Metabolic Effects of Epinephrine in the Perfused Rat Heart

I. Comparison of Intracellular Redox States, Tissue pO_2, and Force of Contraction

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SUMMARY

A direct fluorometric technique for recording the intracellular oxidation-reduction state has been applied to the perfused rat heart. The fluorescence changes were calibrated in terms of the content of analytically determined NADH and NADPH. Simultaneous measurements of the contractile force and tissue oxygen tension were also made.

The kinetics of the fluorescence response to epinephrine were determined under a variety of conditions which allowed an initial oxidation of pyridine nucleotide to be distinguished from the subsequent predominant increase of reduced pyridine nucleotide. The change of the pyridine nucleotide toward the more oxidized state coincided with increased respiratory activity and an increased force of contraction. This effect is interpreted as a mitochondrial response to the increased ADP formed by the increased work of the heart. The increased formation of reduced pyridine nucleotides was transient, and completely inhibited by iodoacetate. This response is interpreted as being due to the rapid formation of NADH at the glyceraldehyde-P dehydrogenase step as glycogenolysis is initiated.

Log dose-response curves for the contractile force and increase of fluorescence are reported together with data showing inhibition of both these effects by the β-receptor blocking agent, Nethalide. A comparison of the tissue oxygen tension changes in hearts subjected to anoxia and epinephrine suggests that respiratory linked reduction of pyridine nucleotide does not occur during the cycle of pyridine nucleotide reduction following epinephrine administration. The results demonstrate a temporal separation between the positive inotropic action of epinephrine and its glycogenolytic effect.

INTRODUCTION

It has previously been reported (1) that epinephrine stimulated the oxidative metabolism of the perfused rat heart over at least 30 min but had only a transient effect on the glycogenolytic process. With low concentrations of epinephrine, oxidation of exogenous substrates was favored over glycogenolysis. It was suggested that the increased fuel utilization was a secondary consequence of the positive inotropic effect of epinephrine, and the results were interpreted as indicating a poor correlation between glycogen metabolism and increased contractility.

This possibility has been investigated further by means of a more direct kinetic approach. A convenient monitor of the metabolic effects of epinephrine is provided by the continuous observation of the state of oxidation-reduction of the pyridine nucleotides in the intact tissue by the microfluorometric techniques developed by Chance and co-workers (2–4). We have used this approach, in conjunction with the use of metabolic inhibitors, in an attempt to distinguish between oxidation-

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reduction changes in the cytoplasmic and mitochondrial compartments. These studies, in conjunction with the simultaneous measurement of the tissue pO_2 and contractile force, have provided evidence indicating that the effects of epinephrine on respiration and the contractile system precede glycogen breakdown, and they support the concept that the different epinephrine responses reflect separate effects on the various intracellular systems. Some of the concepts developed in this paper have been presented in preliminary form elsewhere (5).

METHODS

Heart perfusion. Male, fed, Wistar albino rats (220–260 g) were anesthetized with a 50% Co_2–50% O_2 gas mixture, and the heart was rapidly removed and transferred to the perfusion apparatus. The method of heart perfusion was basically the same as that described previously (*1*, *6*), but the perfusion apparatus was modified in some details, since it was necessary to perfuse the hearts with fluid which could either be recirculated or discarded. In the recirculation apparatus, two water-jacketed 15-ml capacity fritted glass filter funnels with pore size 10–15 μ (Arthur Thomas and Co., Philadelphia) were placed 65 cm above the heart to provide the perfusion pressure and serve as fluid reservoirs. Water-jacketed leads were passed to the cannula on which the heart was mounted through a small stainless steel 3-way tap. A second 3-way tap was mounted below the heart chamber so that fluid which had passed through the heart could be returned to the appropriate reservoir (4). One reservoir contained perfusate equilibrated with 5% CO_2 in oxygen, and the other contained similar fluid equilibrated with 5% CO_2 in nitrogen. The gas pressure served to return fluid which had passed through the heart up to the reservoir in the manner described by Bleehen and Fisher (7). The heart chamber was maintained empty of fluid, and, by means of short, narrow-bore connecting leads, fluid saturated with either the oxygen or the nitrogen gas mixtures could be rapidly supplied to the heart and returned to the appropriate reservoir by turning the 3-way taps. For continuous perfusion with oxygenated fluid, a water-jacketed chamber of 500 ml capacity was used as an upper reservoir, and the fluid flowed into the cannula after passing through a fritted glass filter. Epinephrine was injected into the perfusion circuit immediately below the reservoir, while other compounds were added directly to the reservoir. Substrate additions to the basic perfusate of Krebs bicarbonate medium (8), modified to contain half the usual calcium content, are indicated in the figures.

Force of contraction. In most of the studies, the heart chamber was inverted in a manner similar to that described by Zachariah (*9*), and the heart was attached to the strain gauge by a nylon thread tied to a stainless steel hook which was inserted into the apex. The fluid passed out of the base of the heart chamber, through a one-way valve, and was either returned to the upper reservoir or allowed to run to waste. The force of contraction was measured with a Decker transducer (Model 902-1) or in later experiments with a Statham transducer (Model 91-4-250), which was found to be more convenient.

Tissue oxygen tension. This was measured with a 50-μ diamel-coated gold wire as the cathode and a silver-silver chloride wire as the anode (10, 11). The gold wire (Johnson-Mathey Co., London, England) was further insulated with 3 coats of Moffelak resin (Phillips Electrical Co., Eindhoven, Holland) except for the freshly cut tip, and was inserted into the ventricular muscle through a small hole so that the tip was situated some distance away from the damaged tissue and 0.5–1 mm below the surface. The electrical lead to the cathode passed through the rubber stopper holding the heart cannula, while the anode was immersed in the perfusate entering the heart. A polarizing potential of —0.6 volt was applied to the cathode. Calibration of such electrodes in saline has been previously described (11). Even with these small electrodes, there is extensive microscopic tissue damage, and the recorded tissue oxygen tension is consequently a value

representative of the capillary A–V difference.

Tissue fluorescence. A microfluorometer similar to that first used by Chance and co-workers (3), and described in detail elsewhere (2, 4), was used to measure the intensity of the fluorescence emission at approximately 480 mμ upon excitation of a small area of the heart ventricle with light of wavelength 366 mμ from a high intensity mercury arc lamp. Although only a small area close to the surface of the heart is monitored with this technique, changes in the intensity of the fluorescence emission from the aerobic steady state level have been found to correspond closely with the total content of reduced pyridine nucleotides in the heart as determined by tissue analysis (4, 12). In some experiments the heart was entirely enclosed in a water-jacketed heart chamber of low fluorescence Pyrex glass, but better discrimination of the heart fluorescence was obtained by viewing the tissue directly through a hole in the double-walled heart chamber, and this technique was used in later experiments.

Analytical techniques and materials. Enzymic methods used for the analysis of the metabolic intermediates and the oxidized and reduced forms of the pyridine nucleotides were the same as those previously used (12). All enzymes and cofactors necessary for these assays were obtained from Sigma Chemical Co., or from C. F. Boehringer and Sons through the California Corporation for Biochemical Research. Epinephrine chloride (Parke, Davis and Co.) was diluted from the stock 1 mg/ml solution for each experiment. Nethalide, 1-(2-naphthyl)-2-isopropylaminoethanol hydrochloride, was obtained from the Imperial Chemical Industries, Ltd.

RESULTS

Effects of Epinephrine and Anoxia

Figure 1 shows the effects of 2 μg epinephrine and a brief cycle of anaerobiosis on the levels of the tissue fluorescence (upper trace) and the oxygen tension (lower trace) in the perfused rat heart. The magnitude of the fluorescence signal in the normal oxygenated heart is arbitrarily taken as 100 units, and all fluorescence changes are expressed as percentages of this initial

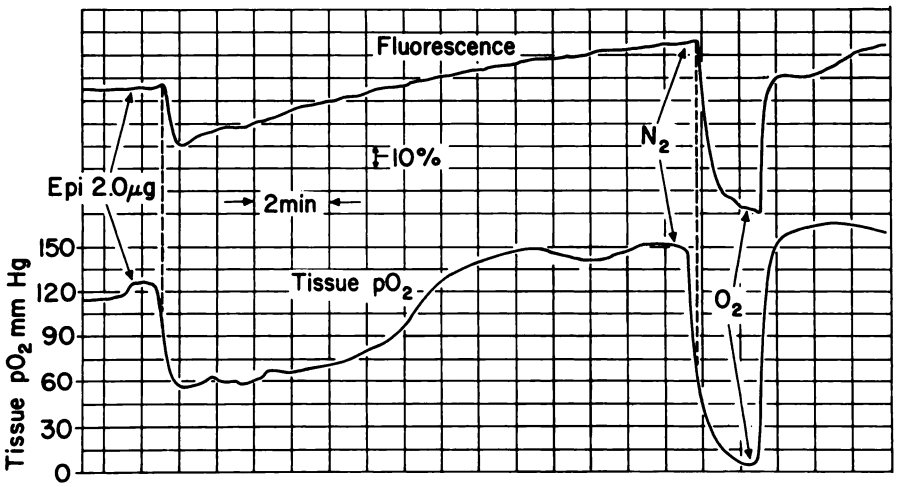

FIG. 1. *Fluorescence and tissue oxygen tension changes (upper and lower traces, respectively) after epinephrine and during anaerobiosis*

Fluorescence increase, corresponding to pyridine nucleotide reduction, is denoted by a downward deflection of the trace. The heart, after 15 min perfusion with 5 mM glucose, was transferred to a recirculation apparatus containing 20 ml of a similar perfusate. Epinephrine (2 μg) was injected into fluid flowing to the heart at the point marked by the arrows.

value. Shortly after the addition of 2 μg epinephrine, the tissue pO_2 decreased abruptly to 60 mm Hg, from an initial level of about 120 mm Hg, and slowly recovered after an interval of 2 min. The fluorescence intensity showed a small decrease occurring simultaneously with the pO_2 decrease, followed by a sharp transient increase of 28% starting after 15 sec. When the perfusate was changed from oxygenated medium to fluid equilibrated with nitrogen, the tissue pO_2 fell rapidly almost to zero, and the fluorescence started to increase shortly after the onset of the decrease in the pO_2. Upon switching to perfusate equilibrated with oxygen, the fluorescence and pO_2 returned to their pre-anaerobic levels.

It may be seen from Fig. 1 that, after the addition of epinephrine, the fluorescence commenced to increase when the tissue pO_2 was about 100 mm Hg; while with nitrogen equilibration, the fluorescence did not change until the pO_2 had fallen to about 60 mm Hg. Analysis of 26 similar traces over a range of epinephrine doses from 0.02 to 4 μg showed that, during hypoxia, the fluorescence started to increase when the tissue pO_2 was 63 ± 6 mm Hg, while with epinephrine, the corresponding value for the pO_2 at the commencement of the fluorescence increase was 114 ± 14 mm Hg, and the minimum pO_2 reached was 62 ± 2 mm Hg. These results suggest that it is unlikely that reduction of respiratory linked pyridine nucleotide in the mitochondria can account for the fluorescence increase induced by epinephrine.

The normal heart rate at 37° under the conditions of these experiments was about 300 beats/min, with a range of 260–340 beats/min (34 hearts). These values are somewhat higher than those reported by Zachariah (9), possibly due to a difference of perfusate temperature. The maximum contraction rate increase during epinephrine stimulation was found to be less than 15%, but a possible effect of a change in heart rate on the fluorescence response was investigated by comparing the fluorescence response to a submaximal dose of epinephrine in the same heart beating either at its natural rate or at a higher imposed rate. Almost identical percentage increases of fluorescence were obtained with an epinephrine dose of 0.2 μg, irrespective of whether the heart was stimulated or not. The imposed heart rate (350 impulses/sec at 0.5 volt for 30 msec) was made 30% greater than the natural rate, and under these conditions the rate did not change during epinephrine stimulation. Control experiments showed that with epinephrine doses in this range, reproducible responses could be obtained in the same heart with up to 4 successive additions, provided that the hearts were perfused with nonrecirculating fluid containing glucose.

Other experiments showed that no clear relationship could be established between the rate of stimulation and the contractile force, since the latter tended to change with time when the heart was stimulated at a constant rate. Consequently, the stimulation potential and the duration of the impulse were carefully chosen to be the minimum necessary for the imposed rate to be followed. Under these conditions, the contractile force remained constant to within 10% for several minutes at different stimulation rates up to 450 impulses/min.

Separation of the Florescence Response into Mitochondrial and Cytoplasmic Components

Several lines of evidence indicate that the fluorescence increase with epinephrine is largely of cytoplasmic origin. It can, for instance, be abolished or greatly diminished by pretreatment of the heart with iodoacetate, by depletion of the glycogen stores by a short period of anoxia, or by successive large doses of epinephrine administered to hearts perfused with substrate-free medium. Figure 2 illustrates the effect of glycogen depletion in an experiment in which the heart was stimulated at a constant frequency of 400 beats/min, and perfused in the absence of glucose without recirculating the medium. Prior to the traces showing in the figure, two additions of 2 μg epinephrine had been made which produced fluorescence changes similar to those shown in Fig. 1. In Fig. 2A, the response after a third dose of 2 μg epinephrine is

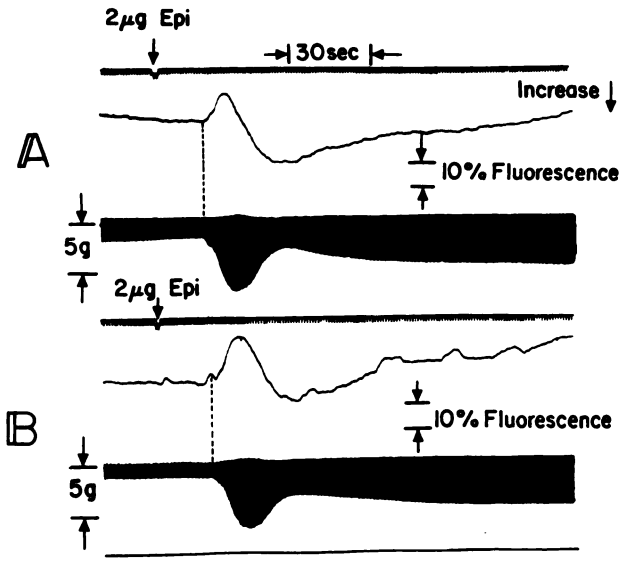

FIG. 2. *Effect of repeated large doses of epinephrine*

A time marker in seconds is shown at the top of each recording. The upper trace denotes fluorescence, and the lower trace shows the contractile force. The heart was perfused, without recirculation, with fluid containing 5 m*M* glucose and 5×10^{-5} M EDTA. The heart was stimulated at a rate of 400 beats/min (0.5 volt for 30 msec). Figure 2A shows the effect of the third addition of 2 μg epinephrine, and Fig. 2B, the effect of a similar fourth addition. The doses were spaced 10 min apart.

shown, and it is seen that the fluorescence trace is triphasic, the normal fluorescence increase being preceded by an initial large decrease of fluorescence (upward deflection of the trace), commencing with the onset of the increased force of contraction. With a further dose of epinephrine 10 min later (Fig. 2B), when the effect of the previous dose had worn off, the initial fluorescence decrease was magnified in extent and duration, and there was only a slight secondary increase of fluorescence. Analysis of five hearts for glycogen after three successive doses of 2 μg epinephrine showed that the glycogen level had been decreased to 16 ± 5 μmoles/g dry wt (from an initial level of 113 ± 9 μmoles/g dry wt).

This triphasic fluorescence response suggests that there are at least two components contributing to the fluorescence change which have a different phase relationship and are opposite in direction. The magnitude of the initial NAD oxidation has been found to depend on the metabolic state of the heart and appears to be of mitochondrial origin. If the contractile activity of the heart does in fact influence the oxidation-reduction state of the mitochondrial pyridine nucleotide, it should be possible to observe oxidation-reduction cycles coupled with each heart beat. Normally, the rate of heart contractions is too rapid compared with the time response of the fluorescence detector and recorder for this phenomenon to be observed. In addition, it is necessary to prevent possible interference due to pyridine nucleotide changes in the cytoplasm. Lastly, the mitochondrial pyridine nucleotides should be poised in a relatively reduced state so that the oxidation changes may more readily be detected. These conditions have been fulfilled in the experiment illustrated in Fig. 3. Preliminary experiments showed that after the addition of 1 mM iodoacetate or a similar concentration of iodoacetamide, the heart initially beat arrhythmically, but thereafter, the contractions became slow and regular for a period of about 20 min. Over this period epinephrine still produced a positive inotropic response. Prior to the recording shown in Fig. 3, a heart had been treated

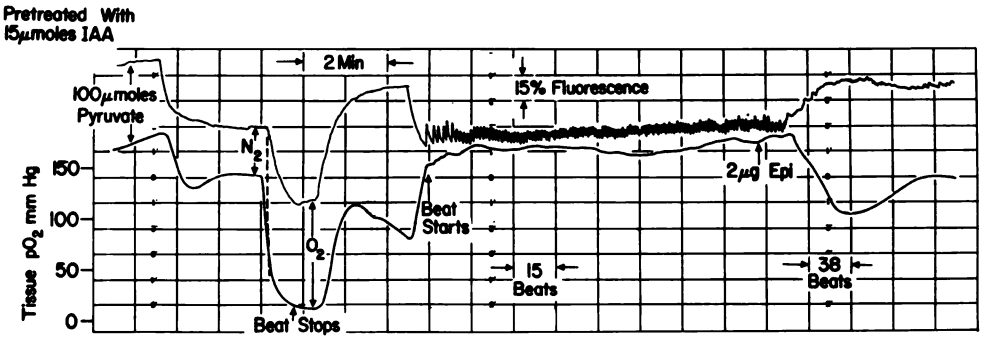

FIG. 3. *Effects of pyruvate, anoxia, and epinephrine in a heart pretreated with 1 mM iodoacetate*
The fluorescence is shown in the upper, and the tissue oxygen tension in the lower, trace. An increase of fluorescence is denoted by a downward deflection. The heart was perfused with 15 ml fluid to which 15 μmoles iodoacetate had been added 15 min prior to the recording shown.

with iodoacetate for 15 min. Addition of 100 μmoles pyruvate to the circulating perfusate volume of 15 ml produced a prompt increase of fluorescence (upper trace) after a short time interval necessary for the pyruvate to reach the heart. Upon changing to a perfusate equilibrated with nitrogen, the fluorescence increased further, the tissue pO_2 fell rapidly, and the heart beats ceased. On returning to perfusate containing iodoacetate and pyruvate, the fluorescence first decreased to an intermediate level and then abruptly increased to a level approximately the same as that prior to the anaerobic transition. Heart contractions resumed at this point at a rate of 10–15 beats/min, and small fluorescence cycles of oxidation and reduction were recorded for several minutes, oxidation occurring with each systole. Addition of 2 μg epinephrine caused the usual decrease in the tissue pO_2 as the rate of respiration increased, a change of the pyridine nucleotides towards the fully oxidized state, and an increase of heart rate to 38 beats/min. At this heart rate, oxidation-reduction cycles of the tissue fluorescence could no longer be recorded. It may be noted that in the presence of iodoacetate, the biphasic response of the tissue fluorescence to epinephrine was abolished. This is considered to be due to the absence of the cytoplasmic pyridine nucleotide component, due to inhibition of glycolysis at the glyceraldehyde-P dehydrogenase step. That this enzyme step was in fact inhibited under the conditions of this experiment was established separately: first, by the addition of test doses of epinephrine at different times after the addition of iodoacetate, and second, by measurements of glucose uptake, lactate production, and the tissue content of glycolytic intermediates.[1] These experiments showed that 20–25 min were required for the inhibitory effect of 1 mM iodoacetate to be fully effective whereas iodoacetamide at the same concentration reacted somewhat faster.

Addition of acetate of pyruvate to the perfusate prior to a test dose of epinephrine has provided another method of distinguishing between changes in the redox state of the cytoplasmic and mitochondrial pyridine nucleotides. An example of the epinephrine response in the presence of acetate has been given in a previous publication (4), and it was shown to be triphasic like that obtained with glycogen-depleted hearts. Additional experiments showed that the onset of the initial fluorescence decrease coincided with a diminution of the tissue pO_2. A somewhat different fluorescence response to epinephrine is obtained in the presence of pyruvate as illustrated by Figs. 4 and 5. Figure 4 shows that the addition of 0.05 μg epinephrine prior to pyruvate produced only an increase of fluorescence, in conjunction with a transient decrease of

[1] J. R. Williamson, unpublished experiments.

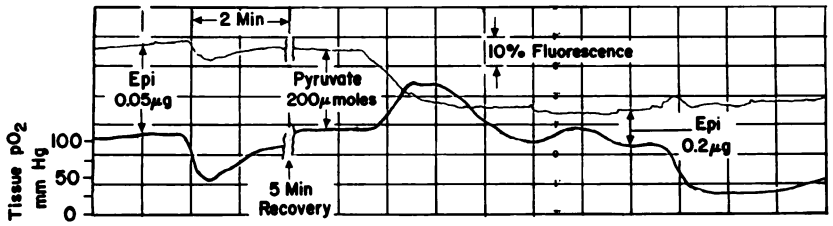

FIG. 4. *Comparison of the effects of epinephrine in the absence and presence of 10 mM pyruvate*
The fluorescence is shown in the upper, and the tissue oxygen tension in the lower, trace. The heart was perfused initially with 20 ml fluid in the absence of substrate.

the tissue pO_2. Upon the addition of pyruvate, the fluorescence increased to a new steady-state level (an effect similar to that seen in the presence of iodoacetate, Fig. 3) while the pO_2 increased for a minute or two before returning to the initial level. Addition of 0.2 μg epinephrine at this point produced a transient diminution of fluorescence, starting at the onset of the pO_2 decrease, and the normal increase of fluorescence was abolished. This effect is more clearly demonstrated in Fig. 5, which shows simultaneous recordings of fluorescence and the force of contraction. The left-hand side of Fig. 5 shows that in a heart, perfused in the presence of 10 mM pyruvate, the addition of 0.2 μg epinephrine produced a fluorescence decrease which started with the onset of the increased force of contraction and lasted for 15 sec. Thereafter, the fluorescence returned to the initial level although the force of contraction remained elevated. Thus, by starting with a more reduced state of the pyridine nucleotides, we have succeeded in enhancing the initial oxidation phase of the pyridine nucleotide oxidation-reduction cycle at the expense of the subsequent reduction. The right-hand side of Fig. 5 shows that after reequilibration of the heart with a substrate-free perfusate, the same concentration of epinephrine produced only the usual fluorescence increase. On the time scale shown, it is seen that the start of the fluorescence increase is delayed by about 15 sec compared with the onset of the increased force of contraction.

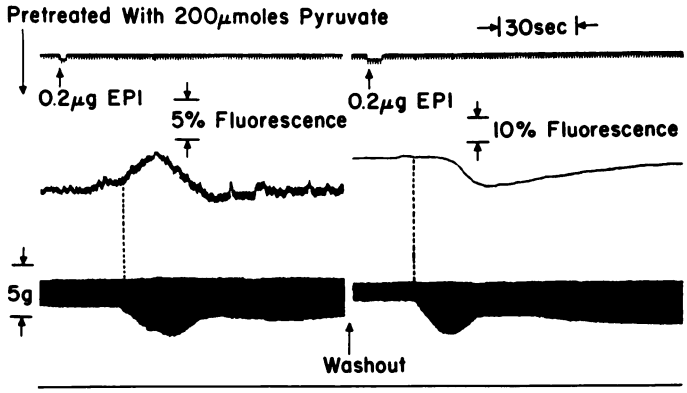

FIG. 5. *Effects of epinephrine in the presence and absence of pyruvate*
The upper trace shows the fluorescence, and the lower trace the contractile force. The heart was perfused initially with 20 ml recirculating fluid containing 10 mM pyruvate. After the first addition of 0.2 μg epinephrine, the heart was perfused for a further 15 min with nonrecirculating fluid containing 5 mM glucose (washout) prior to the second addition of 0.2 μg epinephrine.

It has previously been shown (12) that 0.1 mM arsenite was able to reverse the effect of pyruvate on the pyridine nucleo-

tides. This effect is interpreted as due to inhibition of electron transfer from reduced lipoic acid to flavoprotein in the pyruvic oxidase enzyme complex (13), thereby, inhibiting pyruvate oxidation. Figure 6 shows that the effect of a low concentration of epinephrine in a heart perfused with 10 mM pyruvate and 0.1 mM arsenite was similar to that obtained in a normal heart perfused in the absence of pyruvate. The decrease in the tissue pO_2 after the addition of epinephrine showed that the heart responded with an increased oxygen uptake despite the presence of arsenite. This was a highly reproducible response, and parallel experiments showed that the force of contraction was also increased by epinephrine under these conditions.

A triphasic response of the fluorescence to epinephrine can also be obtained in the presence of lactate, the initial fluorescence decrease coinciding with the onset of the increased force of contraction (5). Lactate itself when added to the perfusate produced a prolonged fluorescence increase and, as shown in Fig. 7, caused a temporary decrease of the contractile force. A similar

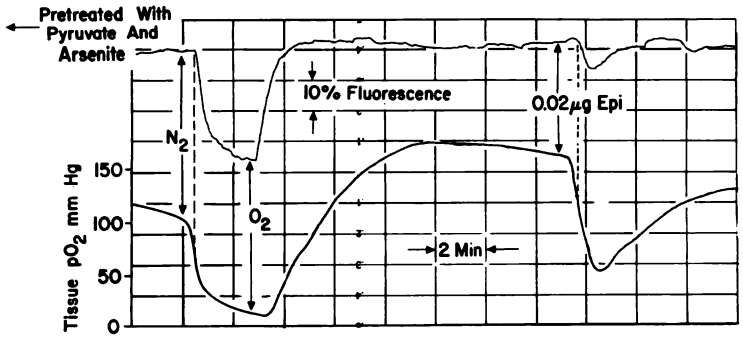

FIG. 6. *Effect of epinephrine in a heart pretreated with pyruvate and arsenite*

The heart was perfused initially with 15 ml fluid to which 100 μmoles pyruvate and 2 μmoles arsenite were added successively. The response of the fluorescence (upper trace) and tissue oxygen tension (lower trace) to 0.02 μg epinephrine added 10 min after the arsenite is shown after a cycle of anoxia.

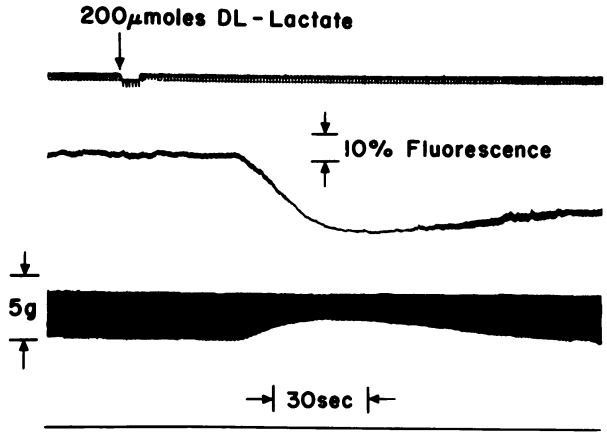

FIG. 7. *Effect of lactate on the fluorescence (upper trace) and contractile force (lower trace) in a heart perfused initially with 20 ml of substrate-free Krebs bicarbonate Ringer*

Increase of fluorescence is shown as a downward deflection of the trace.

effect on the force of contraction was observed when acetate or pyruvate, but not glucose, were added to the perfusate. This transient negative inotropic effect probably explains the increased tissue pO_2 levels obtained immediately on adding acetate or pyruvate.

Assessment of the Cytoplasmic NAD/NADH Potential by Means of Metabolite Redox Couples

In the manner described by Bücher and co-workers (14, 15), the ratio of the oxidant to the reductant of the cytoplasmic NAD-linked dehydrogenase may be used to provide an assessment of the cytoplasmic NAD/NADH redox potential. Figure 8 shows the changes in the ratios of malate/oxaloacetate, lactate/pyruvate, and α-glycerophosphate/dihydroxyacetone phosphate in hearts perfused up to 2 min after the onset of the increased force of contraction subsequent to the addition of 1 μg epinephrine to a perfusate containing 10 mM glucose. In this figure, each point represents analyses performed on separate hearts. The ratios of malate/oxaloacetate and lactate/pyruvate followed a similar pattern: no marked effect of epinephrine was apparent until after 20 sec, when both ratios increased to give sharp maxima at about 30 sec before returning to their initial values. These changes corresponded to a decrease of the cytoplasmic NAD/NADH redox potential, from an initial mean value of −244 mV to −259 mV, after 30 sec. The ratio of α-glycerophosphate to dihydroxyacetone phosphate, on the other hand, changed very little after the addition of epinephrine and showed no sharp maximum after 30 sec. Similar changes of the redox couples were obtained in a separate experiment in which hearts were perfused in the absence of glucose.

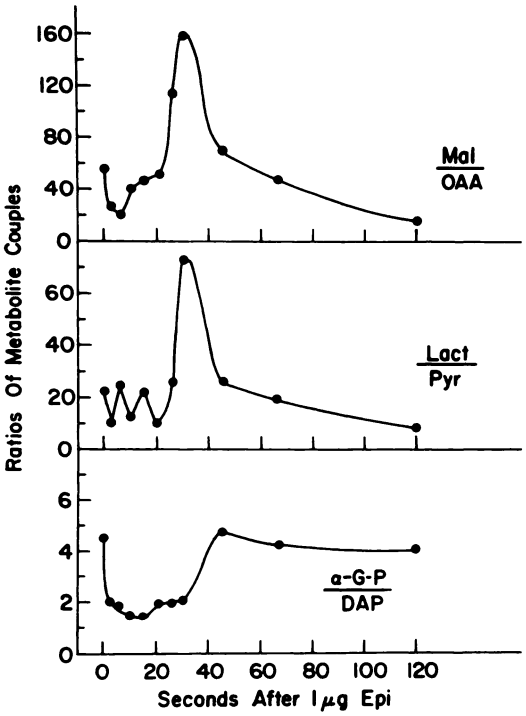

FIG. 8. *Effect of epinephrine on the ratios of the metabolite pairs associated with malate dehydrogenase, lactic dehydrogenase, and α-glycerophosphate dehydrogenase in a series of hearts perfused, without recirculation, with fluid containing 10 mM glucose.*

Zero time on the abscissa represents the time at which the contractile force started to increase. Each heart was rapidly frozen at the time indicated. The abbreviations used are: Mal, malate; OAA, oxalo-acetate; Lact, lactate; Pyr, pyruvate; α-G-P, α-glycerophosphate; DAP, dihydroxyacetone phosphate.

Total Content of Pyridine Nucleotides by Tissue Analyses

The amounts of NADH, NADPH, and NADP found in hearts perfused for up to 3 min, after the addition of 1.5 μg epinephrine, are shown in Fig. 9. It may be seen that there was a large increase in the level of NADH, a smaller and slower increase in the level of NADPH, with a corresponding decrease in the level of NADP. Maximum increases of NADH were observed after 30 sec, and maximum levels of NADPH were not attained until about 50 sec, after the addition of epinephrine. The levels of NADP and NADPH returned to their initial values after about 90 sec, but NADH remained elevated for the 3-min duration of the experiment. Mean changes of NADH and NADPH in a group of five hearts, perfused for 30 sec with epinephrine, have been presented previously (5), and these values are in the same range as the

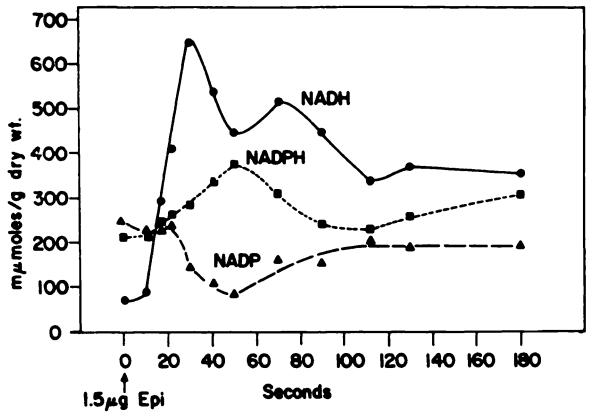

FIG. 9. *Effect of epinephrine (1.5 μg) on the content of NADH, NADP, and NADPH in a series of hearts perfused with 15 ml fluid containing 5mM glucose and 5 × 10⁻⁵ M EDTA*

Zero time on the abcissa represents the time at which the first visible effects of epinephrine were observed. (Contractile force was not measured.) Hearts were rapidly frozen at the times indicated.

corresponding values shown in Fig. 9. The sum of the values of NADH and NADPH, as determined by tissue analyses, followed a similar curve to the fluorescence intensity change with epinephrine depicted in Fig. 1, thereby, providing excellent evidence that the fluorescence changes give an accurate kinetic monitor of changes in the tissue content of reduced pyridine nucleotides.

Log Dose-Response Relationships

Figure 10 shows an example of the relationship between the maximum increase in the force of contraction and the maximum increase of fluorescence obtained with a series of epinephrine additions to the same heart over the range of 5×10^{-4} μg to 2 μg. Fresh oxygenated solution containing 5 mM glucose perfused the heart, and the epinephrine passed through the coronary circulation only once. The heart was allowed to recover fully between each successive addition of epinephrine. The force of contraction increased by 20% with the lowest dose used, but the fluorescence showed no increase with epinephrine doses less than about 10^{-2} μg. Between 2×10^{-2} and 2×10^{-1} μg, the responses of both the contrac-

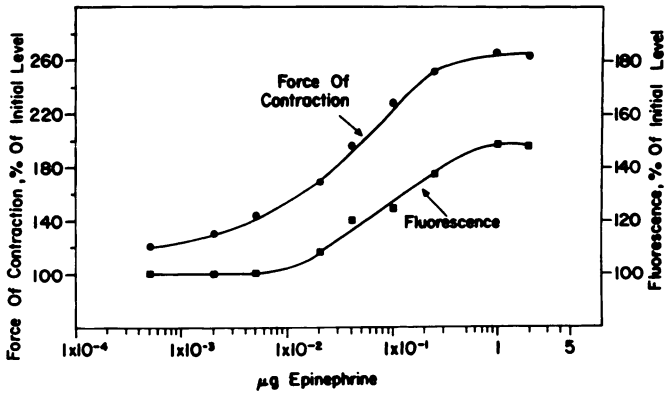

FIG. 10. *Log dose-response curves for the maximum increase of contractile force and the maximum increase of fluorescence following epinephrine additions*

Data were obtained from a single heart perfused with 5 mM glucose without recirculation.

tile force and the fluorescence were approximately linear, and maximum changes were obtained with 1 μg. The horizontal displacement of the two curves illustrates the different sensitivities of the effects on the contractile mechanism and on the biochemical events causing the fluorescence increase. Each phase of the curve was repeated, using different hearts, with essentially the same results as those shown in Fig. 10.

Inhibition of Epinephrine Effects with Nethalide

The stimulatory effect of epinephrine on the force of contraction and on the accumulation of reduced pyridine nucleotides in the heart can be abolished by prior administration of Nethalide. However, the sensitivity of the two effects to Nethalide inhibition appears to be somewhat different. force doubled after 15 sec. Figure 11B shows the effects of the same concentration of epinephrine added to the heart after 3 min perfusion with 15 ml of recirculating medium containing glucose and 5 μg Nethalide. The fluorescence and contractile force increases were both inhibited. In Fig. 11C the effect of a third dose of 0.5 μg epinephrine is shown after the recirculation perfusion circuit was washed out and the heart equilibrated for 3 min with 15 ml of fresh medium containing glucose and a higher concentration of Nethalide (20 μg). As seen from the figure, the effects both on the fluorescence and the force of contraction were completely abolished. After a high dose of epinephrine (2 μg) in the presence of 20 μg Nethalide, Fig. 11D shows that the positive inotropic effect of epinephrine was converted to a slightly negative inotropic response, but a considerable fluores-

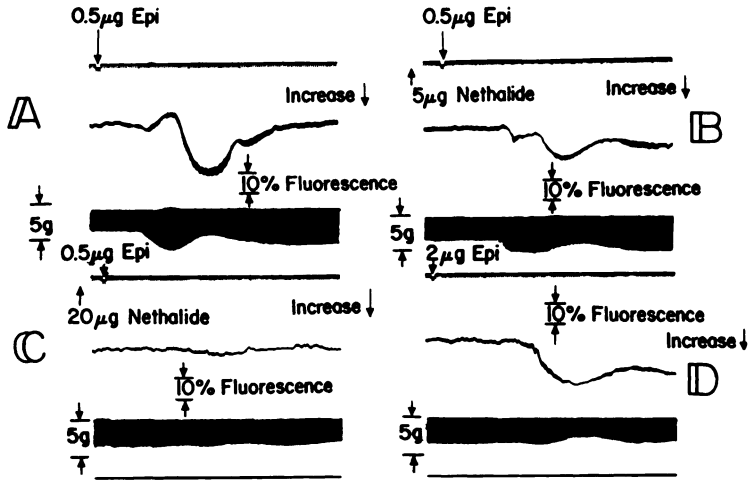

FIG. 11. *Effect of Nethalide on the fluorescence and contractile force response to epinephrine* See text for perfusion details.

Figure 11A shows the normal response of the tissue fluorescence and contractile force to 0.5 μg epinephrine in a heart perfused with nonrecirculating medium containing 5 mM glucose. After an initial small decrease, the fluorescence increased to a maximum value 21% greater than the initial steady state level 34 sec after the onset of the epinephrine effects, and the contractile cence increase was recorded. A tentative interpretation of this result, on the basis of the fluorescence increase being due to cytoplasmic NADH, is that the glycogenolytic effect was not fully blocked with the higher epinephrine dose. Other experiments with different concentrations of Nethalide and epinephrine showed that between 10 and 50 times as much Nethalide

as epinephrine was needed for the complete inhibition of both effects.

DISCUSSION

Fluorescence techniques have been applied to the measurement of the state of oxidation-reduction of the pyridine nucleotides in a number of whole-cell or intact-tissue preparations (3, 16–18). The main advantage of this method is that it provides a kinetic monitor of pyridine nucleotide changes without the need for disrupting the tissue. In principle, a whole series of metabolic transitions may be imposed successively on the system, and the effects of metabolic stimuli involving dehydrogenase reactions can be measured in much the same way as electrical or physical events. A limitation of the method is that there is no means of determining directly which particular dehydrogenase reaction is being affected or whether the fluorescence change is due to NADH or NADPH. However, the use of metabolic inhibitors provides a means of locating the site of action of the pyridine nucleotide change, and confirmation may be achieved by analysis of the tissue for critical intermediates.

Results reported in this paper show that the characteristic response of the perfused rat heart to epinephrine is a small decrease of fluorescence, occurring simultaneously with the onset of the increased force of contraction and oxygen consumption, followed by a transient larger fluorescence increase 30–35% as great as the anoxic response. These changes are interpreted in terms of separate responses of the pyridine nucleotides in the mitochondrial and cytoplasmic spaces, with the initial fluorescence decrease corresponding mainly to an oxidation of the mitochondrial component, and the subsequent fluorescence increase being due to an increase of cytoplasmic NADH. The studies presented here confirm and extend those previously published (4, 5).

Inhibition of NAD reduction at the glyceraldehyde-3-P dehydrogenase step by iodoacetate has allowed the mitochondrial changes to be observed without interference from the cytoplasmic component. Under these conditions, the rapid changes of the pyridine nucleotides toward the more oxidized state with each heart beat, and the slower sustained change to a more oxidized state with epinephrine, are reminiscent of the changes produced in excised frog sartorius muscle at different rates of stimulation (19). Further studies with frog and toad muscles (20–23) showed that, with low rates of stimulation, there was an increased oxidation of cytochrome *b* in addition to pyridine nucleotide; and by analogy with studies on respiratory control in isolated mitochondria (24), the conclusion was reached that the changes were due to stimulation of mitochondrial respiration by ADP and phosphate released during the muscle twitch. A similar conclusion would appear to be true for the rat heart and is supported by the observation that the total content of ADP and phosphate increases from the onset of the increased respiratory and mechanical activity during epinephrine stimulation (5, 25). The possibility of calcium ions competing with ADP for the respiratory carrier during heart contractions has been discussed by Chance (26), but the relative effectiveness of increases of ADP and Ca^{++} *in vivo* in causing a stimulation of respiratory activity remains an open question since the true intracellular concentrations of both ADP and Ca^{++} appear to be much lower than those obtained from calculations based on total tissue content (20, 21).

The pronounced change of the pyridine nucleotide toward the more oxidized state, with a diminution of the subsequent pyridine nucleotide reduction in glycogen-depleted hearts and in hearts perfused with acetate or pyruvate as substrates, lends support to the concept that the epinephrine induced cycle of fluoresence increase is due to the rapid reduction of cytoplasmic NADH by glyceraldehyde-3-P dehydrogenase, followed by reoxidation of the NADH by cytoplasmic dehydrogenases. In hearts perfused with acetate or pyruvate, the level of glycogen is the same or even greater than when glucose is the sole substrate, but flux through the glycolytic path-

way is greatly impaired (12). There is thus less of an imbalance between the rate of NADH production and its removal, and the transient NADH accumulation is of lesser magnitude.

An alternative possible explanation for the observed epinephrine-induced NADH increase is that it is due to tissue hypoxia. This is considered unlikely for the following reasons. First, the fluorescence increase, but not the diminution in tissue pO_2, is abolished if hearts are perfused in the presence of iodoacetate for a short time prior to the addition of epinephrine. Reduction of mitochondrial NAD is not inhibited by iodoacetate per se as shown by the large fluorescence increase during nitrogen anoxia. Hence, the fluorescence increase with epinephrine would seem to be caused by a pyridine nucleotide reduction only in the extramitochondrial compartment. Second, the observation that the pyridine nucleotide change under favorable redox conditions is initially toward a more oxidized state, at the same time as the tissue pO_2 is falling, excludes the possibility of hypoxia as a primary event. If the primary metabolic effect of epinephrine was the development of hypoxia, there would be a simultaneous reduction of pyridine nucleotide in both cytoplasmic and mitochondrial spaces with no oxidative phase. Third, the lowest plateau level of tissue pO_2, after epinephrnie, is not low enough to produce appreciable intracellular hypoxia since it does not fall below the critical level for induction of a fluorescence increase during nitrogen anoxia.

The changes in the ratio of lactate/pyruvate during the first 60 sec of epinephrine action confirm, at least qualitatively, that cytoplasmic NADH increases during the period of maximum fluorescence increase. Cytoplasmic redox couples provide an accurate monitor of the ratio of NADH to NAD only if each of the enzymes catalyzing the different NAD-linked reactions remains close to equilibrium as the flux changes. With a decreased glycolytic flux, caused by the addition of acetate or pyruvate to perfusates containing glucose, a reasonably consistent relationship was obtained between lactic dehydrogenase and α-glycerophosphate dehydrogenase (12). In the present experiments, in which the flux increased greatly, α-glycerophosphate dehydrogenase clearly was not sufficiently active to remain near equilibrium during the flux transition.

The determination of the total amount of reduced pyridine nucleotides in the cell, by analytical procedures (Fig. 9 shows that NADH increased rapidly about 10 sec after the onset of the epinephrine effect on the contractile process, to give a peak at 30 sec, and diminished in an oscillatory manner, reaching a secondary peak at about 70 sec. The kinetics of the NADH changes have not been investigated further; hence, it is probably premature to speculate about the significance of the small sceondary peak. However, the kinetics would seem to indicate that the coupling of the oxidation-reduction reactions may be more complicated than interaction solely between glyceraldehyde-P dehydrogenase and lactic dehydrogenase although these are probably the most important steps. NADPH increased to a lesser extent than NADH and reached a maximum after about 50 sec. The increase of NADPH may represent a transient stimulation of the pentose-phosphate pathway, due to an increase of glucose-6-P levels, although the activity of this pathway is very low in rat heart (27, 28). Other possibilities are that the changes reflect an increase of flux through the malic enzyme or the NADP-specific isocitrate dehydrogenase. However, it was not possible to correlate the increase in the ratio of NADPH/NADP with changes in the ratios of malate/pyruvate or isocitrate/α-ketoglutarate.

The analytical determinations of the NADH content allow a rough estimate of the percentage reduction of the cytoplasmic NAD. Klingenberg (29) has shown that, of the total NAD in the heart, about 53% is in the cytoplasm. Since the cell contains about 4.0 μmoles/g dry wt NAD (27), it may be calculated that, if all the observed NADH increase is indeed cyto-

plasmic, one-half to one-third of the cytoplasmic NAD is reduced during maximum epinephrine stimulation.

The concentration of epinephrine, used for the studies in which the tissue levels of reduced pyridine nucleotides were increased, was approximately maximal, as seen from the dose-response relationship (Fig. 10). Half-maximal effects on the force of contraction were obtained with epinephrine doses in the range 0.02–0.05 μg while 0.08–0.1 gave a half-maximal fluorescence increase. The force of contraction increased appreciably with the lowest amount used (5×10^{-4} μg), but threshold concentration for an increase of fluorescence was 20-fold greater. Both the force of contraction and the fluorescence responses were inhibited by Nethalide, a β-receptor blocking agent, but with different sensitivities. These studies are in agreement with previous suggestions (1, 30, 31) that in cardiac muscle the response of the contractile mechanism is more sensitive to epinephrine than the steps leading to glycogen breakdown. In addition, the results indicate that there is a temporal separation between the two effects of epinephrine, with the inotropic action preceeding the glycogenolytic by 10–15 sec. This conclusion is in agreement with analytical data, describing the changes of the glycolytic intermediates in hearts after epinephrine treatment, presented in the accompanying paper (25), where the significance of the observations in relation to the kinetics of cyclic AMP and phosphorylase changes are discussed.

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