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REDUCTION OF PYRIDINE NUCLEOTIDES BY SUCCINATE IN TISSUE SLICES OF LIMA BEAN HYPOCOTYL

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Abstract—Reversed electron transport in tissue slices of lima bean hypocotyl was studied by fluorometry. The fluorescence emission spectrum at 450 nm, after excitation at 350 nm, is presumed to be due to bound NADH. Succinate (5 mM at pH 7·5) increased fluorescence of the tissue slice about 30 per cent. A further 25 per cent increase in fluorescence of the tissue slice was obtained on addition of a solution of 5 mM succinate and 1·5 mM ATP. The increase in fluorescence of the tissue slice usually observed upon addition of succinate, was blocked by 5×10^{-8} M rotenone, when rotenone was pre-incubated with the tissue slice prior to treatment with succinate. A solution of 6×10^{-5} M 2,4-dinitrophenol (DNP) and 5 mM succinate did not increase fluorescence of the tissue slice. These data are taken as evidence for reversed electron transport in bean hypocotyl tissue.

INTRODUCTION

In animal mitochondria[1–3] and tissues,[4,5] there is a reduction of bound pyridine nucleotides on addition of exogenous succinate. Considerable evidence supports the conclusion that this type of reduction involves reversed electron transport within the respiratory chain, driven by high-energy intermediates.[6,7] Although the function of reversed electron transport in metabolism is unknown, it could possibly be a major source of reduced pyridine nucleotides utilizable in pyridine nucleotide-dependent metabolic processes, and therefore be of universal biological significance.[8]

In plants, however, reversed electron transport has been reported once, and only in isolated mitochondria.[9] Therefore, it is of interest to determine whether reversed electron transfer occurs in intact plant cells. These experiments present evidence that bound NAD is reduced by succinate in bean hypocotyl slices and that this reduction represents reversed electron transport mediated by a high-energy intermediate.

We made a survey of various plant tissues, including roots of sweetpotato and turnip, tuber of white potato, coleoptiles of corn and barley, stem of asparagus, and hypocotyls of pea, soybean, peanut, green bean and lima bean, to determine their suitability for studies of reversed electron transport. Etiolated lima bean hypocotyl was chosen because the tissue

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contains no pigment which interferes in the measurement of fluorescence, and also because it has a relatively high content of pyridine nucleotides.

RESULTS

Validity of Instrumentation: Fluorescence Emission of Free and Bound NADH

The instrumentation system used to obtain the fluorescence excitation spectra in these experiments is shown in Fig. 1. The instrument was designed to determine fluorescence by

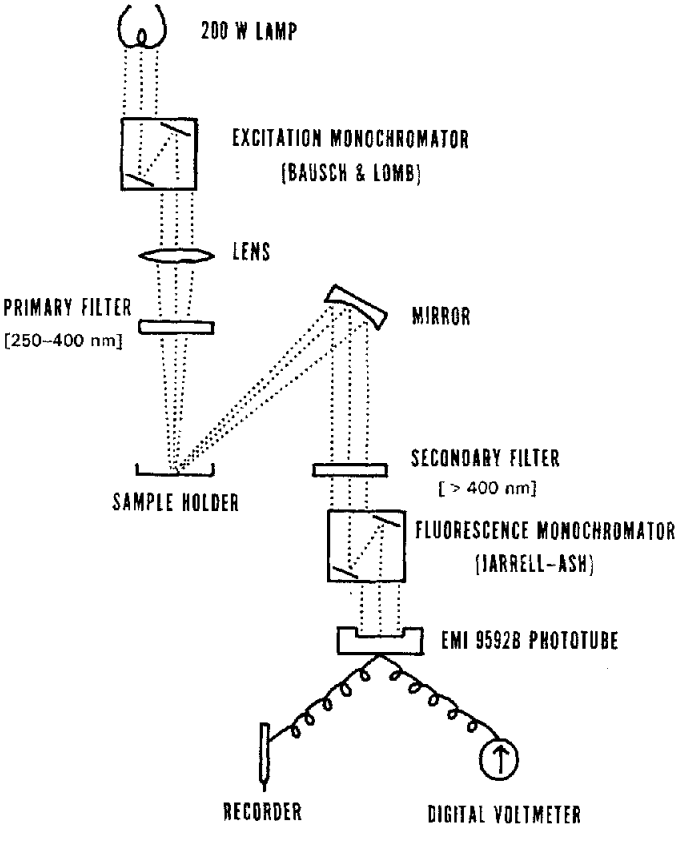

FIG. 1. ESSENTIAL PARTS OF THE FLUOROMETER.
Details are presented in the Experimental section.

reflectance. This technique was considered more suitable than conventional methods for determining fluorescence in 2 mm slices of plant tissues which contain relatively low concentrations of pyridine nucleotides.

The validity of the fluorescence spectra obtained with this instrument was studied with samples of free and bound NADH. The fluorescence spectrum of a solution of NADH excited with radiation at 350 nm, shows a broad band with two apparent maxima at approximately 440 nm and 470 nm (Fig. 2). The maximum at 470 nm is somewhat greater than the one at 440 nm. However, addition of bovine serum albumin, to bind the pyridine nucleotide, caused an increase in fluorescence and a shift of the maximum. The band at 440 nm was now

slightly higher than the one at 470 nm (Fig. 2). Duysens and Kronenberg[10] also recorded a broad fluorescence spectrum for NADH in solution in the 430–500 nm range, with the maximum at about 470 nm. Their spectrum is reminiscent of the spectrum shown in Fig. 2A. These workers also reported that the reaction of NADH with yeast alcohol dehydrogenase caused an increase in fluorescence intensity, and an approximate 20 nm shift of the fluorescence maximum to shorter wavelength. Boyer and Theorell[11] obtained similar results after complex formation between NADH and pyridine nucleotide dehydrogenases.

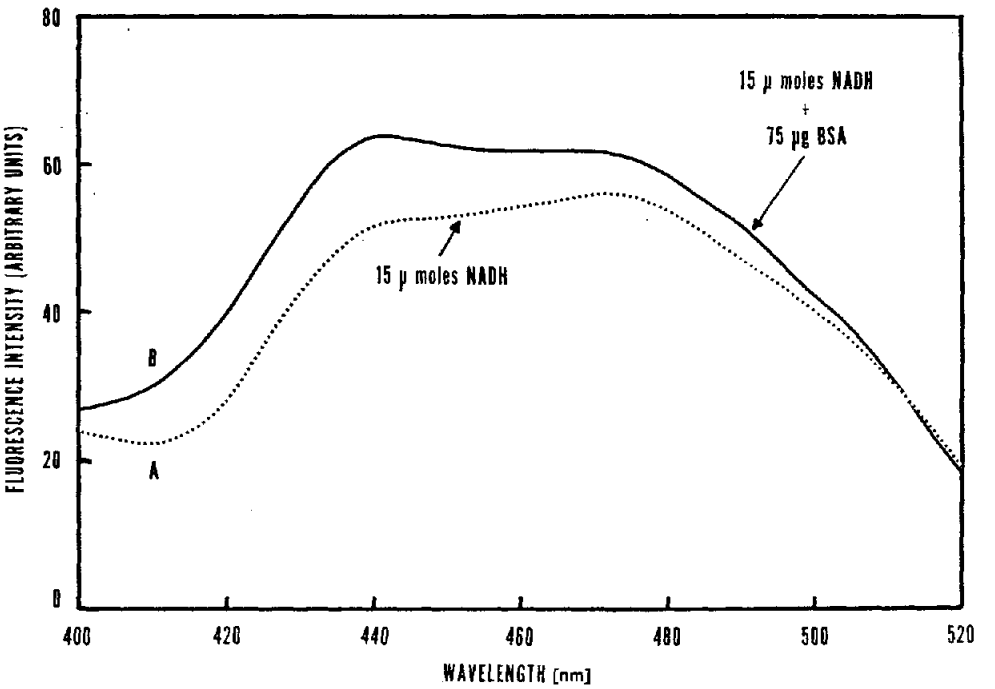

FIG. 2. FLUORESCENCE EMISSION SPECTRA OF (A) 0·2 ml SOLUTION OF 15 μ moles NADH AND (B) AFTER ADDITION OF 0·1 ml SOLUTION OF 75 μg BOVINE SERUM ALBUMIN (BSA) TO (A).

Excitation was effected with radiation at 350 nm.

The increase in fluorescence intensity and shift of the fluorescence maximum of NADH, after binding with bovine serum albumin (Fig. 2), is not as great as that observed with the NADH-linked dehydrogenases. This is to be expected since the binding of NADH to an NADH-linked dehydrogenase takes place at a specific site on the enzyme which apparently forms a complex with better fluorescence characteristics. When alcohol dehydrogenase was reacted with NAD^+ in the presence of ethanol, the fluorescence spectrum obtained showed a sharp peak at 440 nm (Fig. 3). This fluorescence spectrum is similar to that observed by Duysens and Kronenberg.[10]

When the fluorescence spectrum for sweetpotato (Ipomoea batatas (L.) Poir. Var. Yellow Jersey) mitochondria or a lima bean hypocotyl slice was recorded in this laboratory, after

[10] L. N. M. DUYSENS and G. H. M. KRONENBERG, *Biochim. Biophys. Acta* **26**, 437 (1957).
[11] P. D. BOYER and H. THEORELL, *Acta Chem. Scand.* **10**, 447 (1956).

excitation with light at 350 nm, the maximum appeared at 450 nm. There is therefore agreement in the maximum fluorescence (450 nm) obtained after excitation with 350 nm, between sweetpotato mitochondria, a lima bean hypocotyl slice, and rat-liver mitochondria.[12] Chance and Baltscheffsky[12] attributed this fluorescence maximum to bound NADH, and we likewise consider this fluorescence to be associated with bound NADH in the tissues and mitochondria. Our instrumentation system (Fig. 1) therefore, responds to fluorescence of pyridine nucleotides in the expected manner.

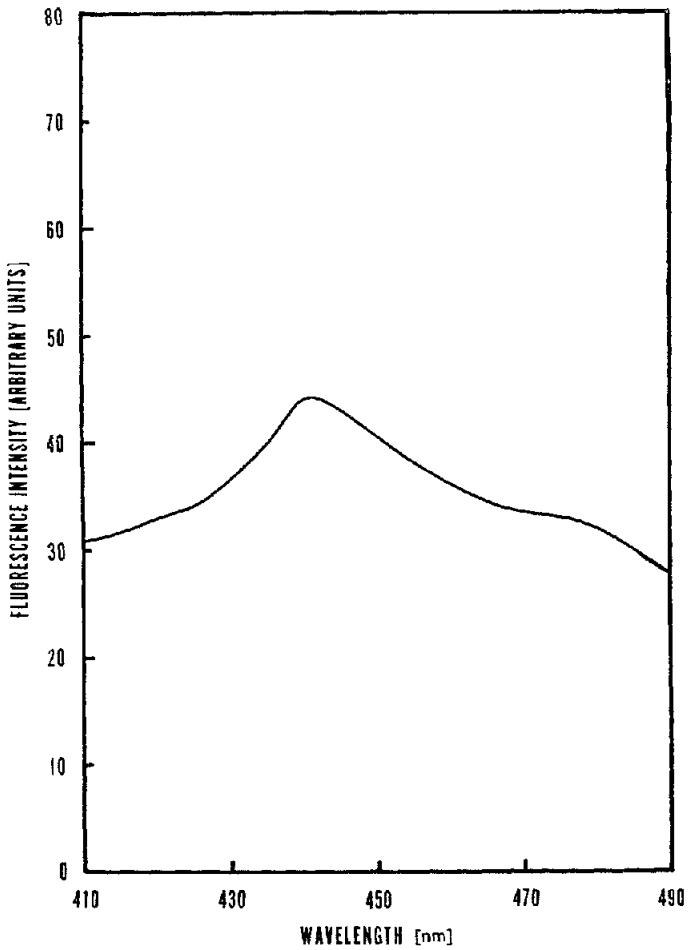

FIG. 3. FLUORESCENCE EMISSION SPECTRUM OF NADH FORMED BY YEAST ALCOHOL DEHYDROGENASE AFTER 1 min.

Solution contained 5 μmoles NAD^+, 0·5 mg alcohol dehydrogenase (approximately 1·5 units), and 0·1 ml of 5 per cent ethanol. Excitation wavelength was 350 nm.

Characteristics of Fluorescence Emission of a Lima Bean Hypocotyl Slice

The fluorescence excitation spectrum of a bean hypocotyl slice was taken in response to various excitation wave-lengths, to find the maximum response induced by a given excitation

[12] B. CHANCE and H. BALTSCHEFFSKY, *J. Biol. Chem.* **233**, 736 (1958).

wavelength in this instrumentation system. A range of excitation energy from 330 nm to 360 nm was used and the fluorescence emission recorded at 450 nm, the approximate region in which bound NADH fluoresces maximally.[11] The excitation spectrum shown in Fig. 4 has been corrected for the energy distribution of the light source, and for the excitation monochrometer and filter characteristics. Maximum fluorescence, at 450 nm, was obtained

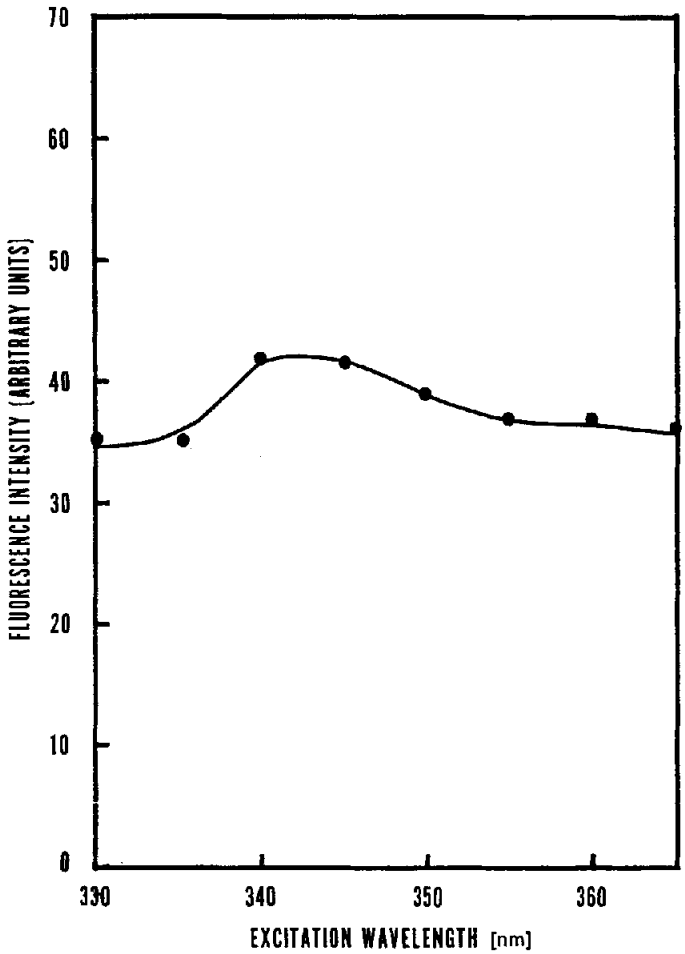

FIG. 4. FLUORESCENCE EXCITATION SPECTRUM AT 450 nm, OF A SLICE OF LIMA BEAN HYPOCOTYL. Corrections were made for the energy distribution of the light source, excitation monochromator and filter (250–400 nm) in the excitation beam.

with an excitation wave-length of about 340 nm (Fig. 4). However, although 340 nm irradiation was most effective in exciting fluorescence in this system, our experiments were performed with excitation at 350 nm to conform with most previous experiments of this kind.[5, 12] Furthermore, the difference in fluorescence caused by excitation at 340 nm and 350 nm is small (Fig. 4).

Figure 5 shows the difference spectrum in fluorescence emission, after excitation at 350 nm, between a slice of lima bean hypocotyl equilibrated in oxygen and in nitrogen. The tissue

slice was first washed in 250 mM sucrose solution and bubbled with oxygen for 50 min before the "oxygen spectrum" was obtained. The same slice was again placed in 250 mM sucrose solution and bubbled with nitrogen for 20 min before the "nitrogen spectrum" was obtained. As a result of bubbling with nitrogen, there was a general increase in fluorescence of about 25 per cent throughout the wavelength region studied. However, in the 450 nm region the difference spectrum showed a maximum which was about 7 per cent above average background. This peak is taken to be due to reduced pyridine nucleotides which are known to fluoresce maximally at 450 nm after excitation by light in the 350 nm region of the spectrum.[12]

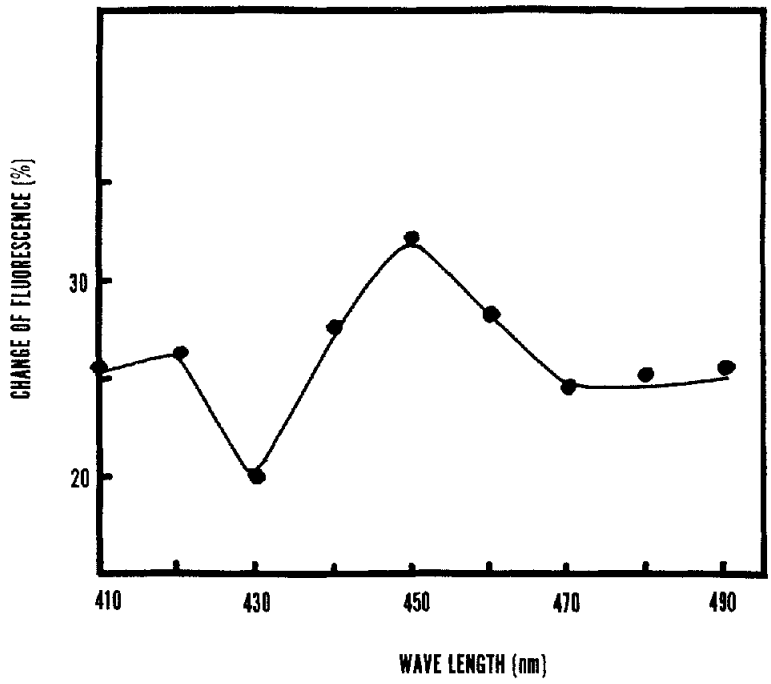

FIG. 5. DIFFERENCE SPECTRUM OF FLUORESCENCE EMISSION BETWEEN AN AEROBIC AND ANAEROBIC SLICE OF LIMA BEAN HYPOCOTYL, AFTER EXCITATION BY LIGHT AT 350 nm.

Effect of Added Succinate

Addition of succinate to a bean hypocotyl tissue slice, after washing in sucrose for 40 min, increased fluorescence at 450 nm. The extent of increase depended on the concentration of succinate (Fig. 6). The same slice was used for successive 1 min treatments with different concentrations of succinate, and the slice was washed for 10 min with 250 mM sucrose solution after the fluorescence reading was taken for each concentration treatment. At low concentrations of succinate, in the range 5–10 mM, fluorescence increased linearly. However, at concentrations higher than 10 mM succinate, the increase in fluorescence leveled off and was no longer proportional to the concentration of succinate (Fig. 6). This fluorescence increase caused by addition of succinate to the lima bean hypocotyl slice could be due to reduction of NAD^+ as a result of reversed electron transport. To test this hypothesis the experiments decribed below were performed with ATP, DNP, and rotenone.

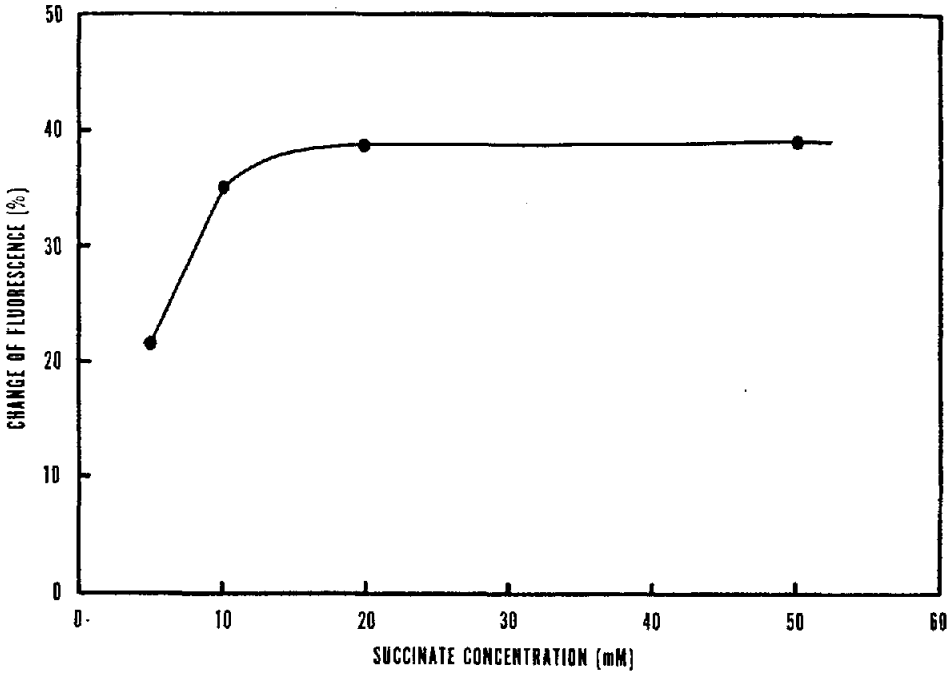

FIG. 6. EFFECT OF SUCCINATE ON THE FLUORESCENCE OF LIMA BEAN HYPOCOTYL SLICE AT 450 nm. A 100 per cent fluorescence change is that obtained in tissues after 40 min washing, immediately after cutting, followed by 10 min bubbling in N_2 in a solution containing 25 mM succinate.

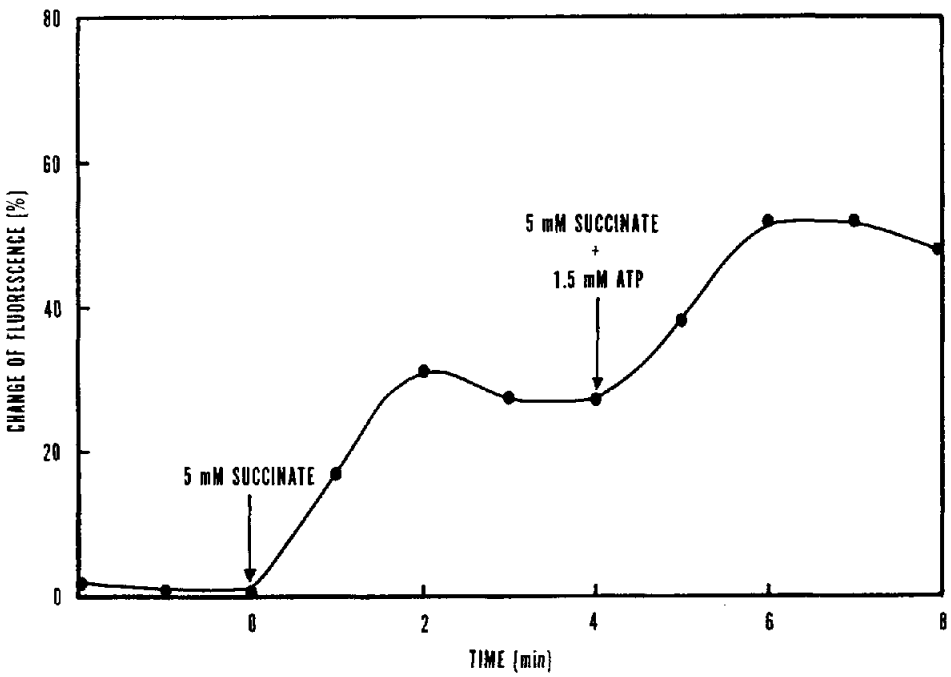

FIG. 7. FLUORESCENCE RESPONSE OF THE TISSUE SLICE OF LIMA BEAN AT 450 nm TO TREATMENTS OF 5 mM SUCCINATE, AND 5 mM SUCCINATE+1·5 mM ATP.
The change of fluorescence is expressed the same as in Fig. 6.

Effect of Added ATP

The kinetics of the fluorescence changes of the tissue slice, induced by treatment with either succinate or succinate plus ATP, are shown in Fig. 7. After washing for 40 min in 250 mM sucrose, the tissue slice was treated with 5 mM succinate solution to give a fairly high rate of increase of fluorescence (Fig. 6). The fluorescence increased immediately upon addition of succinate, and reached a steady state within 2 min (Fig. 7). Then, the slice was treated with a solution containing 5 mM succinate and 1·5 mM ATP and the fluorescence again read at 1-min intervals (Fig. 7). The increase in fluorescence after 2 min was about 25 per cent, as a result of treating with succinate and ATP, after achieving a steady state with succinate alone. This latter increase with ATP and succinate, is considered presumptive evidence of energy-dependent reduction of NAD^+ (reversed electron transport). The initial increase with succinate alone may be due to both reversed electron transport, utilizing endogenous high

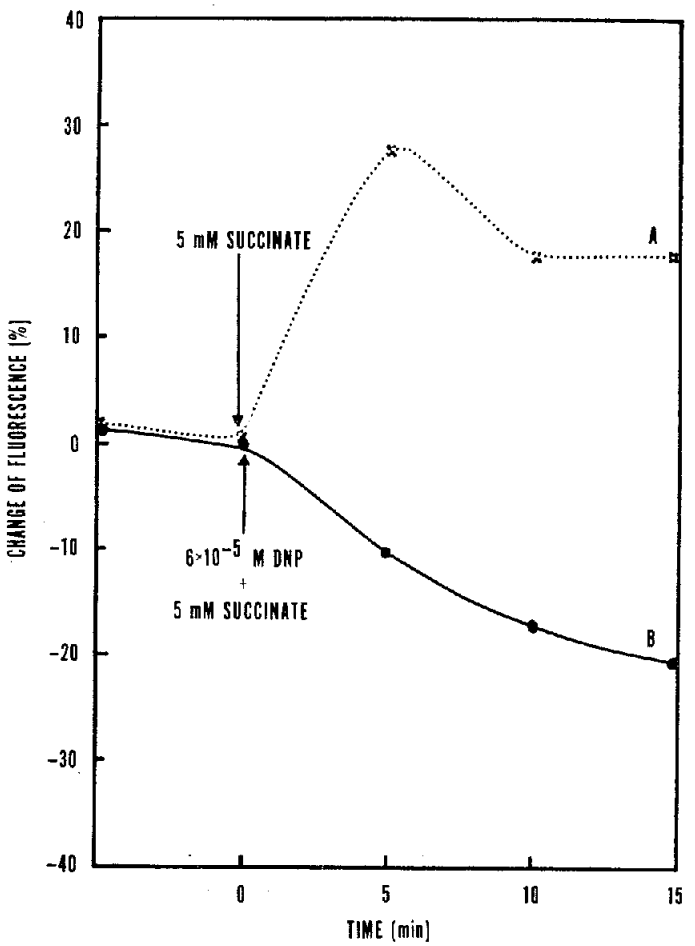

FIG. 8. FLUORESCENCE RESPONSE OF THE TISSUE SLICE OF LIMA BEAN HYPOCOTYL AT 450 nm TO TREATMENTS OF (A) 5 mM SUCCINATE ALONE, AND (B) 6×10^{-5} M DNP+5 mM SUCCINATE. The change of fluorescence is expressed the same as in Fig. 6. Changes in fluorescence at 450 nm caused by DNP alone, were negligible at the concentrations used in these experiments.

energy intermediates, and the reduction of NAD^+ by malate derived from succinate. The further increase in fluorescence resulting from addition of ATP plus succinate implicates a high energy intermediate as the driving force for the reduction of NAD. However, ATP alone at concentrations of 1·5 to 5 mM did not cause reduction of NAD^+. This indicates that either insufficient endogenous electron flow is available for reverse electron transport to NAD^+, or that NADH-linked pathways oxidize NADH faster than it is formed by ATP-driven NAD^+ reduction, in the absence of exogenous succinate.

Influence of Inhibitors

To further demonstrate that reduction of NADH by succinate in the bean hypocotyl slice may be due to reversed electron transport, the uncoupling agent, 2,4-dinitrophenol (DNP), was employed. Since DNP is known to inhibit oxidative phosphorylation without inhibition of respiration, DNP should inhibit reversed electron transport by discharging the high energy intermediate which drives the electrons backwards in the respiratory chain. The time course of fluorescence change of the tissue slice due to treatment with 6×10^{-5} M DNP is shown in Fig. 8. Whereas 5 mM succinate alone induced a rapid increase in fluorescence (about 30 per cent) which then decreased slowly to a plateau value (about 20 per cent) after 10 min, (Fig. 8A), when DNP was added with succinate, fluorescence decreased rapidly and continuously (Fig. 8B). DNP treatment decreased fluorescence about 20 per cent in 15 min.

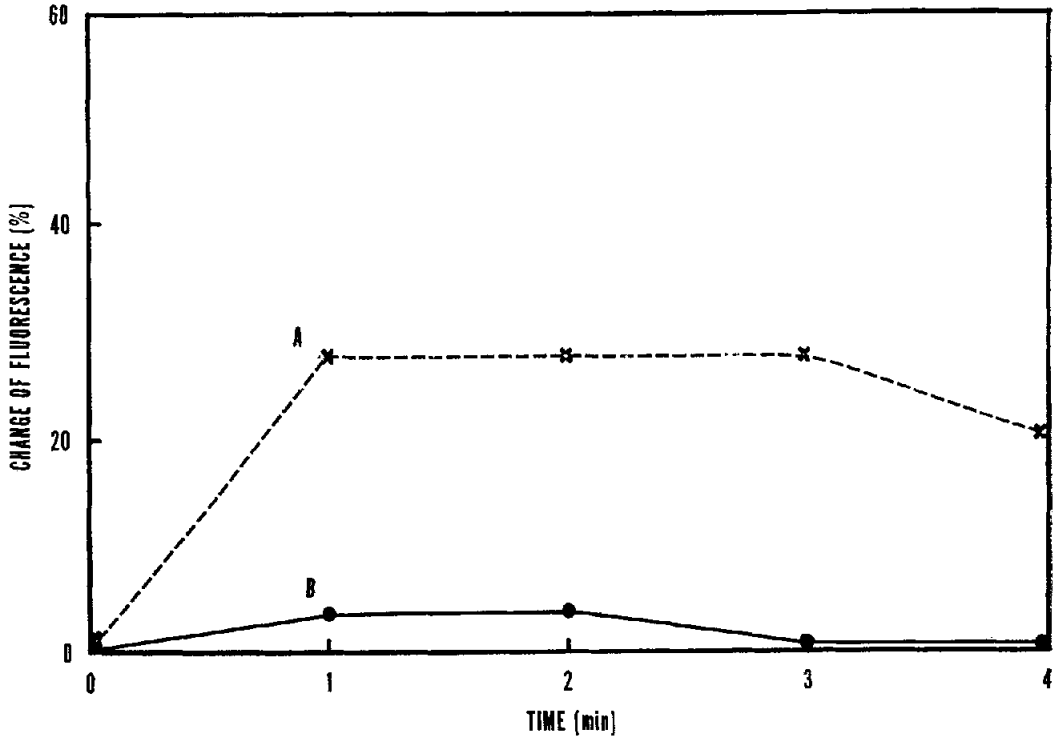

FIG. 9. FLUORESCENCE RESPONSE OF THE TISSUE SLICE OF LIMA BEAN AT 450 nm TO TREATMENTS OF (A) 5 mM SUCCINATE AND (B) 5 mM SUCCINATE AFTER 10 min PRE-INCUBATION WITH 5×10^{-8} M ROTENONE.

The change in fluorescence is expressed as in Fig. 6.

The decreased fluorescence observed upon treatment with DNP may be caused by increased electron flow to oxygen (respiration) with elimination of concomitant energy production (uncoupling), resulting in a lowering of the steady state level of reduced pyridine nucleotides. Thus it appears that reduction of NAD^+ by succinate is energy-linked and is dependent on oxidative phosphorylation.

Further evidence implicating reversed electron transport in this system was obtained when rotenone, which inhibits electron transport between NADH and the flavoprotein-cytochrome b region of the respiratory chain,[13, 14] was used to prevent electron flow. The increase in fluorescence caused by succinate alone is shown in Fig. 9A. When the tissue was incubated with rotenone for 10 min followed by succinate treatment, there was no increase in fluorescence after the initial rise due to endogenous electron flow to pyridine nucleotides (Fig. 9B). These results indicate that rotenone blocks reversed electron flow from succinate to NAD in the tissue slice.

DISCUSSION

Addition of succinate to slices of etiolated bean hypocotyl causes an increase in fluorescence at 450 nm, in response to excitation at 350 nm, which is the type of fluorescence associated with reduced pyridine nucleotides. The fluorescence was shown to be influenced by O_2 and N_2 atmospheres and by rotenone and DNP. Changes in tissue fluorescence after reaction with DNP or succinate and ATP indicates that the fluorescence is energy dependent. The magnitude of the change in fluorescence in response to succinate is of the order of 20–30 per cent increase. These data support the conclusion that an energy-linked reversed electron transport pathway exists in these tissues.

Although reversed electron transfer is readily observed in isolated mitochondria from animal tissues, we could not observe reversed electron transfer in isolated plant mitochondria. Furthermore, we could not induce reversed electron transport in the tissue slice by ATP alone. Therefore, it is possible that the reversal of electron transfer involves a more delicate system in plant tissues, which is disturbed during extraction of mitochondria. However, Bonner[9] did observe reversed electron transfer in mitochondria isolated from mung bean seedlings.

It was not possible to observe reversed electron transport in sweetpotato root, turnip, or white potato tuber tissue slices, but reversed electron transport was observed in seedling tissues. This may signify that the phenomenon of reversed electron transport in plants is characteristic of young tissues. The physiological significance of reversed electron transport is yet to be determined.

EXPERIMENTAL

Lima bean seeds (Phaseolus lunatus L. var. Fordhook 242), were sterilized by wiping with paper toweling saturated with Clorox bleaching solution (approx. 5 per cent NaOCl). The seeds were germinated at 25° for 4 days in the dark, using the rolled towel technique.[16] A cylinder of tissue 5 mm in dia. was made by boring the hypocotyl of the germinated bean, with a No. 2 cork borer, and slices 2 mm thick were then cut with a razor blade. The slice was washed for 40 min by stirring gently with a magnetic stirrer in 250 mM sucrose solution, to reach a stabilized fluorescence reading. The slice was then transferred to an aluminium sample holder, which contained a circular indentation, 2 mm deep and 5 mm dia., for the sample. The sample was illuminated with light of 350 nm and fluorescence emission was read from 400 to 500 nm, to obtain the fluores-

[13] L. ERNSTER, G. DALLNER and G. F. AZZONE, *J. Biol. Chem.* **238**, 1124 (1963).
[14] H. IKUMA, *Science* **158**, 529 (1967).
[15] Y. YAMAMOTO, *Plant Physiol.* **38**, 45 (1963).
[16] B. M. POLLOCK and V. K. TOOLE, *Plant Physiol.* **41**, 221 (1966).

cence emission spectrum. Care was taken to position the sample exactly as in the previous measurement when successive readings were made on the same sample.

All reagents were prepared with 250 mM sucrose solution, and the solution was adjusted to pH 7·5. Treatments with reagents were carried out by washing the tissue slice in a solution of the reagent for various lengths of time with gentle stirring. After specific reaction times the fluorescence emission spectrum of the treated slice was taken.

Solutions of rotenone, 10^{-5} M, were prepared in 95 per cent alcohol, for use on the same day. Final concentration of rotenone was 5×10^{-8} M in 250 mM sucrose containing 2 per cent ethanol. All other reagents were commercial products and were used as received.

Further experimental details are given in the legends of figures. The fluorescence characteristics of all reagents were determined to account for any contribution to the total fluorescence of the combined reagent and treated sample.

Instrumentation

Essential parts of the fluorometer built for these experiments are illustrated in Fig. 1. A quartz iodide lamp (200 W) was used as a source of radiation energy to excite reduced pyridine nucleotides in the sample. The lamp was operated from a regulated power supply to obtain a stable light output. A Bausch and Lomb monochromator with grating, 2700 grooves/mm, equipped with a Corning 7-54 filter (250–400 nm) was used for selecting the excitation wavelength.

A mirror reflected the fluorescence emitted by the sample onto the fluorescence monochromator. The fluorescence monochromator used to obtain the fluorescence emission spectra was a Jarrell-Ash grating model 82-410 with Optical Technology Cut-Off filter (< 400 nm). An EMI 9592B phototube was installed to measure incident intensity of fluorescence. Either a Varian G-10 graphic recorder or a USC digital DC voltmeter model Z-200A, was used as the measuring device for recording or reading the fluorescence.

This fluorometer can detect a concentration of $7{\cdot}5 \times 10^{-10}$ moles of NADH solution, or 6×10^{-12} moles o NADH formed by the yeast alcohol dehydrogenase system, which is assumed to be bound NADH.[10] We estimate that about 1 to 5×10^{-10} moles of bound pyridine nucleotide is present in a bean hypocotyl slice 2 mm thick and 5 mm dia.[15]

Acknowledgements—We are grateful to Mr. K. H. Norris of the Instrumentation Research Laboratory, Plant Industry Station, Beltsville, Maryland, for constructing the spectrofluorometer and for many helpful suggestions. We are also grateful to Mrs. V. K. Toole for providing facilities for germinating the lima bean seeds.