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SPECTROPHOTOMETRIC AND MANOMETRIC STUDIES OF NADPH AND NADH OXIDASE ACTIVITIES OF RAT LIVER MICROSOMES: EFFECTS OF DRUGS AND ADENINE NUCLEOTIDES*

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Abstract—In view of the extensive interest in microsomal NADH and NADPH oxidases —for example, in the relationship of NADPH oxidase to drug-metabolizing enzymes— optimal conditions for the assay of these enzymes were established. Optimal pH was found to depend upon the buffer employed. Variation of microsomal concentration did not give strictly linear rates; high concentration of microsomes caused a decrease in observed reaction rate. In high concentration, both substrates were inhibitory. The reported ADP-stimulated oxidation of NADPH by rat liver microsomes could be confirmed only when particular reaction conditions were employed. The buffer used is crucial, and manometric and spectrophotometric assays may yield divergent results. Poor correlation was found between the extent of metabolism of drugs by NADPH-linked microsomal enzymes and the ability of the drugs to block the stimulation by ADP of oxygen uptake by microsomes in the presence of NADPH. This uptake has previously been suggested to involve the peroxidation of microsomal lipid.

ALTHOUGH microsomal NADH and NADPH oxidases are the subject of many investigations, and the latter enzyme has been implicated in the oxidative metabolism of drugs[1–7] and in the peroxidation of lipids,[8, 9] optimal conditions for the assay of these enzymes have not been described. In this paper we have attempted to define such conditions for spectrophotometric assays and to describe various factors that may affect the assay. We have contrasted a spectrophotometric and manometric assay of NADPH oxidase and have studied the effects of adenine nucleotides and drugs on the activity of this enzyme.

EXPERIMENTAL

Materials

NADH, NADPH (type I, chemically reduced), UDP-N-acetylglucosamine, G6-P,§ cytochrome c (type III, from horse heart), ATP (disodium salt), AMP, and

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§ The abbreviations used are: DCIP, 2,6-dichlorophenolindophenol; G6-P, glucose 6-phosphate; ADPR, adenosine diphosphate ribose; ADPR, adenosine diphosphate ribose; UDPG, uridine disphosphate glucose; UDPGA, uridine diphosphate glucuronic acid; UDPAG, uridine diphosphate N-acetylglucosamine.

adenosine were purchased from Sigma Chemical Co., St. Louis, Mo. ADP (disodium salt), ADP-ribose, and UDP-glucose were obtained from Pabst Laboratories, Milwaukee, Wis.; nicotinamide and aminopyrine from Merck & Co., Rahway, N.J.; G6-P dehydrogenase from C. F. Boehringer & Soehne, Mannheim, Germany; 4-aminoantipyrine from The Matheson Co., Norwood, Ohio; sodium Amytal from Eli Lilly & Co., Indianapolis, Ind.; UDP-glucuronic acid from Calbiochem, Bethesda, Md.; sodium barbital from Mann Research Laboratories, New York, N.Y.; and DCIP and iodoacetic acid from Eastman Kodak Co., Rochester, N.Y. DCIP was chromatographed on Dowex-50, H^+ form, and iodoacetic acid was recrystallized from chloroform prior to use. Zoxazolamine was a generous gift from Dr. Peter G. Dayton, Goldwater Memorial Hospital, New York, N.Y. Rats were purchased from Harlan Small Animals Industries, Harlan City, Ind.

METHODS

Preparation of microsomes. Male Wistar rats were fasted for 24 hr prior to sacrifice. Livers were perfused with ice-cold 0·25 M sucrose and homogenized in 10 volumes of 0·25 M sucrose. Microsomes were prepared as described by Beloff-Chain *et al.*[10] The microsomal pellet was resuspended in 0·25 M sucrose or 0·15 M KCl with gentle homogenization with a hand-operated Ten Broeck homogenizer and was diluted to a volume such that 1 ml of the suspension contained the microsomes equivalent to 1 g liver for spectrophotometric experiments and 2 g for manometric experiments. After perfusion of the livers, all subsequent operations were performed at 4°.

Spectrophotometric assays. Unless otherwise indicated, all assays were performed in a Cary recording spectrophotometer, model 11, at a temperature of 22 ± 1°. Quartz cuvettes of 1·5-ml capacity and with a light path of 1 cm were used.

NADH oxidase. A 1·0-ml reaction mixture contained 150 μmoles potassium phosphate buffer (pH 7·0), microsomal suspension equivalent to 10 mg liver, and 50 mμmoles NADH. NADH was omitted in the blank cuvette. Absorbancy changes at 340 mμ were followed for 5 min, and the reaction rate was calculated from the linear region of the curve.

NADPH oxidase. The assay conditions were as for NADH oxidase, except that 100 mμmoles NADPH was used as the substrate and the pH was 7·2.

NADH and NADPH dehydrogenases. A 1·0-ml reaction mixture contained 150 mμmoles potassium phosphate buffer (pH 7·4), 5 μmoles potassium cyanide, 0·060 mg DCIP, 50 mμmoles NADH or NADPH, and 10 μliters diluted microsomal suspensions. The microsomal preparation, containing 1 g equivalent of liver/ml, was diluted 1 to 10 for the NADH dehydrogenase assay and 1 to 5 for NADPH dehydrogenase. NADH or NADPH was omitted from the blank cuvette. Absorbancy changes were measured at 340 mμ.

When potassium ferricyanide was used as the electron acceptor, absorbancy changes were measured at 340 mμ or 410 mμ, and a correction was applied for the slow rate of nonenzymic oxidation of NADH or NADPH by ferricyanide. The reaction mixture contained, in a total volume of 1·0 ml, 150 μmoles potassium phosphate buffer (pH 7·4), 3 μmoles potassium ferricyanide, and microsomes equivalent to 2·0 mg liver. The substrate was omitted from the reference cuvette.

Cytochrome c reductase. Absorbancy changes were followed at 550 mμ. The complete system contained, in a total volume of 1·0 ml, 150 μmoles potassium phosphate

(pH 7·4), 5 μmoles potassium cyanide, 50 mμmoles NADH or NADPH, 1·0 mg cytochrome c, and microsomes equivalent to 1·0 mg liver.

Manometric experiments. Oxygen consumption was measured with Warburg manometers. The center well contained 0·2 ml of 2 N KOH. To the main compartment were added microsomal suspension (equivalent to 1·6 to 2·4 g liver), 100 μmoles potassium phosphate buffer (pH 7·8), 200 μmoles KCl, and 120 μmoles nicotinamide. The side arm contained 10 μmoles ADP, 1 μmole NADPH, and other compounds as indicated. In control flasks ADP and/or NADPH was omitted. The flasks were gassed with oxygen for 5 min and equilibrated at 37° until a constant rate of endogenous respiration was observed. After tipping the contents of the side arm, the total volume of the reaction mixture was 3·0 ml.

RESULTS

Spectrophotometric studies

pH Optima. In phosphate buffer, the optimal pH for oxidation of NADH was 7·0 and for NADPH was 7·2 (Fig. 1). This latter finding is near the value of 7·5 reported by Staudinger and Zubrzycki.[11] Different results were found when the reaction was carried out in Tris buffer, for in this case the pH optimum was 10 for both NADH and NADPH oxidation (Fig. 2).

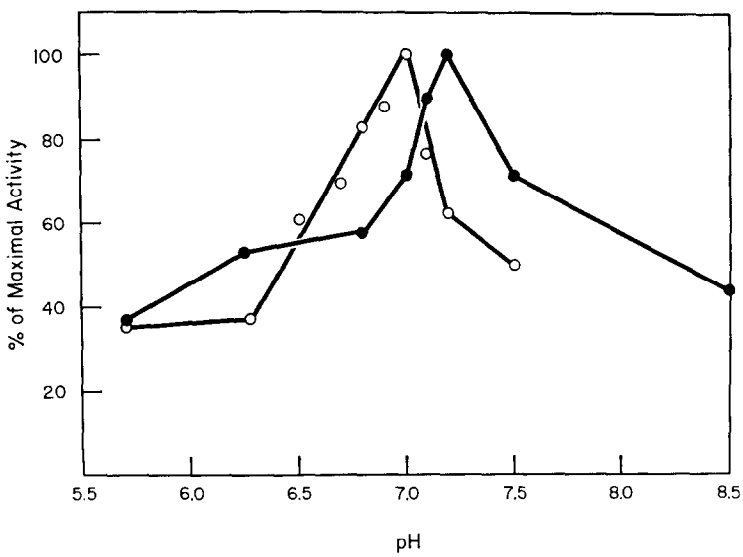

FIG. 1. pH Optima for NADH and NADPH oxidase activities in phosphate buffer. The spectrophotometric assays were performed as described in the Experimental section, except that the pH was varied as indicated. Buffers were prepared by the addition of HCl to K_2HPO_4 to the desired pH. The pH of the reaction mixture was measured; ○——○, NADH oxidase; ●——●, NADPH oxidase.

Effect of microsome concentration. The rates of oxidation of NADH and NADPH were not linear over the range of microsomal concentrations employed in our assay (Fig. 3). The rates approached linearity for NADH oxidation when the concentration of microsomes was 10 to 20 mg liver equivalent/ml and for NADPH between 10 and

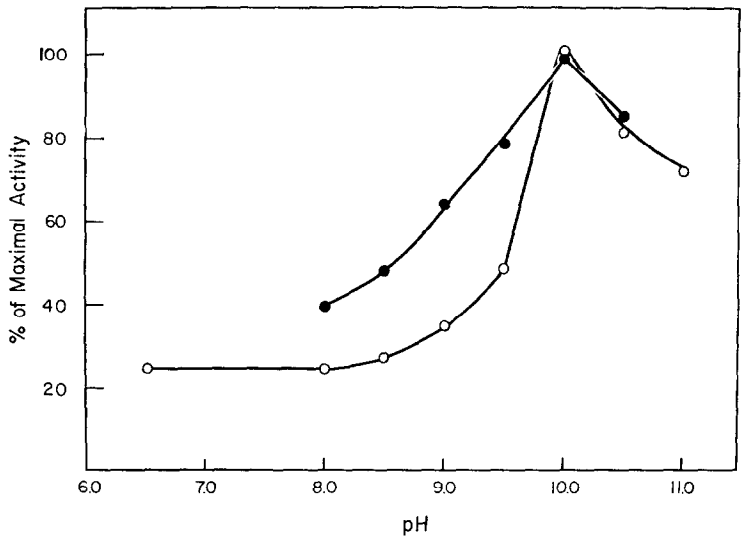

FIG. 2. pH Optima for NADH and NADPH oxidase activities in Tris buffer. The spectrophotometric assays were performed as described, except that 150 μmoles Tris buffer was used instead of phosphate, and the pH was varied as indicated. Buffers were prepared by the addition of HCl or NaOH to Tris to the desired pH. The pH of the reaction mixture was measured; ○——○, NADH oxidase; ●——●, NADPH oxidase.

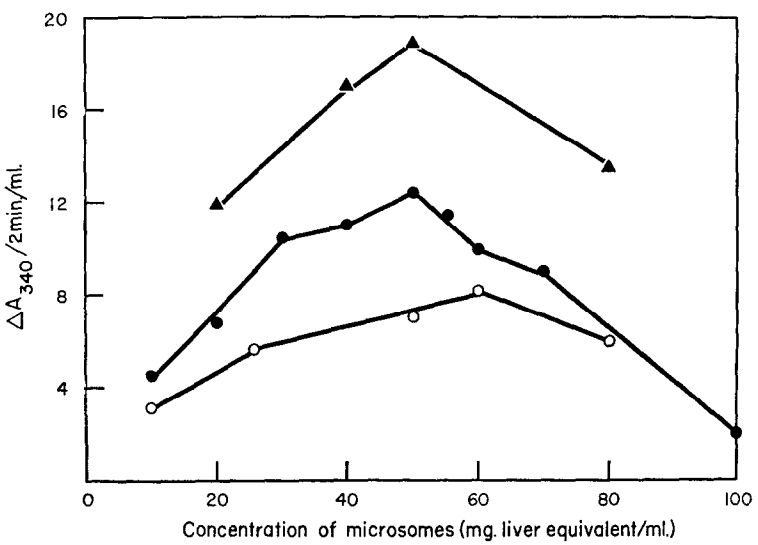

FIG. 3. Effect of concentration of microsomes on the oxidation of NADH, NADPH, and NADH + NADPH. The spectrophotometric assays were performed as described, except that the concentration of microsomes was varied as indicated; ○——○, NADH; ●——●, NADPH; ▲——▲, NADH + NADPH as substrates.

30 mg liver equivalent/ml. When both NADH and NADPH were present at concentrations well above Km values, the observed rates were approximately the sums of the individual rates, which indicates two separate enzymic activities. For example, at a microsomal concentration of 50 mg liver equivalent/ml, the sum of the individual rates

was $A_{340} = 18{\cdot}7/2$ min, and the observed rate in the presence of both NADH and NADPH was $A_{340} = 18{\cdot}8/2$ min.

When the concentration of microsomes was greater than 50 or 60 mg liver equivalent/ml of reaction mixture, the further addition of microsomes caused a decrease in the reaction rate. In these turbid suspensions the extinction of NADH or NADPH was greatly reduced, which would thus diminish the observed rate of oxidation. For example, when a quantity of NADH that gave an A_{340} of 0·315 against a water blank was assayed at the same concentration in the presence of liver microsomes (200 mg liver equivalent/ml reaction medium), the observed A_{340} was only 0·010. That this decrease in A_{340} was not caused by rapid metabolism or by binding of the nucleotide to microsomes was shown by the following observations. When a preparation that had been stored for 5 days and was virtually devoid of NADPH activity was used, a similar decrease was obtained. Moreover, when NADPH was added to a suspension of aged microsomes and the mixture centrifuged at 105,000 *g* for 60 min, 90% of the A_{340} absorption was recovered in the supernatant fraction.

Effect of concentration of NADH and NADPH. The rates of oxidation were enhanced by increasing concentrations of NADPH to approximately 0·05 mM and of NADH to approximately 0·10 mM, above which concentrations both substrates were inhibitory (Fig. 4). The apparent Michaelis constants, calculated by the method of Lineweaver and Burk, were 0·02 mM for NADPH and 0·05 mM for NADH. This K_m value for NADPH is similar to that reported by Staudinger and Zubrzycki.[11]

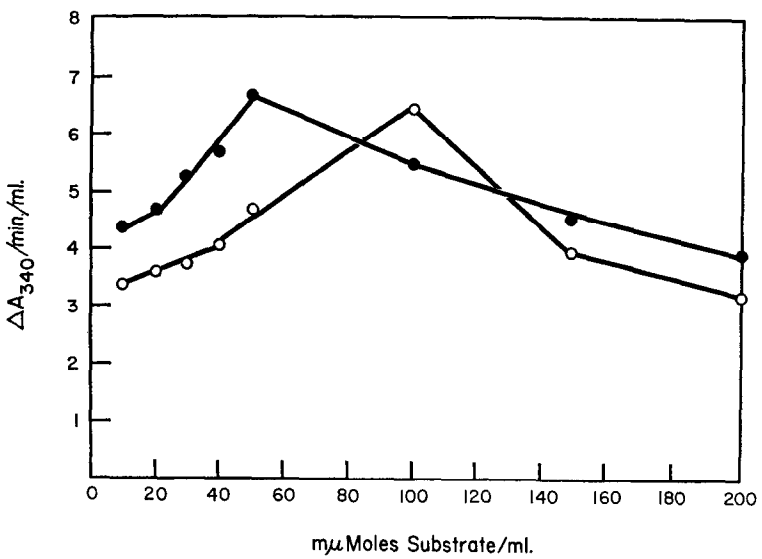

FIG. 4. Effect of substrate concentration on NADH and NADPH oxidase activities. The spectrophotometric assays were performed as described, except that the concentration of NADH or NADPH was varied as indicated; ○——○, NADH; ●——●, NADPH as substrate.

Stability. Incubation at 37° led to rapid inactivation of NADH and NADPH oxidases (Fig. 5). Inactivation was not so rapid at room temperature, and at 4° the activities were stable for about 2·5 hr. Even at 4°, however, 35% to 40% of the activities were lost in 22 hr. Freezing of the microsomes led to considerable losses, regardless of

whether the freezing was carried out slowly in a deep freeze at −20° or rapidly in a solid CO_2–acetone bath in an evacuated Thunberg tube.

Effects of electron acceptor dyes. The rates of oxidation of NADH and NADPH were greatly enhanced by the addition of electron acceptor dyes. The concentration of the added dye was crucial for the measurement of maximal activity, and the observed

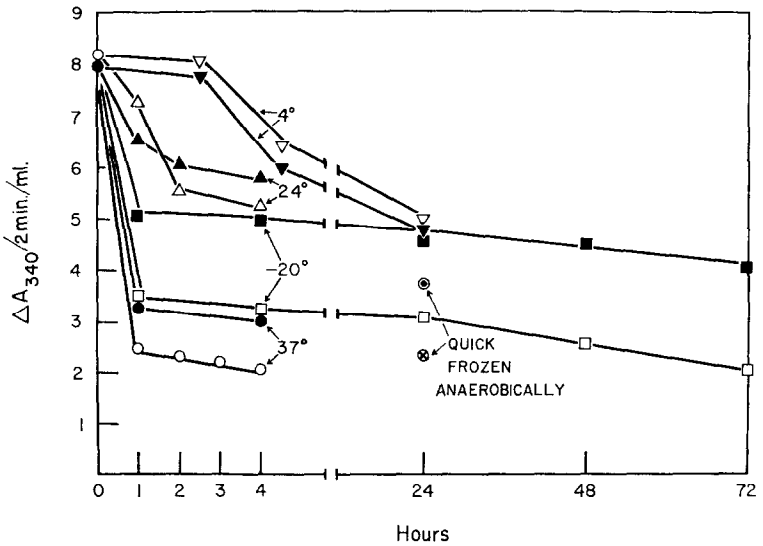

FIG. 5. Inactivation of NADH and NADPH oxidase activities at 37°, 24°, 4°, and −20°. Microsomes were prepared as described at a concentration of 1 g equivalent of liver/ml and were incubated at the temperatures indicated. Also, other samples were evacuated in Thunberg tubes, rapidly frozen in a solid CO_2-acetone bath, and stored at −20°. Samples were taken at various time intervals as indicated and were assayed for NADH and NADPH oxidase activities by the spectrophotometric procedure described. Each sample used after storage at −20° represents one that had not been previously thawed; ○———○, NADH oxidase, stored at 37°; ●———●, NADPH oxidase, stored at 37°; △———△, NADH oxidase, stored at 24°; ▲———▲, NADPH oxidase, stored at 24°; ▽———▽, NADH oxidase, stored at 4°; ▼———▼, NADPH oxidase, stored at 4°; □———□, NADH oxidase, stored at −20°; ■———■, NADPH oxidase, stored at −20°; ⊗, NADH oxidase, quick-frozen anaerobically and stored at −20°; ◉, NADPH oxidase, quick-frozen anaerobically and stored at −20°.

V_{max} varied with different preparations. This indicates partial inactivation of enzyme, and for accurate measurements the V_{max} should be determined by the double-reciprocal method.[12] The addition of 0·2 mM DCIP (0·060 mg/ml) stimulated NADH oxidation 22-fold and NADPH oxidation 8-fold (Fig. 6).

The optimal concentrations of cytochrome c for NADH and NADPH oxidation were similarly determined, and were found to be 1 mg/ml for both activities (Fig. 7). With ferricyanide as electron acceptor, oxidation of NADPH was seen over a broad range of dye concentration from 1 to 3 mM (Fig. 8). Oxidation of NADPH was greatest at a 3 mM concentration of ferricyanide. Relative rates of oxidation of NADH with various electron acceptors were oxygen, 1; cytochrome c, 34; DCIP, 22; and ferricyanide, 57. For the oxidation of NADPH they were oxygen, 1; cytochrome c, 9; DCIP, 8; and ferricyanide, 11. The molar extinction coefficient for cytochrome c (reduced-oxidized) was taken[13] as $\epsilon = 2{\cdot}10 \times 10^4$ liter $mole^{-1}$ cm^{-1}, and it

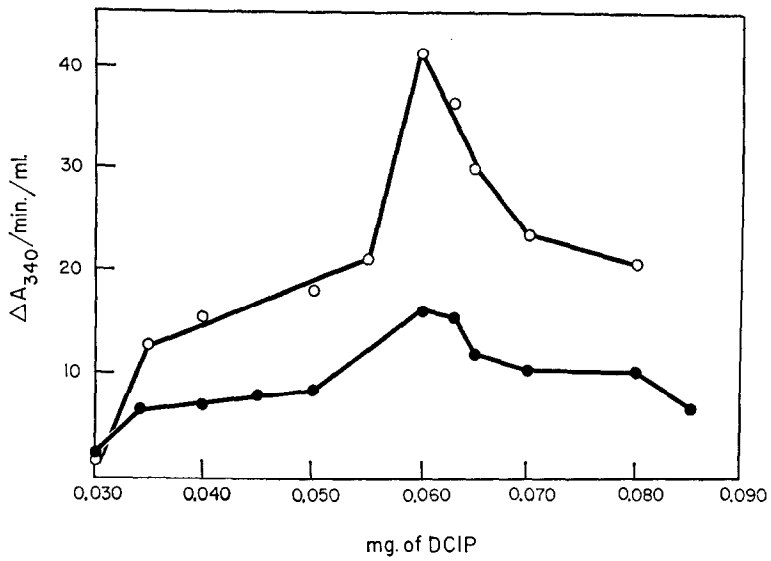

FIG. 6. Effect of concentration of DCIP on the rate of NADH and NADPH oxidation. The assays for NADH dehydrogenase and NADPH dehydrogenase with DCIP as electron acceptor were performed as described, except that the concentration of DCIP was varied as indicated; ○——○, NADH; ●——●, NADPH as substrate.

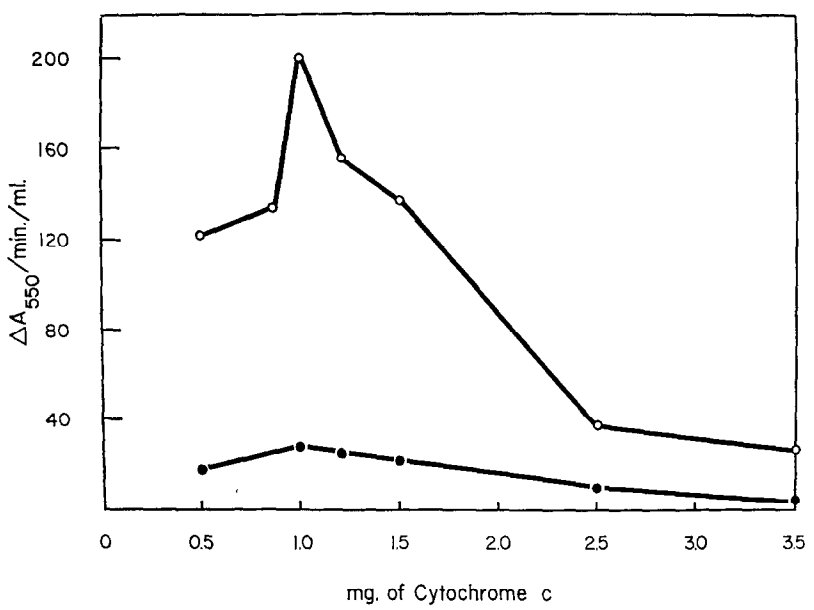

FIG. 7. Effect of concentration of cytochrome c on the rate of NADH and NADPH oxidation. The assays for NADH- and NADPH-cytochrome c reductase activities were performed as described, except that the concentration of cytochrome c was varied as indicated; ○——○, NADH; ●——●, NADPH as substrate.

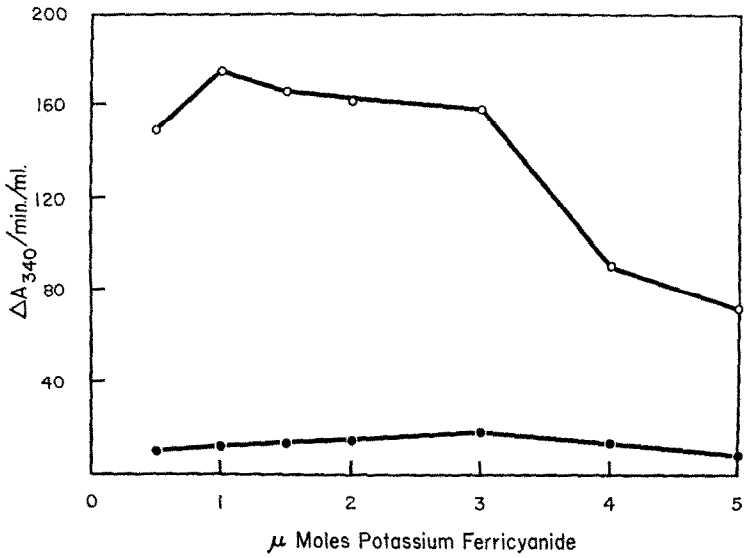

FIG. 8. Effect of concentration of potassium ferricyanide on the rate of NADH and NADPH oxidation. The assays for NADH dehydrogenase and NADPH dehydrogenase with potassium ferricyanide as electron acceptor were performed as described, except that the concentration of ferricyanide was varied as indicated; ○——○, NADH; ●——●, NADPH as substrate.

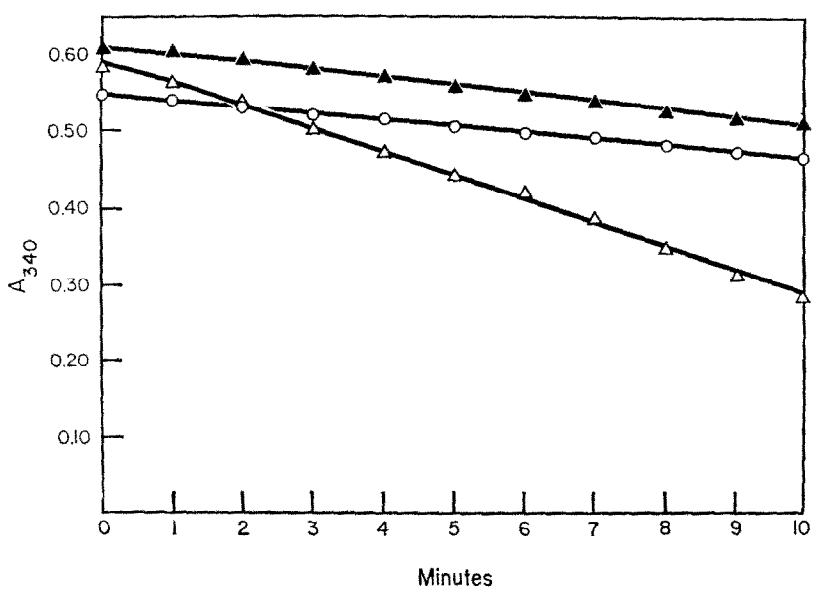

FIG. 9. Effect of ADP on the rate of NADPH oxidation in phosphate buffer. The conditions of assay were as described in Table 1; △——△, control; ▲——▲, 10 mM ADP; ○——○, 20 mM ADP.

was assumed that 2 moles of cytochrome c were reduced per mole of NADH or NADPH oxidized.

Effects of nucleotides on NADPH and NADH oxidation. While this work was in progress, an ADP-stimulated oxidation of NADPH by rat liver microsomes had been described in two laboratories.[6, 8–10] Both of these studies were based primarily upon polarographic measurements of oxygen uptake with silver-wire or platinum electrodes. It was of interest to correlate studies of oxygen uptake with spectrophotometric measurements.

In the latter studies we consistently observed an inhibition of NADPH oxidation by ADP in our assay system (Fig. 9, Table 1). The absorbancy changes were linear during the initial 5 min (Fig. 9). With the phosphate buffer system, ADP inhibited 54% at a 10 mM concentration and 69% at 20 mM. It has been reported by Hochstein *et al.*[9] that ferrous ion is required for the ADP-activated peroxidation of lipids. When ferrous sulfate was present in our phosphate assay system at a concentration of 0·015 μmole/ml, the reaction rate was enhanced by approximately 33% (Table 1). However, in the presence of ferrous sulfate and ADP, inhibition was still observed.

TABLE 1. EFFECTS OF ADP AND FERROUS ION ON NADPH OXIDATION IN SYSTEMS BUFFERED WITH PHOSPHATE OR TRIS

Assay conditions: all reactions were measured at 30° in a Gilford model 2000 recording spectrophotometer. The reaction mixture, in a total volume of 1 ml, contained 50 μmoles of either Tris or potassium phosphate buffer (as indicated), pH 7·4, 50 mμmoles NADPH, 25 μmoles nicotinamide, and 10 μliters microsomes (equivalent to 10 mg liver). Other additions, ADP and/or $FeSO_4$, were made as indicated. NADPH was omitted from the blank cuvette.

System	(mμmoles NADPH oxidized/min/g liver)
Phosphate buffer	432, 445, 455
Phosphate buffer + ADP (10 μmoles)	203
Phosphate buffer + ADP (20 μmoles)	138
Phosphate buffer + ADP (10 μmoles) + $FeSO_4$ (0·015 μmole)	178
Phosphate buffer + $FeSO_4$ (0·015 μmole)	590
Tris buffer	175
Tris buffer + ADP (10 μmoles)	366
Tris buffer + ADP (10 μmoles) + $FeSO_4$ (0·015 μmole)	350

This apparent discrepancy between our results and those of Orrenius *et al.*[6] was resolved by varying the conditions of assay. In the reaction system of these workers, 50 mM Tris buffer, pH 7·4, was employed. It is quite clear that under these assay conditions, the addition of 10 mM ADP results in an enhanced oxidation of NADPH (Table 1, Fig. 10). It should be emphasized, however, that in the absence of ADP, the substitution of Tris buffer for phosphate at this pH decreases the reaction rate by approximately 60%. Also, we were unable to obtain an additional enhancement by ferrous ion when ADP was present in the Tris system.

Manometric studies. When oxygen uptake was measured with Warburg manometers, the marked ADP-enhancement described by Beloff-Chain *et al.*[10] and Hochstein and Ernster[8] was observed (Fig. 11). That NADPH was rate limiting was indicated by the stimulation by G6-P and G6-P dehydrogenase (Fig. 11). Hochstein and

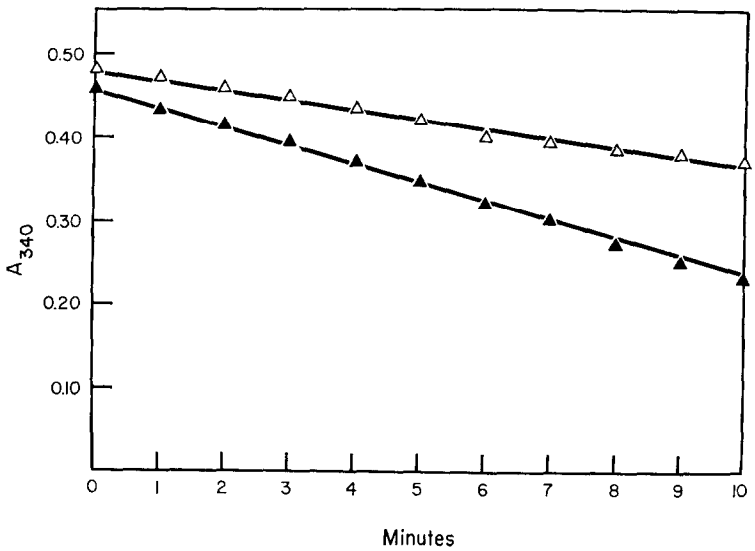

FIG. 10. Effect of ADP on the rate of NADPH oxidation in Tris buffer. The conditions of assay were described in Table 1; △———△, control; ▲———▲, 10 mM ADP.

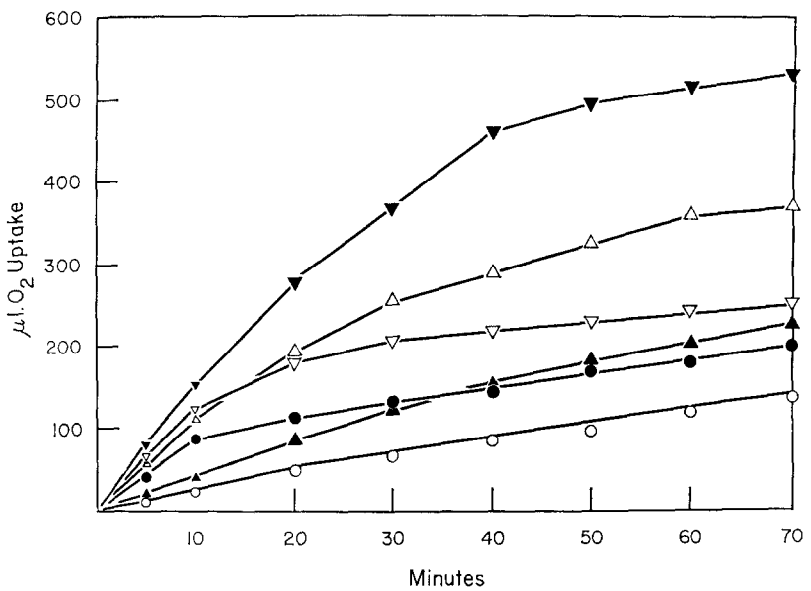

FIG. 11. Effect of ADP, NADPH, G6-P, and G6-P dehydrogenase on oxygen uptake by rat liver microsomes. For details of the manometric assay see Experimental section. NADPH, ADP, G6-P (20 μmoles), and G6-P dehydrogenase (0·01 mg) were added from the side arm as indicated; ○———○, control flask; ●———●, NADPH; △———△, NADPH, ADP; ▲———▲, G6-P, G6-P dehydrogenase; ▽———▽, NADPH, G6-P, G6-P dehydrogenase; ▼———▼, NADPH, ADP, G6-P, G6-P dehydrogenase.

Ernster showed that after oxygen consumption had ceased, it could be started again by the addition of G6-P and G6-P dehydrogenase. It will be noted that the addition of G6-P and G6-P dehydrogenase resulted in a significant enhancement of oxidation in the absence of added ADP (Fig. 11).

The ADP-enhancement of oxygen uptake was stimulated further by the addition of sodium fluoride (Fig. 12). For the initial 16 min there was little difference between

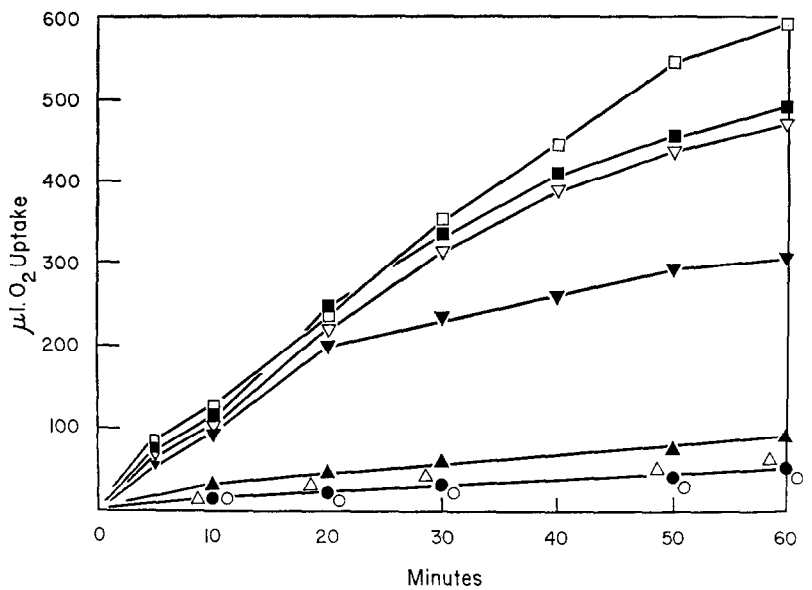

FIG. 12. Comparison of ADP and ATP enhancement of oxygen uptake with microsomes and NADPH; effect of fluoride. For details of the manometric assay see Experimental section. When present, ATP (10 μmoles) and fluoride (300 μmoles) were added from the side arm; ○———○, control flask; ●– – –●, ADP; △– – –△, ATP; ▲———▲, NADPH; ▽———▽, NADPH, ADP; ▼———▼, NADPH, ATP; □———□, NADPH, ADP, fluoride; ■———■, NADPH, ATP, fluoride.

the oxidation rates in the presence of ADP or ATP. Using more sensitive polarographic methods, Beloff-Chain *et al.*[10] and Hochstein and Ernster[8] initially reported that ATP did not enhance oxygen uptake. This finding is somewhat surprising in view of the known microsomal ATPase.[14–16] In a later communication, Hochstein *et al.*[9] found a stimulation with ATP when iron was added. In our experiments, although the rate of oxygen uptake with ADP decreased after 20 to 30 min, it was maintained at a higher rate in the presence of sodium fluoride. The mechanism of this enhancement is unclear. However, it may reflect an inhibition of a pyrophosphatase.[17]

In manometric experiments the addition of cytochrome c did not enhance oxygen uptake in the presence of NADPH (Fig. 13). However, the addition of cytochrome c inhibited by 49% the ADP-enhancement of NADPH oxidation. In the same experiment the drug barbital inhibited by 57%. Other drugs which were substrates for microsomal enzymes were tested for effect on the stimulation by ADP. Amytal did not inhibit, whereas zoxazolamine, 4-aminoantipyrine, and aminopyrine were found to inhibit (Table 2). Iodoacetate and arsenate were also tested and did not inhibit.

Oxygen uptake was markedly reduced when EDTA was added. This inhibition was observed both in the presence and absence of an organic pyrophosphate (Fig. 14).

No enhancement of NADPH oxidation was given by adenosine, AMP, or ADPR, whereas a small stimulation was seen with UDPGA and UDPAG, and a marked

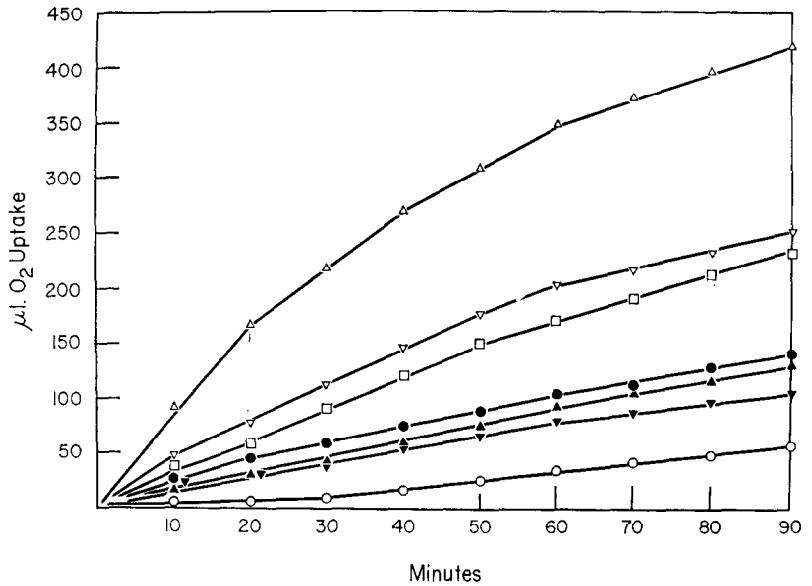

FIG. 13. Effect of cytochrome c and sodium barbital on the ADP, NADPH-stimulation of oxygen uptake by liver microsomes. For details of the manometric assay see Experimental section. When present, cytochrome c (10 mg) or sodium barbital (10 μmoles) was added from the side arm; ○——○, control flasks; ●——●, NADPH; △——△, NADPH, ADP; ▲——▲, NADPH + cytochrome c; ▽——▽, NADPH, ADP, cytochrome c; ▼——▼, NADPH, sodium barbital; □——□, + NADPH, ADP, sodium barbital.

TABLE 2. EFFECT OF DRUGS AND METABOLIC INHIBITORS ON THE ADP-, NADPH-LINKED OXYGEN UPTAKE BY RAT LIVER MICROSOMES

Oxygen consumption was measured with Warburg manometers. The assay conditions were as described in the Experimental section. The control rate was that measured with ADP, NADPH, and microsomes in the absence of inhibitor. The inhibitor in the appropriate concentration, as indicated below, was added from the side arm with the ADP and NADPH after thermal equilibration.

Inhibitor added	Inhibition (%)
Zoxazolamine (0·17 mM)	2
Zoxazolamine (1·7 mM)	37
Zoxazolamine (17 mM)	100
Aminopyrine (1·7 mM), expt. 8	48
4-Aminoantipyrine (1·7 mM), expt. 8	76
Aminopyrine (1·7 mM), expt. 3	77
Barbital (3·3 mM)	57
Iodoacetate (5 mM)	6
Arsenate (33 mM)	4
Amytal (3·49 mM)	0

increase occurred with UDPG (Fig. 13). After 30 min the rate with UDPG declined more sharply than with ADP, and at 90 min the total oxygen uptake with UDPG was 74 per cent of that observed with ADP. Conversion of UDPG to UDP, via UDPG-pyrophosphorylase and UTPase, could account for the stimulation, since the stimulatory effect is not specific with respect to nucleoside diphosphate.[10]

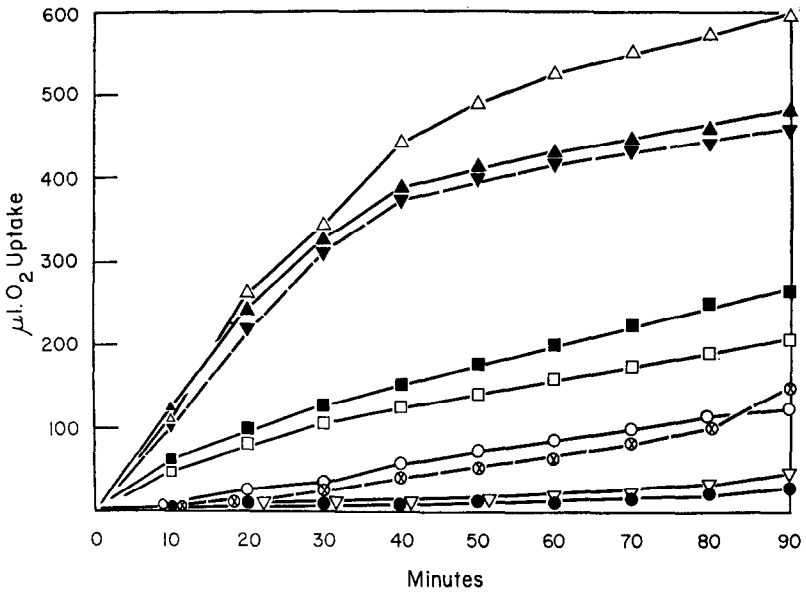

FIG. 14. Effect of EDTA on the ATP, NADPH-stimulation of oxygen uptake by liver microsomes; substrate specificity with respect to organic pyrophosphate. For details of the manometric assay see Experimental section. When present, EDTA (10·6 μmoles), ATP (10 μmoles), UDP-glucose (10 μmoles), UDP-glucuronic acid (10 μmoles), UDP-N-acetylglucosamine (10 μmoles), or ADP-ribose was added from the side arm; ○———○, control flask; ●———●, EDTA; △———△, ADP, NADPH; ▲———▲, ATP, NADPH; ▽———▽, ATP, NADPH, EDTA; ▼——▼, UDP-glucose, NADPH; □———□, UDP-glucuronic acid, NADPH; ■———■, UDP-N-acetylglucosamine, NADPH; ⊗——⊗, ADP-ribose, NADPH.

DISCUSSION

The studies described in this paper define the optimal conditions for the assay of microsomal NADH and NADPH oxidases, both of which are the subject of many current investigations. The concentrations of substrates and of microsomes are particularly crucial, if maximal rates are to be observed.

The enhanced oxygen uptake by microsomes in the presence of ADP and NADPH, which had been observed initially by Beloff-Chain *et al.*,[10] was explained by Hochstein and Ernster[8] on the basis of an enzymic peroxidation of lipid, and it was shown that 1 mole of malonaldehyde accumulates per mole of NADPH utilized. Orrenius *et al.*[6] have suggested that the NADPH-oxidizing system is the NADPH–cytochrome c reductase[18] and functions in the oxidative demethylation of drugs.[7] It has been suggested that NADPH oxidase is involved in the hydroxylation of drugs.[3, 4, 6, 7]

Several of the results described in this paper are pertinent to these proposals. The NADPH (and NADH) oxidase activity, as assayed spectrophotometrically with oxygen as the terminal electron acceptor, was extremely labile to storage. Orrenius

et al.[6] found that storage for 72 hr did not significantly reduce the rate of lipid peroxidation. It is obvious that the spectrophotometric method would have indicated no NADPH oxidase activity at this time. Therefore, cytochrome or factor(s) which links NADPH oxidation with molecular oxygen under the conditions of the spectrophotometric assay is not necessary for the lipid peroxidation. In fact, the cytochrome-coupled pathway may shift electrons away from the lipid in a side reaction, as suggested by the original scheme of Hochstein and Ernster[8] and by our results in which cytochrome c inhibited the ADP-stimulated, NADPH-linked oxygen uptake.

If drug substrates and lipid compete for a common source of electrons, drugs would be expected to reduce the rate of lipid peroxidation. Orrenius *et al.*[6] found this to be the case for codeine and aminopyrine, both of which are substrates for microsomal demethylating enzymes. Our results indicated an inhibition of oxygen consumption by zoxazolamine and aminopyrine. However, we were unable to demonstrate a correlation between the ability of drugs to serve as substrates for microsomal enzymes and their activities as depressors of oxygen uptake. For example, amytal, a substrate for hydroxylation, did not inhibit, while barbital, which is only very slowly metabolized, gave a 57 per cent inhibition. When aminopyrine and its demethylation product 4-aminoantipyrine were studied in the same experiment, greater inhibition was observed with the latter compound.

Inhibition of NADPH oxidation by ADP in spectrophotometric measurements and stimulation of oxygen uptake in manometric experiments illustrate the difficulty in correlating results obtained under dissimilar methods of assay. Under conditions of high oxygen tension and at pH 7·5 in Tris buffer, Orrenius *et al.*[6] observed a 5-fold stimulation of NADPH utilization by ADP. Under our assay conditions, we observed an inhibition by ADP in phosphate buffer and an enhancement in Tris buffer. Since the ADP-stimulation of oxygen consumption may be demonstrated with both buffer systems, the relationship between the spectrophotometric and manometric results is unclear.

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