The Photo-oxidation of Succinate by Chromatophores of *Rhodospirillum rubrum*

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1. The stoicheiometry of the photo-oxidation of succinate by chromatophores has been investigated with [2,3-$^{14}C_2$]succinate. It was found that there is a stoicheiometric relationship between the amount of succinate oxidized and the NAD reduced, and that fumarate is the only product of succinate oxidation. 2. The possibility of a direct hydrogen transfer from succinate to NAD in this reaction was investigated with tritiated substrates. With tritiated succinate less than 3% of the activity expected if direct hydrogen transfer occurred was recovered in the $NADH_2$, and this was due to contamination with the substrate. In experiments with tritiated water, $NADH_2$ was labelled, and had half the specific activity of the water, as expected if water was the source of protons. It was also found that chromatophores catalyse an exchange reaction between $NADH_2$ and water. 3. It is concluded that the exchange reaction makes it impossible to interpret these results as indicating either a hydrogen-transfer or an electron-transfer mechanism for the photoreduction reaction.

Frenkel (1958) showed that the oxidation of succinate by chromatophores from *Rhodospirillum rubrum* could be coupled to the reduction of NAD in a light-dependent reaction. He showed that succinate could be replaced by $FMNH_2$ and that in this case the $FMNH_2$ oxidized and the NAD reduced were stoicheiometrically related. Frenkel (1954) had previously shown that chromatophores phosphorylate ADP in the light. The photoreduction and photophosphorylation reactions have been interpreted by Arnon (1959) in terms of the cyclic and non-cyclic electron-flow mechanisms that he has proposed for green-plant photosynthesis. An alternative mechanism has been proposed by Bose & Gest (1963), in which a light-initiated cyclic electron-flow results in the formation of a 'high-energy' intermediate that may be used either for photophosphorylation or for photoreduction.

The mechanism proposed by Arnon (1959) involves a transfer of electrons, the protons from the electron donor being liberated into the medium on oxidation and other protons being taken from the medium on reduction of the final electron acceptor. The mechanism proposed by Bose & Gest (1963) might also involve a similar electron-transfer process; it is possible, however, that if this mechanism is correct a transfer of hydrogen atoms from the donor to the acceptor could occur, mediated by the 'high-energy' intermediate.

The possibility of a direct transfer of hydrogen can only be investigated in a photoreduction reaction in which the hydrogen of the hydrogen donor and hydrogen acceptor are not in equilibrium with the protons of the medium. The coupled photo-oxidation of succinate and photoreduction of NAD catalysed by chromatophores of *R. rubrum* fulfils this requirement. In an attempt to distinguish between hydrogen-transfer and electron-transfer mechanisms for photosynthesis this reaction has been investigated with ^{14}C- and ^{3}H-labelled substrates.

MATERIALS AND METHODS

Materials. Radioactive compounds were obtained from The Radiochemical Centre, Amersham, Bucks. [2,3-$^{14}C_2$]-Succinic acid was neutralized with NaOH and dissolved in water; [2,3-$^{3}H_2$]succinic acid was found to contain an impurity labile to freeze-drying, and this was removed by repeated freeze-drying before use. The acid was neutralized with NaOH and dissolved in water. Tritiated water was used as supplied. ADP and NAD (sodium salts) and muscle lactate dehydrogenase were obtained from C. F. Boehringer und Soehne, G.m.b.H., Mannheim, Germany. $NADH_2$ was from Sigma Chemical Co., St Louis 18, Mo., U.S.A. All other reagents were British Drug Houses Ltd. (Poole, Dorset) AnalaR grade, or May and Baker Ltd. (Dagenham, Essex) reagent grade. Tris was recrystallized from ethanol before use.

Growth of R. rubrum. Stock cultures were maintained in stab culture as described by Elsden & Ormerod (1956).

The medium used contained Marmite (0·3%), Oxoid peptone (1·0%), Difco yeast extract (0·5%), NaCl (0·5%) and agar (2%). Large-scale cultures were grown photosynthetically under semi-anaerobic conditions in the following medium: DL-malic acid, 2·7g., monosodium glutamate, 3·8g., Difco yeast extract, 2·0g., $(NH_4)_2HPO_4$, 800mg., K_2HPO_4, 500mg., KH_2PO_4, 500mg., $MgSO_4,7H_2O$, 200mg., and $CaCl_2$, 40mg./l. of distilled water, adjusted to pH6·8 with NaOH before autoclaving. Cultures were maintained in tubes containing 15ml. of this medium and transferred every 3–4 days. A tube (after 48hr. growth) was used to inoculate 1l. of medium in a Roux bottle, and the bottle filled to the neck with sterile distilled water. The cells were harvested after growth for 40hr. in the light at 25°.

Preparation of chromatophores. Chromatophores were prepared by a modification of the method of Geller & Lipmann (1960). Cells were harvested by centrifugation at 0°, washed once in 0·1M-tris–HCl buffer, pH7·5, and resuspended in the same buffer at 300mg. wet wt./ml. They were then disrupted by exposure to ultrasonic oscillation in a Mullard 500w 25kcyc./sec. ultrasonic generator. Cell debris was removed by centrifugation at $25000g_{av.}$ for 30min. in a Spinco model L preparative ultracentrifuge. The chromatophores were then sedimented by centrifugation at $105000g_{av.}$ for 30min., washed once and resuspended in the same buffer. They were stored unfrozen at 0° under nitrogen.

Chromatography and radioautography. The 80%-ethanol-soluble fraction obtained from experiments with ^{14}C-labelled substrates was analysed by chromatography on Whatman no. 4 paper washed as described by Knight (1962). Two-dimensional chromatograms were run in the phenol–water and butan-1-ol–propionic acid solvent systems of Benson *et al.* (1950).

Dicarboxylic acids were chromatographed one-dimensionally on unwashed Whatman no. 1 paper in the AnalaR pentan-1-ol–5M-formic acid system of Buch, Montgomery & Porter (1952). They were further identified by co-chromatography on a Celite column as described by Knight (1962).

Radioautographs were made by exposing the chromatograms to Ilford Industrial G X-ray film, the period of exposure being determined by the total radioactivity on the chromatogram. The films were developed in Ilford ID19 X-ray developer.

Paper electrophoresis. High-voltage paper electrophoresis in ammonium carbonate buffer, pH8·9 (7·9g./l.) (Gross, 1959), was used for the primary separation of succinate and fumarate. It was found that this buffer could also be used for the separation of NAD and $NADH_2$ from each other and from succinate and fumarate. Whatman 3MM paper 60cm. long was used with an applied voltage of 1600v. The paper was cooled between two aluminium blocks through which tap water circulated as coolant; it was insulated from the blocks with polythene sheeting.

Measurement of radioactivity. Radioactivity on chromatograms and electrophoresis strips was measured with a thin end-window tube (General Electric Co. Ltd., type 2B2) connected to a Panax D657 scaler.

The ^3H and ^{14}C–^3H mixtures were assayed by scintillation counting. The scintillation mixture used contained 4g. of 2,5-diphenyloxazole and 0·05g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene/l. of toluene. Succinate and fumarate samples dissolved in 50μl. of ethanol were added to 10ml. of this mixture for counting. Aqueous samples were counted in a mixture of 0·5ml. of water, 4·5ml. of ethanol and 5·0ml. of double-strength scintillation mixture. Succinate and fumarate samples and aqueous samples from experiments with tritiated succinate were counted with a Technical Measurement Corp. TMC model L P2A liquid-phosphor coincidence counter. External standards were used to estimate the counting efficiency with this counter. Aqueous samples from experiments with tritiated water were counted in a Packard Tri-Carb model 314EX. Internal standards of tritiated toluene were added to samples counted in this counter to estimate the counting efficiency.

Samples containing mixtures of ^{14}C and ^3H were counted in the TMC counter by using two counting channels, the amounts of each isotope in the sample being calculated from the counting efficiency for each isotope in the two channels.

Photophosphorylation. Photophosphorylation was assayed as described by Geller & Lipmann (1960). Warburg manometers filled with nitrogen were used as anaerobic vessels and were incubated at 30° in an illuminated water bath. Dark controls were carried out by wrapping the manometer in aluminium foil before mixing the contents of the side arm and main cup. The decrease in inorganic phosphate observed on illumination was used as a routine measure of photophosphorylation. That this represented the formation of ATP was confirmed with a luciferin–luciferase preparation from firefly tails (Strehler & Trotter, 1952).

Photoreduction. The photoreduction of NAD was followed spectrophotometrically in Thunberg cuvettes designed to fit the modified cell compartment of a Unicam SP.500 spectrophotometer. The 1cm. quartz cells with a graded seal (The Thermal Syndicate Ltd.) were fused to a glass extension with two side arms and a 'female' ground-glass joint, and the cell was sealed with a stopcock on a matching 'male' joint. The cells were made anaerobic by evacuating four times, each followed by flushing with oxygen-free nitrogen (British Oxygen Co. Ltd.). The gas was used without the removal of residual traces of oxygen.

After gassing, the contents of the cell and side arms were mixed and the extinction at 340mμ was read. The cell was then incubated at 30° in an illuminated water bath and the extinction read again to determine the NAD reduced. Extinctions were read against either dark-control cells or reaction mixtures without NAD.

Experiments with ^{14}C-labelled substrate. Experiments with [2,3-$^{14}C_2$]succinate were done in the anaerobic cuvettes by the procedure described under Photoreduction. The procedure used for the isolation of reaction products from experiments was based on that of Moses & Calvin (1958). After incubation the reaction mixture was poured rapidly into ice-cold ethanol to give a final ethanol concentration of 80% (v/v). After 15min. extraction the insoluble material was centrifuged off. The pellet was re-extracted with 10ml. of 80% ethanol for a further 15min. Extraction overnight did not increase the recovery of products. The supernatants from the extractions were combined, reduced to a small volume in a rotary evaporator at 30° and finally freeze-dried. The residue was extracted into 1ml. of water. After removal of insoluble material by centrifugation a portion was analysed by two-dimensional chromatography.

Experiments with ^{3}H-*labelled substrate: isolation of* $NADH_2$. The procedure used in experiments with tritiated substrates was developed for the isolation of $NADH_2$ from the reaction mixture; it was also used for the isolation of succinate, fumarate and water.

After incubation the reaction mixture was transferred with a Pasteur pipette to a Pyrex-glass centrifuge tube standing in a boiling-water bath. The tube was boiled for 2 min., then cooled in ice; 1·5 μmoles of carrier $NADH_2$ were added during the boiling. At the pH of the reaction mixture, pH 8·0, $NADH_2$ is stable to boiling but NAD is destroyed. The precipitated material was centrifuged off and the supernatant freeze-dried.

The freeze-drying apparatus included a double trap, cooled in a mixture of ethanol and solid CO_2, which was essential to prevent the escape of tritiated water into the laboratory; the trap was also used to recover water formed in experiments with tritiated succinate for counting. The reaction products were washed three times to remove exchangeable tritium by taking up in 3 ml. of water followed by freeze-drying. Finally they were taken up in 1 ml. of water and a sample was taken for isolation of $NADH_2$, succinate or fumarate by electrophoresis.

After electrophoresis $NADH_2$ was identified on the paper by its fluorescence under ultraviolet light. The strip of paper containing the $NADH_2$ was dried quickly in a stream of air, and eluted with water into a weighed counting vial. When elution was complete, as shown by the disappearance of fluorescence from the strip, the vial was reweighed and the volume of water made up to 0·7 ml. A portion (0·2 ml.) was then taken for extinction measurement to estimate the $NADH_2$ recovered. The identification of the $NADH_2$ was confirmed in some cases by the addition of acetaldehyde and alcohol dehydrogenase to the sample to oxidize the $NADH_2$, the fall in extinction at 340 mμ being followed. Ethanol and scintillation mixture were then added to the 0·5 ml. of sample remaining in the vial for counting.

The recoveries of $NADH_2$ by this procedure were variable, ranging from 36 to 65%. The losses occurred almost entirely during electrophoresis, the steps before this giving 95–100% recovery.

The electrophoresis gave recoveries of 75% from salt-free mixtures of NAD and $NADH_2$, but with experimental mixtures recoveries were lower owing to interference by salts. Under the experimental conditions no NAD was present on the electrophoresis owing to destruction by boiling at pH 8·0.

A control strip of paper was always taken from the electrophoresis paper from a position adjacent to the strip containing the $NADH_2$ and between it and the part containing the succinate and fumarate. In experiments with tritiated succinate streaking resulted in up to 1% of the total tritium used being recovered in the $NADH_2$. Considerably more tritium was always recovered in the control strip.

Analytical procedures. Inorganic phosphate was estimated by the method of Allen (1940). Bacteriochlorophyll was estimated by the method of van Niel & Arnold (1938) by using the extinction coefficient given by Lascelles (1956). L(+)-Lactic acid was estimated by a method similar to that of Wieland (1963). Yeast lactate dehydrogenase was prepared as described by Boeri, Cutolo & Luzzati (1955). The preparation was taken to the 65% saturated $(NH_4)_2SO_4$ precipitation stage. Lactate was assayed by following the reduction of ferricyanide in the following reaction mixture: $K_4Fe(CN)_6$, 2·0 μmoles; KH_2PO_4 buffer, pH 7·2, 200 μmoles; EDTA, 4·0 μmoles; L(+)-lactic acid, 0–1·0 μmole; enzyme, 0·03 ml. After incubation for 30 min. at 30° the extinction at 420 mμ was read and compared with a blank with no lactate. The extinction coefficient given by Appleby & Morton (1959) for ferricyanide was used.

RESULTS

Fig. 1 shows the photoreduction of NAD by chromatophores with succinate as substrate. The rate of photoreduction was between 19 and 27 μ-moles of $NADH_2$/mg. of bacteriochlorophyll/hr. with a succinate concentration of 6·6 mM. Decreasing the succinate concentration to 0·66 mM resulted in a halving of the rate of photoreduction. Similar

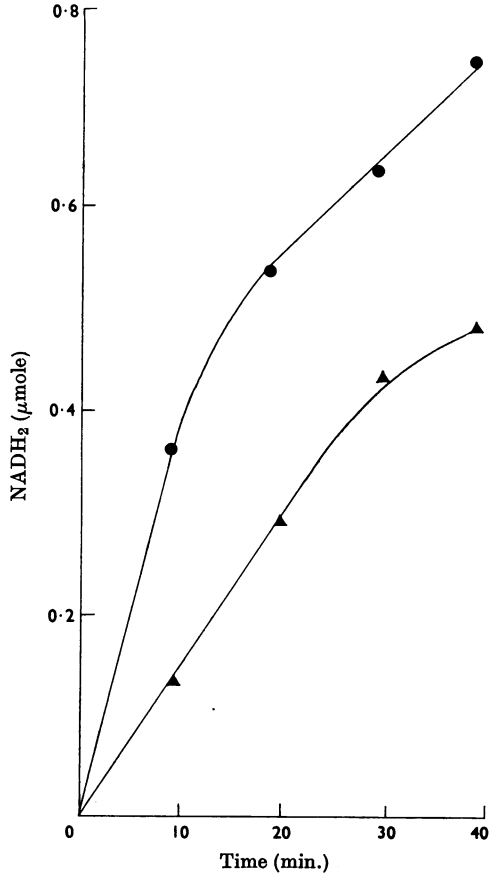

Fig. 1. Photoreduction of NAD. The reaction mixture contained (in 3 ml.): NAD, 2·0 μmoles; $MgCl_2$, 5·0 μmoles; tris–HCl buffer, pH 8·0, 100 μmoles; chromatophores, 50 μg. of bacteriochlorophyll; sodium succinate, 2·0 μmoles (●) or 20·0 μmoles (▲). Incubation was at 30° under oxygen-free nitrogen in the light.

rates were observed if a trapping system of muscle lactate dehydrogenase and pyruvate was used. The lower succinate concentration was used in experiments with isotopically labelled substrates to obtain high specific activities. The pH optimum of both photoreduction and photophosphorylation was pH 8·0. Chromatophores prepared at pH 8·0 were inactive; they were therefore prepared at pH 7·5 and assayed at pH 8·0. The photoreduction activity of the chromatophores fell by up to 50% on standing overnight and freshly prepared chromatophores were therefore used in all experiments with isotopically labelled substrates. The chromatophores catalysed photophosphorylation at rates between 250 and 300 μmoles of ATP/mg. of bacteriochlorophyll/hr.

[2,3-$^{14}C_2$]Succinic acid was used to investigate the oxidation of succinate by chromatophores. The experimental system used is shown in Table 1. After incubation the reaction products were analysed by two-dimensional chromatography. The only radioactive compounds present on the chromatograms were succinate and fumarate. The identity of the fumarate, the only product of the reaction, was confirmed by co-chromatography with authentic fumarate in the pentan-1-ol–formic acid solvent and on a Celite column. Dark-controls were run with all experiments. It was found that a small amount of fumarate was formed in the dark, and in most cases about 5% of the succinate was oxidized in the dark. It is possible that this oxidation in the dark was due to traces of oxygen remaining in the system, as Mr H. J. Somerville in this Laboratory has found that the nitrogen used in these experiments contains traces of oxygen. A measure of the amount of fumarate formed was obtained by counting the succinate and fumarate spots either on chromatograms or paper-electrophoresis strips. The percentage of the total counts in each was taken as a measure of the amount present in the reaction mixture. These were then compared with the $NADH_2$ or lactate formed in the light (Table 1).

These experiments show that the only product of succinate metabolism by chromatophores is fumarate and that there is a stoicheiometric relationship between the succinate oxidized and the NAD reduced in the light. An investigation of the hydrogen-transfer reactions involved in this system may therefore be made without interference from the further metabolism of the substrate.

$NADH_2$ formed in the light with [2,3-$^3H_2$]-succinate as substrate was isolated by paper electrophoresis (Table 2). In all cases the $NADH_2$ was found to be unlabelled, containing less than 3% of the activity expected if a direct hydrogen transfer occurred. In the dark-control experiments (Expts. 5 and 8 in Table 2) a similar activity was eluted with the $NADH_2$ although only carrier $NADH_2$ was present. Control strips of paper cut from between the $NADH_2$ and succinate showed far higher activity (Table 2). These observations suggest that the activity observed in the $NADH_2$ is due to contamination with traces of succinate caused by streaking during electrophoresis.

The failure to observe a transfer of tritium from succinate to NAD may be due to any of three factors: (1) the photoreduction of NAD involves the transfer of electrons from succinate, no direct transfer of hydrogen occurring; (2) tritium is lost

Table 1. *Metabolism of* [2,3-$^{14}C_2$]*succinate by chromatophores: relationship between* $NADH_2$ *or lactate and fumarate formed in the light*

The complete reaction mixture contained (in 3 ml.): sodium [2,3-$^{14}C_2$]succinate, 2·1 μmoles (5·0 μc); NAD, 2·0 μmoles; $MgCl_2$, 5·0 μmoles; tris–HCl buffer, pH 8·0, 30·0 μmoles; chromatophores, 100 μg. of bacteriochlorophyll. In Expts. 3 and 4 NAD was replaced by: sodium pyruvate, 10·0 μmoles; muscle lactate dehydrogenase, 20 μg. of protein; NAD, 0·2 μmole. After incubation for 30 min. at 30° under oxygen-free nitrogen the $NADH_2$ or lactate formed was estimated and an 80%-ethanol-soluble fraction prepared. Succinate and fumarate were isolated by paper chromatography or electrophoresis.

Expt. no.	Conditions	Total radioactivity on paper (counts/min.)	Distribution of radioactivity (%)		Amount present (μmoles)		Fumarate formed (μmole) (light − dark)	Product formed (μmole)
			Succinate	Fumarate	Succinate	Fumarate		
1	Light	11131	60	40	1·25	0·85	0·76	0·71
	Dark	11800	96	4	2·01	0·09		($NADH_2$)
2	Light	10000	69	31	1·45	0·65	0·53	0·50
	Dark	10175	94	6	1·98	0·12		($NADH_2$)
3	Light	1890	78	22	1·66	0·44	0·32	0·35
	Dark	1966	94	6	1·97	0·12		(lactate)
4	Light	1987	66	34	1·38	0·72	0·58	0·61
	Dark	1956	93	7	1·96	0·14		(lactate)

Table 2. *Metabolism of* [2,3-$^3H_2$]*succinate by chromatophores*

The reaction mixture contained (in 3ml.): [2,3-$^3H_2$]succinate, 2·0μmoles (181μc); NAD, 2·0μmoles; $MgCl_2$, 5·0μmoles; tris–HCl buffer, pH 8·0, 30·0μmoles; chromatophores, 100μg. of bacteriochlorophyll. During heat inactivation 1·5μmoles of carrier $NADH_2$ were added. $NADH_2$ was isolated as described in the Materials and Methods section. The specific activity of succinate was $4·12\times10^6$counts/min./μmole. The expected specific activity of $NADH_2$ was $1·03\times10^6$counts/min./μmole.

Expt. no.	Conditions	$NADH_2$ counted (μmole): Total $NADH_2$ isolated	$NADH_2$ counted (μmole): $NADH_2$ formed in the light	Radioactivity of $NADH_2$ (counts/min.)	Sp. activity of $NADH_2$	Percentage of expected sp. activity of $NADH_2$	Radioactivity in control strip (counts/min.)
1	Light	0·25	0·11	1608	14600	1·42	5901
2	Light	0·30	0·14	3224	20310	1·97	21993
3	Light	0·17	0·078	2325	30000	2·9	16351
4	Dark	0·18	0·00	2943			16495
5	Light	0·22	0·11	2074	15200	1·47	15501
6	Light	0·27	0·14	2469	23000	2·23	58913
7	Light	0·17	0·08	1547	19500	1·89	34751
8	Dark	0·27	0·00	2126			29183

Table 3. *Metabolism of* [2,3-$^3H_2$]*succinate by chromatophores: radioactivity of isolated water*

The reaction mixture was as given in Table 2. Water was isolated at the end of the reaction by freeze-drying. In experiments in the light 0·1ml. of the water isolated was diluted to 10ml. and 0·5ml. taken for counting. In experiments in the dark 0·2ml. was diluted to 5ml. and 0·5ml. taken for counting. The specific activity of succinate was $4·12\times10^6$counts/min./μmole. The expected radioactivity in water/μmole of $NADH_2$ was $2·06\times10^6$ counts/min./μmole.

Expt. no.	Conditions	Total radioactivity in water (counts/min.)	$NADH_2$ formed (μmole)	Radioactivity in water (counts/min./μmole of $NADH_2$)
1	Light	$9·65\times10^5$	0·44	$2·2\times10^6$
	Dark	$8·25\times10^4$		
2	Light	$8·95\times10^5$	0·43	$2·08\times10^6$
	Dark	$6·9\times10^4$		
3	Light	$8·9\times10^5$	0·52	$1·71\times10^6$
	Dark	$7·5\times10^4$		
4	Light	$9·35\times10^5$	0·52	$1·80\times10^6$
	Dark	$11·1\times10^4$		

from the succinate by exchange with water; (3) tritium is lost from the $NADH_2$ by exchange with water. The possibility of an exchange reaction occurring between succinate or fumarate and water was investigated in two ways. First, the water of the reaction mixture was isolated and the tritium released as water during the photoreduction reaction estimated. Table 3 shows the specific activity of the water relative to that of the $NADH_2$ formed in the light. The specific activity of the water is close to the value that would be expected if the succinate dehydrogenase of the chromatophores specifically removed two hydrogen atoms from succinate, as the mammalian enzyme has been shown to do by Tchen & van Milligan (1960). Secondly, a mixture of ^3H- and ^{14}C-labelled succinate was used as substrate for the photoreduction of NAD. After incubation, samples of succinate and fumarate were isolated from the reaction mixture by paper electrophoresis followed by paper chromatography in the pentan-1-ol–formic acid solvent. The acids were eluted, dried and taken up in a small volume of ethanol, and 50μl. samples were taken for counting. Two groups of experiments were performed, one in which the $^3H/^{14}C$ ratio was 60:1 and another in which it was 30:1. If no exchange reactions occur the $^3H/^{14}C$ ratio should be the same in succinate before and after incubation. The ratio in the fumarate should be half that in the succinate. The results of these experiments (Table 4) show that the $^3H/^{14}C$ ratios in the succinate and fumarate were as expected if no exchange reactions occur.

The experiments described above show that

Table 4. *Metabolism of mixtures of* [2,3-$^{14}C_2$]- *and* [2,3-$^{3}H_2$]-*succinate by chromatophores: $^{3}H/^{14}C$ ratio in succinate and fumarate isolated from reaction mixtures*

The complete reaction mixture contained (in 3 ml.): [2,3-$^{14}C_2$]succinate, 0·17 μmole (2·5 μc) in series 1, 0·34 μmole (5·0 μc) in series 2; [2,3-$^{3}H_2$]succinate, 2·0 μmoles (181 μc); tris–HCl buffer, pH 8·0, 30 μmoles; $MgCl_2$, 5·0 μmoles; chromatophores, 50 μg. of bacteriochlorophyll; NAD, 2·0 μmoles, as indicated. In some experiments NAD was replaced by: sodium pyruvate, 10 μmoles; NAD, 0·2 μmole; muscle lactate dehydrogenase, 20 μg. of protein. This is referred to below as 'Trap'. The gas phase was nitrogen and the temperature 30°. Incubation was for 30 min. After incubation the mixture was heat-inactivated, and succinate and fumarate were isolated by paper electrophoresis followed by chromatography.

Nucleotide	Conditions	Acid	Radioactivity (counts/min.) Channel A	Radioactivity (counts/min.) Channel B	^{3}H (disintegrations/min.)	^{14}C (disintegrations/min.)	$^{3}H/^{14}C$ ratio
Series 1							
Trap	Light	Succinate	19800	5661	216000	3400	63·5
		Fumarate	4400	2002	46500	1630	28·6
Trap	Dark	Succinate	66785	18805	715000	11100	64·5
Trap	Light	Succinate	34302	9827	350000	6220	61·0
		Fumarate	5505	2469	58300	1990	29·2
Trap	Dark	Succinate	69700	20339	760000	12800	59·0
NAD	Light	Succinate	21600	6160	236000	3760	63·0
		Fumarate	5117	2254	54200	1780	30·4
None	Dark	Succinate	32562	9237	354000	5600	63·0
None	Light	Succinate	16963	4850	184000	2980	61·5
Zero time		Succinate	26668	7789	290000	4950	58·5
Series 2							
NAD	Light	Succinate	33600	13099	358000	11700	30·6
		Fumarate	2959	1993	29500	2160	13·6
NAD	Dark	Succinate	22576	7842	241000	7400	32·6
None	Light	Succinate	35061	13744	369000	12450	29·6
		Fumarate	3237	2195	32400	2380	13·6
None	Dark	Succinate	17053	6647	18000	5970	30·0
Zero time		Succinate	26719	10224	276000	8950	29·8

Counting efficiencies:

	Channel A	Channel B
Succinate ^{3}H	8·85	1·6
^{14}C	17·15	6·3
Fumarate ^{3}H	8·95	2·02
^{14}C	14·2	65·0

chromatophores do not catalyse exchange reactions between succinate or fumarate and water and suggest that the protons of $NADH_2$ formed in the photoreduction reaction are supplied by water. To confirm this the photoreduction reaction was carried out in tritiated water. The $NADH_2$ formed in the light in the presence of tritiated water was isolated. The specific activity of the $NADH_2$ was found to be half that of the water (Table 5). This is the value expected if water is the source of protons, representing the addition of one non-exchangeable hydrogen to NAD.

It was found in control experiments with added $NADH_2$ that an exchange reaction between $NADH_2$ and water is catalysed by chromatophores. This exchange reaction occurs in the presence or absence of succinate and in the light or dark. It does not occur if the chromatophores are first heated at 100° for 2 min. The exchange reaction did not result in a complete equilibration of the $NADH_2$ with the medium in these experiments. The reason for this has not been investigated.

DISCUSSION

The results of the experiments with ^{14}C-labelled succinate as substrate show that the only product of the metabolism of succinate by chromatophores is fumarate, and that the amount of fumarate formed is stoicheiometrically related to the NAD reduced. This confirms and extends Frenkel's (1958) demonstration of a stoicheiometric relationship between $FMNH_2$ oxidized and NAD reduced in the photoreduction reaction.

Table 5. *Photoreduction of NAD in the presence of tritiated water: radioactivity in isolated water*

The complete reaction mixture contained (in 2 ml.): sodium succinate, 2·0 μmoles; NAD, 2·0 μmoles, or $NADH_2$, 1·5 μmoles, as indicated; $MgCl_2$, 5·0 μmoles; tris–HCl buffer, pH 8·0, 30 μmoles; water containing 20 mc of ^3H_2O; chromatophores, 100 μg. of bacteriochlorophyll. After inactivation 1·5 μmoles of $NADH_2$ were added as carrier except when $NADH_2$ was in the initial reaction mixture. The gas phase was nitrogen and the temperature 30°. Incubation was for 30 min. $NADH_2$ was isolated as described in the Materials and Methods section. The specific activity of ^3H_2O was $41·5 \times 10^4$ disintegrations/min./μmole. The expected specific activity of $NADH_2$ was $20·7 \times 10^4$ disintegrations/min./μmole. Internal standards were used in all experiments except Expt. 1 to measure the counting efficiency.

Expt. no.	Nucleotide	Substrate	Conditions	$NADH_2$ from reaction counted (μmole)	Radioactivity of $NADH_2$ (counts/min.)	Counting efficiency (%)	Specific activity (disintegrations/min./μmole)
1a	NAD	Succinate	Light	0·05	524	5·0	21×10^4
b	$NADH_2$	Succinate	Light	0·09	707	5·0	15×10^4
c	NAD	Succinate	Dark	—	22	5·0	
2a	$NADH_2$	Succinate	Dark	0·275	935	4·8	$7·1 \times 10^4$
b	$NADH_2$	None	Light	0·18	712	4·8	$8·4 \times 10^4$
c	$NADH_2$	None	Dark	0·23	830	4·8	$7·6 \times 10^4$
3a	NAD	Succinate	Light	0·08	876	5·0	$20·4 \times 10^4$
b	$NADH_2$	Succinate	Light	0·21	790	5·0	$7·5 \times 10^4$
c	$NADH_2$	Succinate	Dark	0·21	1011	5·0	$10·3 \times 10^4$
4a	NAD	Succinate	Light	0·11	600	2·5	$21·8 \times 10^4$
b	$NADH_2$	Succinate	Light	0·25	1323	4·7	$11·2 \times 10^4$
c	$NADH_2$	Succinate	Boiled extract	0·18	20	5·0	$24·4 \times 10^2$

As the oxidation of succinate involves the removal of two hydrogen atoms from the 2,3-positions, the oxidation of [2,3-$^3H_2$]succinate will therefore result in the removal of half the tritium from the molecule. This is not affected by the *cis–trans* specificity of the succinate dehydrogenase, or of the labelling of the succinate. The fumarate formed on oxidation of succinate should therefore have half the specific activity of the succinate. This was found to be the case in the experiments with mixtures of ^{14}C- and ^3H-labelled succinate, indicating that no exchange reaction between succinate or fumarate and water was catalysed by chromatophores. This was confirmed by the finding of a stoicheiometric relationship between the tritium liberated into the water and the NAD reduced.

If a direct transfer of hydrogen from succinate to NAD occurred, analogous to that demonstrated by Vennesland and her co-workers (Vennesland, 1955) in a number of NAD-linked enzymes, one hydrogen would be transferred to the non-exchangeable position 4 of the NAD molecule and one to the ionizable position 1. This latter would be lost by exchange with water.

Since half of the tritium of the succinate remains in the fumarate and half of that transferred to NAD would be lost by exchange, the specific activity of the NAD would be expected to be a quarter of that of the succinate. In fact the activity of the $NADH_2$ isolated from experiments with tritiated succinate was never more than 3% of the expected value. This result is consistent with an electron transfer mediating the reduction of NAD, the protons of $NADH_2$ being supplied by water.

The experiments with tritiated water showed that $NADH_2$ formed in the light had one non-exchangeable hydrogen equilibrated with water, as would be expected if an electron transfer occurred. However, it was also found that the chromatophores catalysed an exchange reaction between $NADH_2$ and water. The exchange reaction was independent of light and succinate and does not appear to be dependent on the overall photoreduction process.

It seems likely that the enzyme catalysing the exchange reaction has the same stereospecificity as the final dehydrogenase involved in the photoreduction reaction. $NADH_2$ reduced in the light had one hydrogen atom equilibrated with the water of the medium. If the exchange reaction had the opposite specificity to the final dehydrogenase both hydrogens on position 4 of the $NADH_2$ would have been equilibrated with the water.

The exchange reaction did not catalyse the complete equilibration of one hydrogen of the added $NADH_2$ with water during the period of incubation used. The reason for this was not investigated. Although added $NADH_2$ was not completely equilibrated with the water, $NADH_2$ formed in the light may have become labelled as a result of the exchange reaction. Similarly, if a

direct hydrogen transfer from tritiated succinate to $NADH_2$ occurred, the tritium would be lost by exchange with the aqueous medium.

It is not therefore possible on the basis of these experiments to distinguish between an electron-transfer and a hydrogen-transfer mechanism for the photoreduction reaction.

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