the action of alcohol dehydrogenase, we investigated the effects of pyrazole on mitochondrial function.

* Supported in part by United States Public Health Service Grant MH-20067.

MATERIALS AND METHODS

Rat liver mitochondria were prepared by a previously described method.[11] The mitochondria were washed and suspended in 0·25 M sucrose–10 mM Tris HCl, pH 7·4–1 mM EDTA. For those experiments in which Ca^{2+} uptake was studied, EDTA was omitted from the final two washings and resuspensions. All radioactive counting procedures were performed in a liquid scintillation counter using PPO, POPOP* and a dioxane base. Protein was determined according to Lowry *et al.*[12]

Oxygen consumption. Oxygen uptake was assayed at 23° using a Gilson oxygraph equipped with a Clark oxygen electrode. The reaction system consisted of: 0·3 M mannitol; 10 mM Tris HCl, pH 7·4; 10 mM potassium phosphate, pH 7·4; 2·5 mM $MgCl_2$; 20 mM KCl and mitochondria equivalent to 2–4 mg protein, in a final volume of 3·0 ml. Substrates included 10 mM succinate, 5 mM ascorbate–0·2 mM TMPD,† 20 mM α-ketoglutarate and 10 mM glutamate. ADP (1·5 mM) was added to initiate state 3 conditions.

Oxidative phosphorylation. Oxidative phosphorylation was assayed at 30° using a Gilson differential manometer as previously described.[13] The reaction mixture consisted of the same medium used for measurement of oxygen consumption, with the addition of 10 mM ATP, 16·7 mM succinate, 25 mM dextrose and 0·5 mg hexokinase. The flasks were incubated for 10 min, ATP was tapped in from the side arm, the system was closed, and manometer readings were taken every 5 min for the next half-hour. The reaction was terminated by the addition of trichloroacetic acid (final concentration, 10%). After centrifugation, the P_i concentration was determined by the method of Sumner.[14] Blanks contained the acid added before the preincubation period.

ATP-^{32}P exchange. The ATP-^{32}P exchange reaction was assayed at 30° as described by Pullman,[15] using $^{32}PO_4$ (20,000 cpm/μmole of P_i) and mitochondrial protein equivalent to 1·2 mg, in a final volume of 1·0 ml. The labeled ATP was extracted with isobutanol–benzene as described by Pullman,[15] and aliquots were counted.

Ca^{2+} uptake. The uptake of Ca^{2+} by the mitochondria was assayed at 30° in the same medium used to study oxygen consumption, but with 0·5 to 1·0 mg mitochondrial protein and 1·0 mM $^{45}Ca^{2+}$ (200,000 cpm/μmole). Either the combination of succinate plus ATP or ATP itself served as the energy source. In the latter system, 5 μg antimycin was also added to prevent endogenous respiration. The reaction was initiated by the addition of Ca^{2+}. After 2 min, the samples were passed through 0·45 μ millipore filters. The filters were washed three times with ice-cold buffer, dried and counted. Blanks were carried out in a similar manner except for the omission of mitochondria.

ATPase activity. ATPase activity was determined as described previously.[13] The released P_i was determined on a 1-ml aliquot by the method of Sumner.[14] Mg^{2+} and DNP-stimulated‡ ATPase activities were assayed by the addition of 3 mM Mg^{2+} or 0·1 mM DNP, respectively, to the incubation mixture.

Transport and oxidation of reducing equivalents (shuttles). The reaction of ethanol with alcohol dehydrogenase generates NADH in the cytoplasm; reoxidation of NADH may then be rate-limiting for the overall metabolism of ethanol.[16,17] In view of the

* PPO = 2,5-diphenyloxazole; POPOP = 1,4-bis-2-(4-methyl 5-phenyloxazolyl) benzene.
† TMPD = *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.
‡ DNP = 2,4-dinitrophenol.

virtual impermeability of the mitochondria to NADH,[18] several shuttle mechanisms have been proposed for the transport of reducing equivalents into the mitochondria, including the malate-aspartate,[19] fatty acid[20] and α-glycerophosphate shuttles.[21] The equilibrium of the alcohol dehydrogenase reaction favors formation of ethanol and NAD^+ from acetaldehyde and NADH. Therefore, the rate of ethanol disappearance would be quite low in the absence of a shuttle mechanism to remove one of the products of the reaction (NADH). Since dissociation and reoxidation of NADH bound to the enzyme probably represent the rate-limiting step in the reaction[16,17] the rate of ethanol disappearance in these systems reflects the rate of passage of reducing equivalents into the mitochondria.[22,23] Mitochondria (5–10 mg protein) were suspended in a medium containing: 300 mM mannitol; 10 mM phosphate buffer, pH 7·4; 10 mM Tris-HCl, pH 7·4; 10 mM KCl; 5 mM $MgCl_2$; 1 mM ADP; and H_2O to a final volume of 3·0 ml. An NADH-generating system was produced by the addition of 0·25 mM NAD^+, 6 mM ethanol and 16 units of alcohol dehydrogenase. The fatty acid shuttle was reconstituted by adding 1 mM ATP, 0·2 mM coenzyme A and 0·1 mM of albumin-bound fatty acid. The malate-aspartate shuttle was assembled by adding 1 mM malate plus 1 mM glutamate, 3 units of malate dehydrogenase and 2 units of glutamic–oxalacetic transaminase. The α-glycerophosphate shuttle was formed by adding 10 mM α-glycerophosphate, 1 mM ATP and 1 unit of α-glycerophosphate dehydrogenase. The components of each shuttle system were added and the flasks were incubated at 30° for 2 min. The reaction was then initiated by the addition of ethanol. The flasks were immediately sealed and maintained at 30° in a Dubnoff metabolic shaker for 20 min. Ethanol oxidation was linear during this period. The reaction was terminated by the addition of trichloroacetic acid (final concentration, 10%), aliquots were removed, and the remaining ethanol concentration was determined by the method of Bonnichsen.[24] Blank flasks contained the acid added before the ethanol. All incubations were carried out in triplicate. There is no oxidation of ethanol in the absence of mitochondria; therefore extramitochondrial dismutations play no role under our conditions (see Results).

Anion uptake. The ability of the mitochondria to take up labeled anions at 15° was assayed essentially according to Chappel and Haarhoff[25,26] in a reaction mixture consisting of: 125 mM KCl; 20 mM Tris-HCl, pH 7·4; 1 mM $MgCl_2$; 0·5 mM EDTA; 2 μg rotenone plus 2 μg antimycin plus 2·5 μg oligomycin per mg of mitochondrial protein. Tritiated H_2O was used to determine total water space, while ^{14}C-sucrose was used to determine the sucrose-permeable space. The low temperature plus the addition of inhibitors of mitochondrial respiration (antimycin plus rotenone) prevented metabolism of the added labeled anion, thereby allowing it to accumulate. The reaction was initiated by the addition of 1 mM ^{14}C-labeled anion (1 μCi). After 3 min, the samples were passed through 0·45 μ millipore filters. The filters were washed three times with ice-cold 125 mM KCl, dried, and counted. Blanks were carried out in a similar manner, except for the omission of mitochondria.

Mitochondrial swelling. The ability of mitochondria to swell in isotonic solutions of their ammonium salts was assayed as previously described.[22] The mitochondria were incubated with 2 mM ADP plus 5 mM potassium phosphate to deplete them of endogenous substrates.

Succinic dehydrogenase. Succinic dehydrogenase activity was assayed at 23° by following the reduction of phenazine methosulfate.[27]

Cytochrome oxidase. Cytochrome oxidase activity was measured at 23° by following the oxidation of reduced cytochrome c.[28]

RESULTS

Oxygen consumption and partial electron transport reactions. The effect of pyrazole and its derivatives on oxygen consumption by rat liver mitochondria depends on the substrate and the presence of ADP. In these experiments, three substrates were used to supply electrons to different parts of the respiratory chain: α-ketoglutarate, an NAD^+-dependent substrate; succinate, which reduces cytochrome b; and ascorbate-TMPD, which reduces cytochrome c. Pyrazole, bromopyrazole and methylpyrazole had little effect on state 4 respiration when α-ketoglutarate was the substrate, but all three compounds inhibited state 3 respiration, bromopyrazole being the most potent (Table 1). Similar results were obtained with other NAD^+-dependent substrates, e.g. glutamate. However, using the succinoxidase or ascorbate-linked systems, state 4 respiration was stimulated by all three compounds, whereas state 3 respiration was slightly inhibited.

TABLE 1. EFFECT OF PYRAZOLE, BROMOPYRAZOLE AND METHYLPYRAZOLE ON OXYGEN UPTAKE ASSOCIATED WITH THE OXIDATION OF α-KETOGLUTARATE, SUCCINATE AND ASCORBATE*

Compound	Concn (mM)	% Change					
		α-Ketoglutarate		Succinate		Ascorbate–TMPD	
		State 4	State 3	State 4	State 3	State 4	State 3
Pyrazole	1	+12	−3	+4	−14	0	0
Pyrazole	3	0	−21	+32	−15	+5	−8
Pyrazole	10	−6	−37	+56	−18	+18	−14
Bromopyrazole	1	0	−39	+31	−2	+9	−13
Bromopyrazole	3	−5	−58	+52	−12	+16	−17
Bromopyrazole	10	−17	−67	+55	−24	+22	−20
Methylpyrazole	1	0	−8	+6	−9	0	+4
Methylpyrazole	3	0	−26	+25	−9	+7	−16
Methylpyrazole	10	+10	−31	+40	−18	+18	−20

* Oxygen uptake was assayed as described under Materials and Methods. State 4 respiration refers to the respiratory rate in the absence of ADP, while state 3 respiration refers to the respiratory rate in the presence of 1·5 mM ADP. Control values (n-atoms oxygen/min/mg of protein) were: α-ketoglutarate, 9·2 to 13·3 (state 4) and 38·5 to 51·8 (state 3); succinate, 21–27 (state 4) and 84–98 (state 3); ascorbate–TMPD, 39–44 (state 4) and 59–74 (state 3).

The lack of stimulation of state 4 respiration with α-ketoglutarate as the substrate suggested that the stimulation observed with succinate or ascorbate may involve some parameter not common to all these substrates. It seemed possible that pyrazole and its derivatives stimulate either the primary dehydrogenases, e.g. succinate dehydrogenase, or that they promote the uptake or accessibility of the substrates, e.g. succinate or ascorbate-TMPD. We, therefore, studied the effect of pyrazole and its derivatives on succinate dehydrogenase and cytochrome oxidase activity. Both of these activities were stimulated by pyrazole and its derivatives, with bromopyrazole having a striking stimulatory effect (Fig. 1). The differences between the extent of stimulation of the

isolated enzymes and of oxygen consumption probably reflect the different assay conditions and the fact that additional factors may play a role in the overall rate of oxygen consumption. These results suggest that the stimulation of state 4 respiration with succinate or ascorbate as the substrate may be caused by stimulation of the primary dehydrogenases (oxidases) or of substrate uptake.

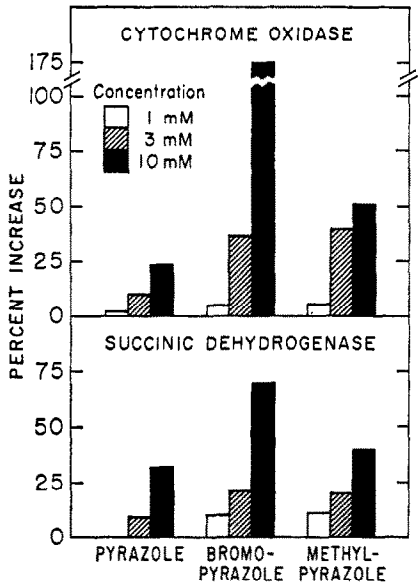

FIG. 1. Effect of pyrazole, bromopyrazole and methylpyrazole on succinic dehydrogenase and cytochrome oxidase activities. The reactions were assayed as described under Materials and Methods. Control values were: succinic dehydrogenase, 31·4 nmoles phenazine methosulfate reduced/min/mg of protein; cytochrome oxidase, 72·3 nmoles cytochrome c oxidized/min/mg of protein.

Effect on energy production and utilization. The respiratory control ratio (state 3/state 4 respiration) is a sensitive index of mitochondrial coupling. Pyrazole and its derivatives inhibited respiratory control with all substrates tested. Bromopyrazole, the most effective inhibitor, decreased the respiratory control ratio as much as 60 per cent. Even the slight respiratory control associated with ascorbate oxidation was nearly completely abolished. With α-ketoglutarate, the inhibition is due to a decrease in state 3 respiration, with little effect on state 4 respiration. Using ascorbate, a decrease in state 3 respiration is accompanied by an increase in state 4 respiration, whereas with succinate, the inhibition is primarily caused by an increase in state 4 respiration.

Pyrazole and its derivatives inhibited mitochondrial energy production and utilization, as evidenced by a decrease in the P/O ratio and the ATP-^{32}P exchange reaction, a partial reaction of oxidative phosphorylation (Table 2). The uptake of Ca^{2+} by mitochondria, whether energized by substrate oxidation or by ATP itself, was also inhibited (Table 2). The greater potency of bromopyrazole compared to pyrazole as an inhibitor of energy-linked reactions correlates with its greater toxicity *in vivo*.[29] When succinate and ATP were omitted, and antimycin plus oligomycin were added to inhibit endogenous energy production, the uptake of Ca^{2+} was reduced by 90 per cent. This non-energy-dependent Ca^{2+} uptake was slightly inhibited by pyrazole.

Similar results were obtained when the Ca^{2+} concentration was lowered to 0·1 mM (so-called "limited loading" conditions).[30]

TABLE 2. EFFECT OF PYRAZOLE, BROMOPYRAZOLE AND METHYLPYRAZOLE ON THE P/O RATIO OF OXIDATIVE PHOSPHORYLATION, THE ATP-^{32}P EXCHANGE REACTION AND ENERGIZED Ca^{2+} UPTAKE*

Compound	Concn (mM)	% Change P/O	% Change ATP-^{32}P exchange	% Change Ca^{2+} Uptake	
				I	II
Pyrazole	1	−8	−12	−17	−5
Pyrazole	3	−22	−15	−37	−20
Pyrazole	10	−40	−30	−45	−36
Bromopyrazole	1	−18	−8	−40	−33
Bromopyrazole	3	−31	−35	−60	−68
Bromopyrazole	10	−58	−70	−89	−90
Methylpyrazole	1	−14	−16	−22	−35
Methylpyrazole	3	−23	−17	−41	−59
Methylpyrazole	10	−30	−35	−70	−69

* The various reactions were assayed as described under Materials and Methods. In the Ca^{2+} uptake experiment, 10 mM succinate plus 5 mM ATP was used as the energy source in experiment I, while the energy source in experiment II was 5 mM ATP. Control values in the absence of pyrazole and its derivatives were: P/O, 1·53 (succinate used as substrate); ATP-^{32}P exchange, 47 nmoles ^{32}ATP formed/min/mg of protein; Ca^{2+} uptake, 251 (I) and 137 (II) nmoles Ca^{2+} bound/min/mg of protein.

Reversibility of pyrazole inhibition. To determine whether inhibition of energy production and utilization results from an interaction with some component(s) of the coupling process, or whether it reflects irreversible damage to mitochondrial structure, ATP-supported Ca^{2+} uptake was studied in the presence of pyrazole and after washing. When mitochondria that had been incubated with 3 and 10 mM pyrazole or 3 and 10 mM bromopyrazole were washed free of these components, full recovery of energy-dependent Ca^{2+} uptake was observed. The addition of pyrazole or bromopyrazole to the washed mitochondria again resulted in strong inhibition of Ca^{2+} uptake. Reversibility of inhibition was about 70 per cent with methylpyrazole. Further evidence that pyrazole and its derivatives do not cause significant mitochondrial disorganization is the finding that these compounds had no effect on latent ATPase activity and Mg^{2+}- or DNP-stimulated ATPase activity. Bromopyrazole caused a slight stimulation of latent ATPase activity (20–40 per cent), but this was much lower than that observed with DNP (400 per cent).

Isolated mitochondria are impermeable to NADH when prepared intact;[18] penetration of NADH is therefore a sensitive index of mitochondrial damage. Pyrazole (1–10 mM) and its derivatives had little effect on the low rate of NADH oxidation observed polarographically or spectrophotometrically (2·3 nmoles/minute/mg of protein).

Anion uptake. Mitochondria accumulate phosphate and dicarboxylic and tricarboxylic anions from the medium by means of specific carriers, which are inhibited by various compounds.[25,26] Citrate uptake was inhibited maximally by pyrazole and its derivatives at a concentration of 1 mM (Table 3). Glutamate uptake was decreased

at higher concentrations of these compounds. By contrast, the uptake of malate and phosphate was stimulated at all concentrations, except for 10 mM methylpyrazole.

TABLE 3. EFFECT OF PYRAZOLE, BROMOPYRAZOLE AND METHYLPYRAZOLE ON THE UPTAKE OF ANIONS BY MITOCHONDRIA*

Compound	Concn (mM)	% Change			
		Citrate	Glutamate	Malate	Phosphate
Pyrazole	1	−26	+27	+62	+30
Pyrazole	3	−29	−17	+31	+86
Pyrazole	10	−32	−21	+27	+62
Bromopyrazole	1	−28	+5	+12	+37
Bromopyrazole	3	−21	−42	+14	+41
Bromopyrazole	10	−28	−49	+16	+22
Methylpyrazole	1	−30	−3	+24	+48
Methylpyrazole	3	−31	−19	+24	+54
Methylpyrazole	10	−30	−45	−6	+68

* The uptake of citrate, glutamate, malate and phosphate was assayed as described under Materials and Methods. Control values for anion uptake (nmoles/min/mg of protein) were: citrate, 6·33; glutamate, 4·66; malate, 6·85; phosphate, 5·75.

Since mitochondria are freely permeable to NH_3, if the anions of ammonium salts transverse the mitochondrial membrane, water enters to maintain isosmotic conditions, and the mitochondria swell.[25,26] Therefore, mitochondrial swelling in isotonic solutions of ammonium salts is an index of mitochondrial permeability to anions. Pyrazole and its derivatives inhibit mitochondrial swelling in isotonic solutions of ammonium citrate (Fig. 2A), whereas these compounds stimulate swelling of mitochondria in the presence of ammonium phosphate (Fig. 2B). This supports the finding that pyrazole inhibits citrate uptake, but stimulates phosphate uptake.

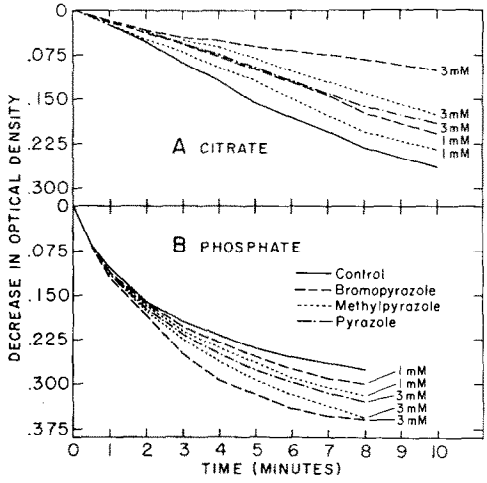

FIG. 2. Effect of pyrazole, methylpyrazole and bromopyrazole on mitochondrial swelling in isotonic solutions of ammonium citrate (A) and ammonium phosphate (B). Swelling was assayed as described under Materials and Methods, using 200 μg mitochondrial protein/ml. The addition of malate (10 mM) and phosphate (10 mM) was required to induce citrate swelling. A decrease in optical density reflects mitochondrial swelling.

Transport of reducing equivalents (shuttles). The inhibition of NAD^+-dependent state 3 respiration by pyrazole and its derivatives suggested that these compounds may also inhibit shuttles which transport reducing equivalents into mitochondria. These shuttles are NAD^+-linked and operate maximally in the presence of ADP.[31] Methylpyrazole and bromopyrazole were found to be potent inhibitors of these shuttles (Table 4). Methylpyrazole and bromopyrazole had no effect on the activities of glutamic-oxalacetic transaminase, α-glycerophosphate dehydrogenase or malic dehydrogenase. If α-ketoglutarate and aspartate are substituted for malate and glutamate as the extramitochondrial components of the reconstituted malate-aspartate shuttle, there is considerable oxidation of ethanol even in the absence of mitochondria, owing to the oxidation of NADH by oxalacetate in the presence of malic dehydrogenase.[31] Methylpyrazole and bromopyrazole had no effect on this extramitochondrial system.

TABLE 4. EFFECT OF METHYLPYRAZOLE AND BROMOPYRAZOLE ON THE ACTIVITIES OF THE MALATE-ASPARTATE, FATTY ACID AND α-GLYCEROPHOSPHATE SHUTTLES FOR THE TRANSPORT OF REDUCING EQUIVALENTS INTO MITOCHONDRIA*

Shuttle	Compound	Concn (mM)	Specific activity (nmoles ethanol/min/mg)	% Change
Malate-aspartate	Control		9·45	
	Methylpyrazole	3	7·58	−20
	Methylpyrazole	10	5·04	−46
	Control		10·79	
	Bromopyrazole	3	5·16	−52
	Bromopyrazole	10	2·81	−74
α-Glycerophosphate	Control		12·19	
	Methylpyrazole	3	8·66	−29
	Methylpyrazole	10	6·33	−48
	Bromopyrazole	3	10·31	−16
	Bromopyrazole	10	4·91	−59
Fatty acid	Control		7·50	
	Methylpyrazole	10	4·45	−41
	Bromopyrazole	10	4·00	−47

*The shuttles were reconstituted as described under Materials and Methods. The endogenous rate (the rate of ethanol disappearance in the absence of the shuttle components) was 1·95 nmoles/min/mg of protein.

In assays of the shuttles, we use ethanol, NAD^+ and yeast alcohol dehydrogenase to generate NADH. Since pyrazole strongly inhibits purified yeast alcohol dehydrogenase (86 per cent at 0·3 mM), it was not possible to test the effect of pyrazole itself on shuttle activity. In agreement with others,[3,32] our data suggest that yeast alcohol dehydrogenase cannot be used to determine blood ethanol concentrations when pyrazole is also present, unless the effective inhibitory concentration of pyrazole is sufficiently diluted during the assay procedure. However, 3 and 10 mM methylpyrazole and bromopyrazole had little effect on yeast alcohol dehydrogenase activity (1–12 per cent inhibition), confirming the data of others.[33] Since a several-fold variation in alcohol dehydrogenase concentration has no effect on shuttle activity,[31] these compounds inhibited shuttle activity directly.

DISCUSSION

Pyrazole, 4-bromopyrazole and 4-methylpyrazole influence several mitochondrial functions *in vitro*. Energy production is decreased, as measured by the respiratory control ratio, P/O ratio and ATP-^{32}P exchange. These compounds also inhibit energy utilization by mitochondria, as evidenced by decreased energized Ca^{2+} uptake. Mitochondrial uptake of Ca^{2+} may play an important role in the excitation–relaxation cycle of skeletal muscles and myocardium,[34,35] in biological calcification processes,[36] and in the maintenance of the ionic milieu of the cell.[30] Thus, caution is mandated when the use of pyrazole or pyrazole derivatives in man is suggested. It has been reported that 0·07–8·82 mmoles pyrazole/kg of body weight inhibited ethanol oxidation 20–90 per cent.[3] In man, 2·5 mmoles methylpyrazole/kg of body weight has been administered.[37,38] Thus, the concentration of pyrazole used *in vivo* (mM) is substantially higher than that required to inhibit purified alcohol dehydrogenase *in vitro* (μM). Twenty-four hr after administration of 2 mmoles pyrazole/kg of body weight to rats (a dose below that which produces maximal inhibition of ethanol oxidation *in vivo*), the concentration of pyrazole in the blood was 2 mM, while that in liver water was 3 mM.* Hence, the concentrations employed in this study *in vitro* are comparable to the concentrations of pyrazole[3,6,8,9,39–41] or 4-methylpyrazole[37,38] employed *in vivo*.

The effects of pyrazole and its derivatives are reversed by washing the mitochondria, indicating that these compounds do not form a strong complex with the mitochondria and do not irreversibly damage mitochondrial structure. The lack of stimulation of latent ATPase activity and the maintenance of permeability barriers to pyridine nucleotides provide further evidence for this concept. However, there are indications that pyrazole and its derivatives do interact with the mitochondrial membrane. These compounds interact with anion carriers of the inner membrane, either promoting or inhibiting the uptake of various anions, and slightly inhibit energy-independent Ca^{2+} uptake, which involves binding sites on the surface of the mitochondria.[42]

The effects of pyrazole and pyrazole derivatives on oxygen consumption are complex. These compounds stimulate state 4 respiration associated with substrates such as succinate and ascorbate. This stimulation was verified by assaying the activities of succinic dehydrogenase and cytochrome oxidase (Fig. 1). The rate of respiration with succinate as substrate has been shown to be controlled by succinate uptake.[43,44] Since pyrazole and pyrazole derivatives stimulate the dicarboxylate carrier which transports anions such as malate and succinate into the mitochondria (Table 3), stimulation of succinate uptake by these compounds may be responsible for the stimulation of state 4 respiration and succinic dehydrogenase activity. The stimulation of dicarboxylate uptake was supported by assaying mitochondrial swelling in isotonic solutions of ammonium malate. A direct stimulatory effect on succinic dehydrogenase, however, cannot be ruled out. The stimulation of ascorbate oxidation (state 4) and cytochrome oxidase activity appears to be due to a direct stimulatory effect in this part of the respiratory chain, since ascorbate-TMPD reduces cytochrome c, which is located at the external surface of the mitochondrial inner membrane;[45] hence, there is no need for actual uptake of the substrate (ascorbate-TMPD). No such stimulation was noticed with α-ketoglutarate as substrate (state 4), because with NAD^+-linked

* F. Deis, G. Lin and D. Lester, unpublished data.

substrates the respiratory rate is limited by the activity of the primary dehydrogenases (α-ketoglutarate or glutamate dehydrogenase) and not the cytochrome oxidase region of the respiratory chain.

Although pyrazole and its derivatives inhibit several energy-linked reactions, these compounds do not act exactly like the true uncouplers of oxidative phosphorylation. State 4 respiration, with α-ketoglutarate as the substrate, and ATPase activity are not stimulated by these compounds, whereas uncouplers cause a large stimulation. These compounds do have some properties in common with the classic inhibitors of oxidative phosphorylation, oligomycin and aurovertin, e.g. inhibition of respiratory control, P/O ratio, ATP-^{32}P exchange and ATP-supported Ca^{2+} uptake.[46,47]

For proper operation of the malate-aspartate shuttle, glutamate and malate must enter the mitochondria, the former having been suggested as the rate-limiting step of the shuttle.[48] Thus, the inhibition of glutamate transport (Table 3) correlates with the inhibition of the malate-aspartate shuttle (Table 4). Since citrate stimulates the fatty acid shuttle,[31] the inhibition of citrate transport by pyrazole may be responsible for inhibition of this shuttle. Inhibition of NAD^+-dependent state 3 respiration by these compounds may also play a role in the inhibition of the transport of reducing equivalents into the mitochondria.

Rats treated chronically with pyrazole plus sucrose had less hepatic triglyceride than those given sucrose alone, leading to the suggestion that pyrazole itself tends to reduce hepatic triglyceride by an unknown mechanism.[9] The inhibition of citrate transport by pyrazole may be a factor in the reduction of hepatic lipid content. Acetyl CoA, the primary precursor for fatty acid synthesis, must exit from the mitochondria to be available for fatty acid synthesis in the cytoplasm. Since the mitochondria are impermeable to acetyl CoA,[49] it may exit from the mitochondria in the form of citrate. The citrate–lyase reaction then regenerates acetyl CoA.[50] Inhibition of citrate transport by pyrazole may thus decrease fatty acid synthesis, thereby lowering hepatic triglyceride content.

Pyrazole and its derivatives inhibit the microsomal ethanol oxidizing system,[8] catalase activity (*in vivo*),[8] various mitochondrial functions and the transport of reducing equivalents into the mitochondria. The mitochondria are important for ethanol metabolism, not only because they oxidize reducing equivalents generated by the metabolism of ethanol, but also because they are responsible for the major part of acetaldehyde oxidation.[51] The inhibition of mitochondrial energy production and utilization by pyrazole may also influence the phosphorylation state of the adenine nucleotide system, which controls the cytoplasmic NAD couple, i.e. the redox state directly affected by ethanol administration.[52,53] Thus, studies in which pyrazole is used to assess ethanol metabolism and its consequences *in vivo* should be interpreted cautiously.

Acknowledgement—We thank Dr. D. Lester, Rutgers, Center of Alcohol Studies, New Brunswick, New Jersey, for his generous gift of 4-methylpyrazole and 4-bromopyrazole.

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