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MULTIPLE ACTIONS OF PHENYLETHYLBIGUANIDE ON RESPIRATION BY RAT LIVER MITOCHONDRIA

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Abstract—The effect of phenformin over a wide range of concentrations on oxidation of several substrates by rat liver mitochondria was studied polarographically. Biphasic responses with both stimulatory and inhibitory phases were observed: their order of appearance depended on the nature of the substrate. Dose–response data for these phenomena are reported. At low phenformin concentrations ($< 10^{-4}$ M) only stimulation occurred, while at high concentrations ($> 10^{-2}$ M) only inhibition was observed. Insensitivity to dinitrophenol and ADP suggested that the stimulation was due to progressive uncoupling. Very high concentrations of phenformin did not inhibit the oxidation of ascorbate/TMPD. Possible sites of action of phenformin are discussed. Although it is not possible to define these with certainty it appears that several sites must be involved.

Although phenformin (phenethylbiguanide, PEBG) and similar biguanides have been used successfully as oral hypoglycaemic agents in certain types of diabetes mellitus for a number of years, their mechanism and even their site(s) of action are unknown. Effects claimed for these drugs are diverse and include inhibition of intestinal glucose absorption [1–5], inhibition of gluconeogenesis [6–9] and of a variety of other biosynthetic processes [9, 10], and stimulation of glycolysis [9, 11, 12]. It has also been suggested that phenformin may promote the release of an intestinal hormone such as glucagon-like-immunoreactivity which may have peripheral effects on carbohydrate metabolism and uptake [13]. Each of these effects, except for the hormonal one, could be expected if cellular ATP levels were depleted.

Morphological damage to mitochondria [14] and inhibition of oxidative phosphorylation [15–18] by phenformin have been well demonstrated. However the actual site(s) and mechanism(s) of the effect on oxidative phosphorylation remain controversial. Sites of inhibition of electron transport between NAD and cytochrome b [17], at cytochrome oxidase [16], and site specific uncoupling [18] have all been proposed. Non-specific interactions with membranes and subsequent changes in electrostatic surface potential have also been suggested [19]. The use of different substrates, phenformin concentrations and methodologies has made it impossible to interpret these findings from different laboratories coherently. Therefore we have investigated the effects of phenformin over a wide range of concentrations on the oxidation of several Krebs Cycle substrates by isolated mitochondria by monitoring polarographically the oxygen uptake over several min. Since the effects were clearly complex we also tested the effects of ADP and 2,4-dinitrophenol on oxygen uptake following treatment with phenformin.

While this type of investigation may shed light on the pharmacological action of the biguanides it may also yield evidence on the mechanism of oxidative phosphorylation. For example it has been claimed that phenformin is a site-specific uncoupling agent [18]. As Schäfer pointed out [18], if this were to be substantiated then it might cast doubt on the validity of Mitchell's chemiosmotic hypothesis [20].

MATERIALS AND METHODS

Mitochondria were isolated according to Hogeboom and Schneider's procedure [21] from the livers of female albino rats weighing 180–200 g. The isolation medium contained sucrose (250 mM), MOPS* (10 mM) and EGTA* (1 mM) at pH 7.2 [21].

Mitochondrial protein was estimated by a biuret method incorporating sodium deoxycholate (0.2 mg/ml) to solubilize particulate matter [22].

Oxygen uptake was monitored in a stirred reaction chamber which was water-jacketed at 35° and equipped with a Clark recording oxygen electrode (Rank Bros, Bottisham, Cambridge). The standard reaction medium contained sucrose (625 μmoles), MOPS (25 μmoles), EGTA (2.5 μmoles), sodium phosphate buffer, pH 7.2 (50 μmoles), plus 0.1 ml mitochondrial suspension (containing on average 2.5 mg protein). Appropriate substrates from the following list were added as aqueous solutions of the sodium salts pre-adjusted to pH 7.0: succinate, citrate, pyruvate or α-oxoglutarate (50 μmoles), malate plus glutamate (25 μmoles of each), α-glycerophosphate (20 μmoles) or ascorbate (3 μmoles) plus TMPD* (0.2 μmoles). Other additions as required were: malonate (50 μmoles), rotenone (1 μg) in ethanol, DNP* (140 nmoles) in ethanol or ADP (0.5 μmoles). The final vol. was normally 2.9 ml.

The reduction of DCPIP* by mitochondria which had been lysed in 0.02 M sodium phosphate buffer,

* MOPS—3-(*N*-morpholine)-propane sulphonic acid; EGTA—1,2-di(2-aminoethoxy)-ethane-*N*,*N*,*N*′,*N*′-tetracetic acid; TMPD—*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylene diamine; DNP—2,4-dinitrophenol; DCPIP—2,6-dichlorophenol indophenol.

pH 7.3, was measured using a recording spectrophotometric technique [23].

Phenformin hydrochloride (PEBG) was donated by Winthrop Laboratories, Surbiton-on-Thames, Surrey, and in all cases was added from a 0.55 M aqueous solution.

RESULTS AND DISCUSSION

Viability and stability of mitochondrial preparations. Any preparations which gave respiratory control index values of less than 4.0 were discarded. The viability with respect to coupling of the preparations was monitored by the determination of P:O ratios before, during and at the end of each experimental session. Mean values (and number of experiments) were as follows: Succinate 1.83 (9), Malate plus glutamate (together) 3.07 (22), α-ketoglutarate 2.83 (3), pyruvate 2.96 (4), citrate 3.20 (3). Since these values are close to the theoretical values of 3.0 for the NAD-linked substrates and 2.0 for the FAD-linked substrates, it appears that the respiring mitochondria were coupled and that they remained so during each experimental session.

In the absence of added phenformin but in the presence of substrate the rate of oxygen consumption remained fairly constant for at least 5 min.

The polarographic results shown in figures in this paper have been constructed by tracing typical traces. In all cases however the features reported were observed on at least two separate batches of mitochondria. In some instances (Figs. 7–10) mean rates of oxygen uptake in nmoles O_2 per mg protein per min are shown in brackets adjacent to the traces.

Effect of phenformin on oxidation of glutamate plus malate. Figure 1 shows the effects of phenformin at several concentrations in the range 4–40 mM on respiration when malate plus glutamate (together) were the substrates. As seen from Fig. 1 a biphasic response was obtained with an initial rapid stimulation of respiration, especially at the lower phenformin concentrations, followed by inhibition of respiration. At 40 mM phenformin no stimulatory phase was seen, but inhibition was noted immediately on addition of the phenformin.

Effect of phenformin on oxidation of succinate. Figure 2 shows the effects of phenformin at several concentrations from 5 to 20 mM on respiration when succinate was the substrate. At the lower phenformin

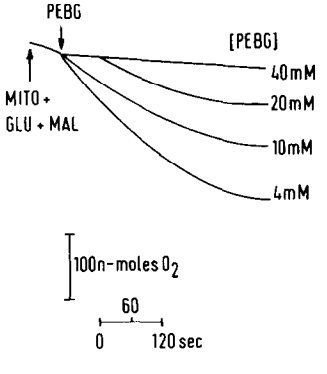

Fig. 1. Oxygen consumption in the presence of glutamate and malate. At the second arrow phenformin was added.

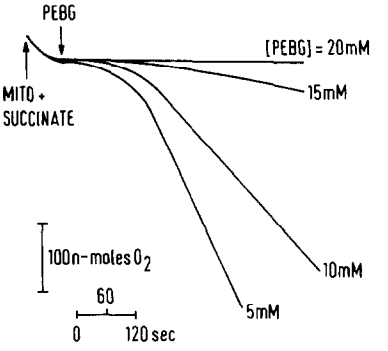

Fig. 2. Oxygen consumption in the presence of succinate. At the second arrow phenformin was added.

concentrations a biphasic response was again seen; however, the immediate effect was inhibition of respiration and this was followed by a stimulatory phase at phenformin concentrations up to and including 15 mM– i.e. the order of these effects was the reverse of that observed with glutamate plus malate as substrate. At 20 mM the phenformin caused inhibition only, and this was immediate. Figures 3 and 4 show the dose–response relationships for the stimulation and inhibition phases respectively for both substrates. There is a sharp peak showing maximum stimulation of both glutamate-plus-malate and succinate oxidation by phenformin at about 4–5 mM phenformin (Fig. 3).

A limited number of experiments were made with other substrates. The oxidation of α-ketoglutarate and pyruvate (both NAD-linked substrates) was affected in a biphasic fashion closely similar quantitatively to that found with glutamate-plus-malate. However no stimulatory phase was seen when citrate, which is oxidised by an NAD-limked dehydrogenase, was the test substrate. The oxidation of α-glycerophosphate (an FAD-linked substrate) however gave a similar pattern to that for glutamate-plus-malate but with only mild stimulation by 5 mM phenformin.

Thus Figs. 3 and 4 show that (i) at low concentrations of phenformin ($< 10^{-4}$ M) only stimulation

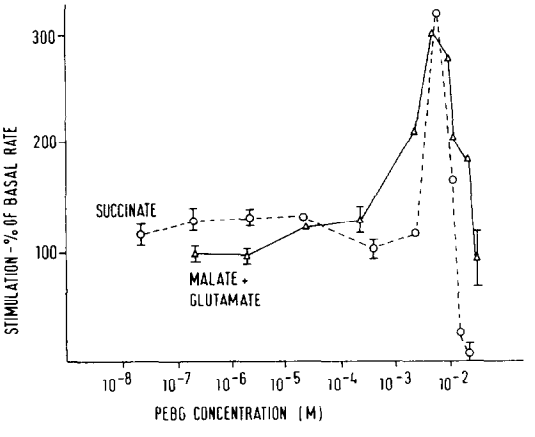

Fig. 3. Maximum stimulation of oxygen consumption produced by various concentrations of phenformin. succinate as substrate; malate plus glutamate as substrates. Each point is the mean of 3–10 observations, and the S.E. of mean is shown in some instances.

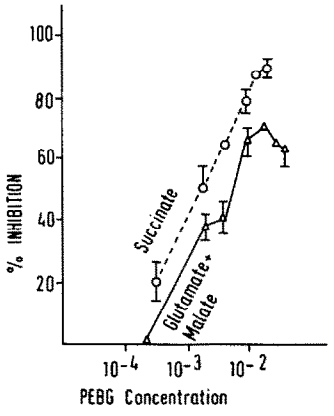

Fig. 4. Maximum inhibition of oxygen consumption produced by various concentrations of phenformin. – – – succinate, ——— glutamate plus malate as substrates.

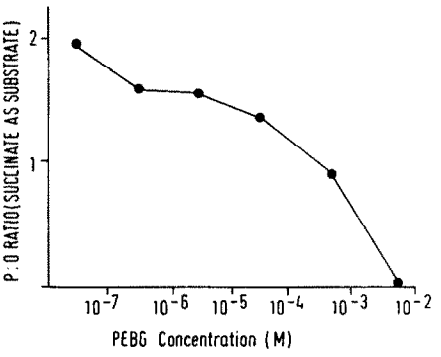

Fig. 6. P:O ratios for succinate oxidation at various phenformin concentrations.

of respiration was observed, (ii) at intermediate concentrations (10^{-4}–10^{-2} M) biphasic responses were observed, and (iii) at high concentrations ($>10^{-2}$ M) inhibition alone was seen. We therefore sought to determine whether the stimulatory effect was due to uncoupling and also at what site(s) the inhibition was located.

Effects of addition of ADP or dinitrophenol after phenformin. In the absence of phenformin, addition of the uncoupling agent DNP caused an immediate and dramatic increase in the rate of oxygen uptake with all substrates. Similarly, addition of ADP resulted in a temporary increase in oxygen consumption (from which the P:O ratio could be calculated).

For all the NAD-linked substrates examined it was found that PEBG at concentrations greater than 2 mM resulted in total insensitivity to added ADP or DNP at all times after the addition of the PEBG. This suggests that the mitochondria have already been uncoupled fully by the phenformin. The absence of stimulation by ADP or DNP is unlikely to be due to inhibition by phenformin since the maximum inhibition caused by 2 mM phenformin was only about 50 per cent (Fig. 4) and oxygen was being consumed when the ADP or DNP was added. At lower phenformin concentrations addition of either ADP or DNP stimulated respiration. This corresponds to previous reports that DNP 'overcomes' the inhibition by phenformin [24]; it appears however that different sites of action are involved (see below). At a phenformin concentration of 0.2 mM the eventual onset of inhibition was slow and progressive. Addition of DNP at varying times after 0.2 mM phenformin showed that the sensitivity to DNP decreased with time; a similar and closely parallel decrease in the respiratory control value with time was observed (Fig. 5).

When succinate was the substrate ADP insensitivity was observed at phenformin concentrations of 1 mM and above. As Fig. 6 shows, at lower phenformin concentrations the apparent P:O ratios decreased progressively as the phenformin concentration was increased. Previous studies had suggested that the P:O ratio was only affected at very high concentrations [9]. It thus appears that the average degree of uncoupling does increase with the phenformin concentration, and that it is complete at phenformin concentrations above about 1 mM. These effects were observed regardless of whether the ADP was added during the initial phase of inhibition or during the subsequent phase of stimulated respiration. On the other hand when DNP was added shortly after the phenformin (5 mM), i.e. during the early phase of inhibition, it did increase the rate of oxygen uptake (Fig. 7). This implies that the mitochondria had not been uncoupled, at least completely. When the DNP

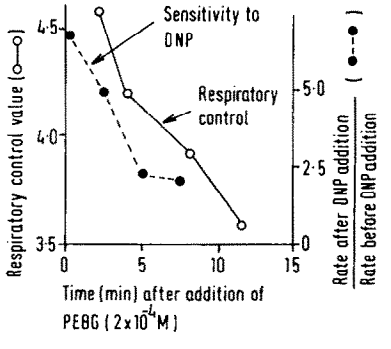

Fig. 5. Respiratory control values and sensitivity to DNP (i.e. ratio of rate of respiration after DNP addition to rate before addition) as a function of time after addition of 2×10^{-4} M phenformin. Succinate as substrate.

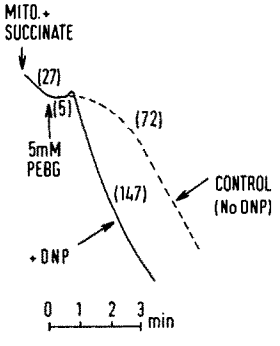

Fig. 7. Effect on oxygen consumption of addition of DNP shortly after phenformin (5 mM)—i.e. during inhibitory phase. Figures in brackets represent mean oxygen uptake in nmoles O_2 per mg mitochondrial protein per min. Succinate as substrate.

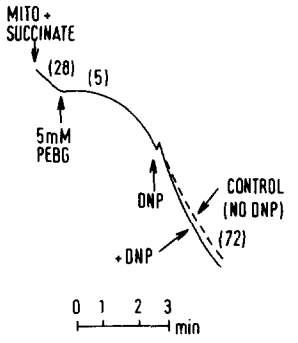

Fig. 8. Effect on oxygen consumption of addition of DNP approx. 3 min after phenformin (5 mM)—i.e. during stimulatory phase. Rates of O_2 uptake (nmoles O_2. mg·protein^{-1}·min^{-1}) shown in brackets. Succinate as substrate.

was added some 2–3 min after the addition of phenformin (5 mM), i.e. during the stimulated phase, no significant effect was observed (Fig. 8). This is consistent with complete uncoupling by the phenformin.

Phenformin inhibition of respiration by uncoupled mitochondria. When phenformin was added after the DNP the respiration of the DNP-uncoupled mitochondria was gradually inhibited and the extent of inhibition increased as the phenformin concentration was increased—see Fig. 9. Thus, marked inhibition was observed, but it was not followed by the phase of stimulation which was seen in the absence of DNP (Fig. 2), presumably because the DNP had already fully uncoupled the mitochondria.

Effect of phenformin on ascorbate oxidation. Ascorbate in the presence of TMPD donates electrons at the cytochrome *c* level in the respiratory chain [25]. No effect on oxygen uptake was observed in the presence of phenformin at concentrations of 20 mM and below. However, phenformin at 40 mM caused slight inhibition, but much less than had been observed with other substrates. Figure 10 shows oxidation of ascorbate proceeding in the presence of 40 mM phenformin which had drastically inhibited oxidation of malate-plus-glutamate and of succinate and had made the mitochondria completely non-responsive to ADP—i.e. they were presumably fully uncoupled. However, oxidation of the ascorbate was abolished by potassium cyanide. Therefore, phenformin inhibition appears to occur at a site other than, and proximal to, that at which KCN inhibits.

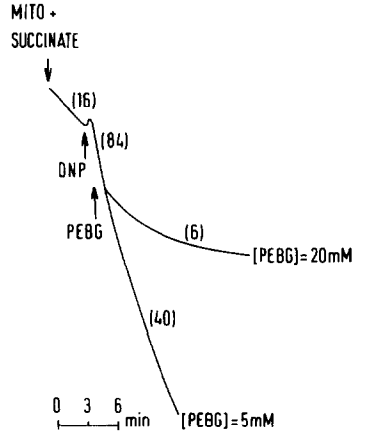

Fig. 9. Effect on oxygen consumption of addition of phenformin (5 or 20 mM) after uncoupling by DNP. Rates of O_2 uptake shown in brackets.

Location of the site(s) of phenformin inhibition. The fact that the dose–response curves for phenformin inhibition of both succinate and glutamate-plus-malate oxidation were similar (Fig. 4) suggests that the site, or the main site, of inhibition is in that part of the main respiratory chain which is common to both NAD- and FAD-linked substrates, namely between ubiquinone and cytochrome oxidase. Since ascorbate oxidation was not affected by the phenformin at concentrations up to 20 mM (Fig. 10 see above), it appears that there is no inhibition between cytochrome *c* and cytochrome oxidase. Therefore the main site of inhibition is likely to be between ubiquinone and cytochrome *c*. This contrasts with the conclusion of Steiner and Williams that cytochrome oxidase was a major site of inhibition [16]. These authors, however, did express the reservation that "It cannot be concluded definitely ... that the observed effects on tissue respiration result from inhibition at this particular enzyme site". On the other hand inhibition of succinate oxidation was marginally greater than that of glutamate-plus-malate oxidation (Fig. 4), and this might indicate one relatively minor site of action to be between succinate and ubiquinone. Steiner and Williams tentatively concluded that phenformin could inhibit succinate dehydrogenase. We examined this further by monitoring succinate oxidation by lysed mitochondria spectrophotometrically with an artificial electron acceptor, DCPIP. This compound has $E'_0 = +0.217$ V [26] and would therefore be expected to accept electrons from the cytochrome *b* and *c* region of the respiratory chain. Inhibition by phenformin (5–20 mM) was observed, but the extent of inhibition was scarcely dependent on the phenformin concentration in this range. Furthermore, the extent of inhibition was significantly less than that determined in the oxygen electrode experiments (Table 1): in the latter experiments the extent of inhibition was dependent on the phenformin concentration. These differences between the two sets of experiments would be consistent with two sites of inhibition by phenformin, with one site proximal and one distal to the point of electron acceptance by DCPIP. In general this is consistent with Steiner and Williams observations on succinate dehydrogenase. In

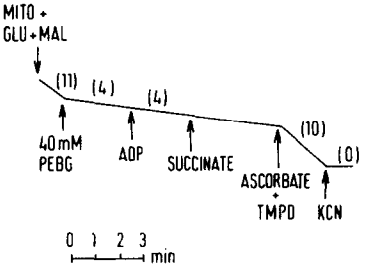

Fig. 10. Ascorbate/TMPD oxidation in the presence of 40 mM phenformin. The mitochondria were already unresponsive to added ADP. Figures in brackets represent oxygen uptake.

Table 1. Maximal percentage inhibition of succinate oxidation by phenformin as determined by (a) the oxygen electrode (polarographically) and (b) DCPIP as electron acceptor (spectrophotometrically)

Phenformin concentration (mM)	% Inhibition (a) Polarographic method	% Inhibition (b) Spectrophotometric method	Significance of difference
5	64 ± 4.8 (6)	54 ± 5.0 (3)	N.S.
10	79 ± 2.6 (5)	52 ± 2.8 (3)	$P < 0.001$
20	89 ± 2.5 (6)	59 ± 3.6 (3)	$P < 0.001$

Values are means ± S.E.M. and the number of observations is given in parentheses.

contrast to the polarographic experiments (Fig. 2) no stimulation was observed. However, caution must be exercised in any comparisons of data from experiments on lysed and intact mitochondria. The evidence is insufficient to identify the site(s) of action of the phenformin with confidence. Inhibition is observed at at least one and possibly two sites, and uncoupling is also observed. Uncoupling is seen especially at the lower phenformin concentrations; at relatively high concentrations uncoupling appears to be complete although stimulation of respiration is not seen probably because it is masked by the predominating inhibitory effect.

Location of the site(s) of uncoupling by phenformin. As seen in Fig. 3 the maximal extent of stimulation of respiration by phenformin is closely similar for both the NAD- and the FAD-linked substrates. This might suggest that the main stimulatory effect of phenformin is located after ubiquinone in the region of the electron transport chain which is common to both classes of substrate—i.e. that there was little or no uncoupling at 'Site I'. However, it must be noted that the data for maximum stimulation shown in Fig. 3 are presumably the *net* result of two opposing effects of stimulation and inhibition, and therefore do not necessarily reflect absolute values of stimulation. (A similar reservation must be placed on the interpretation of the inhibition results discussed above.)

The absence of phenformin stimulation of electron transfer to DCPIP while succinate was being oxidised might imply that the phenformin was not uncoupling at 'Site II'; however as these experiments were made on lysed mitochondria, phosphorylation had probably already been uncoupled and so this interpretation is not valid.

Therefore while these results are fully consistent with site-specific uncoupling they cannot be accepted as firm evidence for this hypothesis.

Although it is not possible to explain fully these findings and to define the sites of action of phenformin, the results reported here suggest that this drug has multiple actions on mitochondria which are more complex than those previously reported. Furthermore, the actions of phenformin are to some extent substrate specific as evidenced particularly by the different types of biphasic responses obtained with NAD- and FAD-linked substrates.

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