of metal phosphatidates, as evidenced by an increase in the ash phosphorus/total phosphorus ratio of the lipid.

5. The enzyme is progressively inactivated in the period between tapping and spontaneous coagulation of the latex, probably owing to bacterial action.

This work forms part of a programme of research on natural rubber undertaken by the Rubber Research Institute of Malaya. The author wishes to thank Mr Yee Shin Meng for able practical assistance and Mr D. R. Westgarth for the statistical analysis.

REFERENCES

- Acker, L., Diemair, W. & Jager, R. (1952). *Biochem. Z.* **322**, 471.
- Altman, R. F. A. (1946). *Rec. Trav. chim. Pays-Bas*, **65**, 919.
- Altman, R. F. A. (1947). *I.R.I. Trans.* **22**, 274.
- Altman, R. F. A. (1948). *Industr. Engng Chem. (Industr.)*, **40**, 241.
- Altman, R. F. A. & Kraay, G. M. (1940). *Arch. Rubbercult. Ned.-Ind.* **24**, 58.
- Bie, G. J. van der (1948). *India Rubb. J.* **113**, 541.
- Cockbain, E. G. (1948). *India Rubb. J.* **114**, 451.
- Cook, A. S. & Sekhar, B. C. (1953). *J. Rubb. Res. Inst. Malaya*, **14**, 163.
- Gils, G. E. van (1947). *I.R.I. Trans.* **23**, 74.
- Glick, D. (1944). *J. biol. Chem.* **156**, 643.
- Hanahan, D. J. & Chaikoff, I. L. (1947a). *J. biol. Chem.* **168**, 233.
- Hanahan, D. J. & Chaikoff, I. L. (1947b). *J. biol. Chem.* **169**, 699.
- Hanahan, D. J. & Chaikoff, I. L. (1948). *J. biol. Chem.* **172**, 191.
- McKibbin, J. M. & Taylor, W. E. (1949). *J. biol. Chem.* **178**, 17.
- McMullen, A. I. (1949). *Nature, Lond.*, **164**, 715.
- McMullen, A. I. (1951). *J. Rubb. Res. Inst. Malaya*, **13**, 129.
- Resing, W. L. (1951). *J. Rubb. Res. Inst. Malaya*, **13**, 141.
- Rhodes, E. & Bishop, R. O. (1930). *Quart. J. Rubb. Res. Inst. Malaya*, **2**, 125.
- Tristram, G. R. (1942). *Biochem. J.* **36**, 400.

The Turnover Rates of the Phosphate Groups of Flavin-Adenine Dinucleotide and Adenosine Triphosphate during Oxidative Phosphorylation

BY L. V. EGGLESTON AND D. H. WILLIAMSON

Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Sheffield

(Received 16 July 1953)

The nature of the reactions by which inorganic phosphate is converted into pyrophosphate groups of adenosine triphosphate (ATP) when reduced diphospho- or triphospho-pyridine nucleotides are oxidized is still unknown. An approach to the problem is a search, with the help of radioactive phosphate, for the formation of any phosphorylated compound in an enzyme system which synthesizes ATP from adenosine diphosphate (ADP) and phosphate. If radioactive phosphate is added to a phosphorylating system any intermediate on the way from phosphate to ATP must become radioactive, its specific activity being at least as high as that of ATP.

Hummel & Lindberg (1949), working with rabbit liver, reported that flavin-adenine dinucleotide (FAD) becomes radioactive about as rapidly as ATP, and they concluded that FAD is closely associated with oxidative phosphorylation. The present paper is a re-investigation of the problem with improved methods of separation of the phosphate fractions. Whilst the experimental observations of Hummel & Lindberg have been repeated

with similar results, evidence is presented which suggests that their technique fails to separate FAD quantitatively from phosphate, and that FAD does not become radioactive under conditions where ATP shows a high turnover rate.

Materials

A commercial preparation of the sodium salt of ATP (Schwarz Laboratories Inc., New York) was used in most experiments. The distribution of total phosphorus was: ATP 68.4, ADP 20.4, AMP 1.44, inorganic pyrophosphate 4.18, and inorganic orthophosphate 5.58% (methods of Eggleston & Hems (1952) and Krebs & Hems (1953)). [³²P] ATP was prepared by R. Hems (Hems & Bartley, 1953); after dilution with carrier, its activity was 1.7×10^6 counts/min./ml. 0.02M ATP solution. Flavin mononucleotide (FMN), of about 95% purity, was supplied by the Sigma Chemical Co. Flavin-adenine dinucleotide was prepared from baker's yeast or brewer's yeast according to Warburg & Christian (1938) up to the stage of the decomposition of the silver salt with H₂S. After aeration to remove H₂S, the solution containing the mixture of nucleotides (about 100 ml.) was neutralized, transferred to phenol, brought back to water (30 ml.), and run on to a charcoal

column ('NuChar C'), which was washed successively with water, 0.5, 1.0, and 5% pyridine. Adenine nucleotides appeared in the last solvent. The flavin nucleotides were eluted from the column with 15–20% aqueous pyridine. The pyridine was removed by three extractions with CHCl_3 . After saturation with $(\text{NH}_4)_2\text{SO}_4$, the flavins were transferred to phenol, and after addition of ether were collected in about 10 ml. water. This aqueous solution contained little or no diphosphopyridine nucleotide (DPN) and no other detectable phosphate compounds, and consisted of a mixture of FAD, FMN, traces of riboflavin, and inorganic salts. The extract was dried on to the starting line of a chromatogram paper (Whatman no. 1, washed according to Eggleston & Hems, 1952), and run downwards with butanol:acetic acid:water solvent (Crammer, 1948). The FAD band was cut out after air drying and eluted with water by the technique described by Hems & Bartley (1953) for the collection of radioactive ATP in a small volume.

Methods

Paper chromatography. The pre-treatment of papers and the equipment used were as previously described (Eggleston & Hems, 1952). Solvents were modified in the present work and sometimes much larger volumes of test solution were spotted on to a small area on the starting line. Out of 204 mixtures tested, the following were selected:

Solvent A: isopropyl ether:90% (v/v) formic acid (3:2, v/v) (Hanes & Isherwood, 1949). Ascending flow was continued for 3–5 hr. at room temperature.

Solvent B: phenol saturated with water at 18°, 0.001 M (final concentration) sodium ethylenediaminetetraacetate. Descending flow at 25°, starting after temperature equilibration, was continued for 8 hr. After drying with a strong current of warm air, the papers were rolled up and kept in this position with paper clips. They were then shaken vigorously in a stoppered, wide-necked 500 ml. cylinder with three successive vol. of 100 ml. anhydrous ether. This was necessary in order to remove residual phenol, which prevents identification of spots absorbent and fluorescent in u.v. light.

Solvent C: 55 parts methanol + 15 parts isobutyric acid + 30 parts water, 0.001 M (final concentration) sodium ethylenediaminetetraacetate. isobutyric acid and methanol were previously redistilled, the latter over 2,4-dinitrophenylhydrazine to bind aldehydes. Descending flow was continued for 16–17 hr. at 15°.

Solvent D: 35 parts isobutyric acid + 15 parts ammonia (sp.gr. 0.880) + 15 parts water, 0.001 M (final concentration) sodium ethylenediaminetetraacetate. Ascending flow was continued for 16–17 hr. at room temperature.

These solvents were used in the dark, to prevent FAD decomposition. The papers were dried in a current of warm air, except when the FAD spots were to be extracted for

quantitative spectrophotometry or for assay with D-amino acid oxidase. In these instances the papers were hung in a cool, dark fume cupboard to allow the organic solvent to evaporate.

The dried papers were examined under u.v. light from a 'chromatolite' lamp (Hanovia Ltd., Slough, Bucks). Absorbent areas (e.g. adenine nucleotides) and fluorescent areas (flavins) were ringed with pencil. The molybdate-spray technique of Hanes & Isherwood (1949) was used for further location of phosphorus-containing compounds.

The distribution of various nucleotides and phosphate after separation on paper with each of the four solvent mixtures is shown in Table 1. Solvent B has the disadvantage that it is easily overloaded; only small quantities are separated satisfactorily. The other solvents are usable even with high salt concentrations. As no one solvent system alone would give a complete separation of all the components, the determination of each compound or group of compounds was made by combining the results of several methods. Thus, solvent A gives data for phosphate and for total nucleotides, the space between these two areas being occupied by hexose and triose phosphates (Eggleston & Hems, 1952; Krebs, Ruffo, Johnson, Eggleston & Hems, 1953). With solvent B, two separate groups of compounds were determined, FAD + FMN, and ATP + ADP + phosphate + hexose and triose phosphates. Solvent C was used mainly for determinations of FAD. Solvent D was used in the latter stages of the work and only for the separation of compounds present in phenol extracts.

Specific radioactivity measurements. Phosphorus determinations were made as described by Eggleston & Hems (1952) and Bartley (1953). Radioactivity was measured on the final, blue isobutanol-ethanol solutions from the P determinations (for limitations of this technique see Ennor & Rosenberg, 1952). A β -counting tube, liquid type M6 of 20th Century Electronics, was used, and after counting was cleaned with N-NaOH followed by water, and finally with N-H₂SO₄. Specific activity is expressed throughout as counts/min./ μg . P.

Spectrophotometric determinations of FAD. A measured volume (0.05–1.0 ml.) of neutralized trichloroacetic acid (TCA) extracts, or flavin solutions purified by phenol extraction (Hummel & Lindberg, 1949), was pipetted into a test tube, stored in ice, and transferred quantitatively on to the starting line of a chromatogram paper with a finely drawn-out Pasteur pipette, and rapidly dried by a hair drier set at room temperature, the area being no greater than 2 cm.². The operations were carried out under low illumination. Solvent C was used for the separation. The FAD spot was cut out and placed in 4 ml. water in the dark for 1–2 hr. with occasional shaking. After filtration through glass wool, the absorption of the solution was read in a Beckman spectrophotometer at 450 m μ , using matched cells. The molecular extinction coefficient of FAD given by

Table 1. R_f values of flavin and adenine nucleotides with various solvents

(For conditions see text. The R_f values concerning solvent C are relative to phosphate, as this solvent leaves the paper in the time required for adequate separation.)

Solvent	Phosphate	ATP	ADP	AMP	DPN	FAD	FMN
A	0.60	0.00	0.00	0.10	0.00	0.0–0.10	0.0–0.10
B	0.12	0.05	0.15	0.50	0.81	0.57	0.68
C	1.00	0.87	0.87	0.94	0.77	0.59	0.80
D	0.88	0.75	0.75	0.75	.	0.45	0.85

Warburg & Christian (1938) as 1.13×10^4 at this wavelength was used as reference standard; an optical density of 0.100 was equivalent to 6.95 μg . FAD/ml. Recovery of standard amounts of FAD run on papers was linear, the range used being 3–35 μg . $(\text{NH}_4)_2\text{SO}_4$ and traces of isobutyric acid remaining in the paper did not interfere.

Enzymic assay of FAD. The assay was used to confirm the identity of FAD. The D-amino acid oxidase was prepared from sheep kidney according to the method of Warburg & Christian (1938). 25–50 μl . of solution to be examined were treated with solvent *C* and the FAD spot was cut out and placed in the main compartment of a Warburg manometer cup. To this were added 1 ml. water, 1 ml. 0.1M sodium pyrophosphate buffer, pH 8.3, and 0.2 ml. enzymic solution. The side arm contained 0.2 ml. 4% (w/v) DL-alanine, the centre well 0.2 ml. 2N-NaOH and a pleated filter paper to absorb CO_2 . The O_2 uptake was then measured, as usual, at 40°. As little as 0.3 μg . FAD gave a measurable increase of O_2 uptake above that of the blank, even though the traces of isobutyric acid remaining in the paper spot were slightly inhibitory. The usual range was an increase of 40–170 μl . O_2 in 30 min. In no case did an FAD spot visible in u.v. light give no extra O_2 uptake. Conversely no uptake was found when no FAD was visible.

RESULTS

Turnover of flavin mononucleotide

As the concentration of naturally occurring flavin nucleotides in liver is very low, additional FAD or FMN was added to dilute liver suspensions. This has the advantages of increasing the accuracy of flavin phosphate turnover measurements, and decreasing the relative concentration of other organophosphates in the liver which might interfere. The experimental conditions were similar to those used by Krebs *et al.* (1953) in which the labile phosphate of adenosine phosphates reached equilibrium with phosphate within 60 min. at 20°, and where the stable phosphate, i.e. AMP phosphorus, did not become appreciably radioactive.

Chopped rat liver was ground up in a chilled, stainless-steel Potter-Elvehjem homogenizer with 6.5 vol. 0.9% (w/v) KCl. A sample (0.5–1 ml.) of suspension was incubated aerobically in Warburg vessels with 1.5–3.0 ml. additions of 0.025M α -oxoglutarate, 0.0025M ATP, 0.005M phosphate buffer, pH 7.4, 0.0025M MgCl_2 , 10–20 μC radioactive phosphate, and 0.001–0.0025M FMN (final concentrations). The reaction was stopped by the addition of 0.1–0.2 ml. 30% (w/v) TCA and centrifuged at 4°. The TCA extracts were neutralized with 0.1–0.2 ml. 2N-NaOH, stored at –15°, and samples run on paper chromatograms.

The very low rate of turnover of FMN phosphate in liver, found by Hummel & Lindberg (1949), was confirmed; after separation of TCA extracts with solvent *B*, the radioactivity in the FMN areas of the papers was no greater than in the same areas from incubated liver controls where no FMN had been added. There was a trace of activity which was almost certainly due to trailing; it constituted only

about 0.2% of the total radioactivity on the paper. The addition of FMN did not affect the high radioactivity found in the adenosine phosphates, the labile P of which reached equilibrium with phosphate within 30 min. at 30°.

Turnover of flavin-adenine dinucleotide in liver suspensions

Experiments with added flavin-adenine dinucleotide. The conditions were essentially those described in the FMN section, except that the only FMN added was that present as a contaminant of FAD preparations. Full details of an experiment in which FAD was added to rat-liver suspensions are shown in Table 2; similar results were obtained in four other experiments of this type. The addition of FAD (below 10^{-4}M) caused partial inhibition of oxygen uptake, and a corresponding reduction of the turnover rate of the labile phosphorus of adenosine phosphates; however, incorporation of [^{32}P] phosphate into adenosine phosphate was still rapid after addition of FAD, and a high radioactivity was also found in the hexose and triose phosphates. The fact that the specific activity of the adenosine phosphate fraction did not always reach 66.6% of that of the inorganic phosphate would indicate either that the labile groups were not yet in equilibrium with phosphate, or that the adenosine phosphate fraction contained ADP and AMP. In contrast, the flavin nucleotide areas of the paper chromatograms were no more radioactive than the similar areas obtained from incubated controls without added flavins when 25 μl . of TCA extract was analysed with solvent *B*. Although the FAD concentration in these areas was very low (0.04–0.08 μg . P of FAD), an additional 60–200 counts/min. would have been found if the specific activities of FAD had been similar to those of phosphate or adenosine phosphates.

When relatively large volumes (150–300 μl .) of neutralized TCA extracts were dried on to a small area on papers subsequently run with solvent *C*, appreciable radioactivity was found in the FAD areas, even when none was visible under ultraviolet light. This was found to be due to trailing of radioactive ATP, and, although chemically and visually undetectable, gave radioactivities amounting to 0.5–2% of the total labile phosphorus of adenosine phosphates. The trailing in the FAD areas due to radioactive phosphate rarely amounted to more than 0.1% of the total radioactivity of the phosphate.

The accuracy of the measurements of the specific radioactivity of FAD was increased by extracting 1 or 2 ml. of the TCA extracts with phenol, as described by Hummel & Lindberg (1949) and transferring the flavin nucleotides to a small

Table 2. *Incorporation of [³²P] phosphate into flavin and adenine nucleotides and other phosphates during incubation of rat-liver suspension*

(Rat-liver suspension 1 in 30: 0.005M phosphate; 0.0025M-MgCl₂; 0.025M-KCl; 0.025M α -oxoglutarate; 0.0025M ATP; in Expt. 1, 20 μ C [³²P] phosphate, 4 ml. final vol.; in Expt. 2, 10 μ C [³²P] phosphate, 2 ml. final vol.; 30 min. incubation at 30°; O₂.)

	Expt. 1			Expt. 2	
Added FAD: concentration—initial (M $\times 10^{-5}$)	0	2.7	5.4	0	6.5
amounts—initial (μ g.)	0	85	170	0	102
final (μ g.)	0	84	151	0	52
O ₂ uptake in 30 min. (μ l.)	232	118	75	171	111
Sp. act. of total adenosine-P (cts./min./ μ g. P)	2140	1800	1460	1755	1583
Sp. act. of inorganic-P (cts./min./ μ g. P)	3065	2816	2980	2610	2428
Ratio: $\frac{\text{sp. act. of adenosine-P}}{\text{sp. act. of inorganic-P}}$	0.698	0.640	0.490	0.672	0.652
<i>FAD and FMN separated with solvent B (25 μl. TCA extract)</i>					
Visibility of FMN spot in u.v.	0	+	+	0	+
Visibility of FAD spot in u.v.	0	+	+	0	+
P in FMN + FAD areas (μ g.)	0.14	0.63	0.70	0.21	0.49
P in FMN + FAD areas (cts./min.)	27	28	27	39	42
<i>FAD separated with solvent C</i>					
FAD area enzymic assay from 50 μ l. TCA extract (see text) (μ l. O ₂ /30 min.)	8	57	64	3	93
FAD areas separated from phenol-extracted flavins of 2 and 1 ml. TCA extracts in Expts. 1 and 2, resp., P (μ g.)	0*	0.74	1.29	.	1.16
P radioactivity (cts./min.)	53†	63†	39†	.	229
P sp. act. (cts./min./ μ g.)	.	85	30	.	197
Ratio: $\frac{\text{sp. act. of P in FAD area}}{\text{sp. act. of inorganic-P}}$	.	<0.030	<0.010	.	<0.081
<i>'Hexose-P + triose-P' separated with solvent A</i>					
μ g. P from 25 μ l. TCA extract	0.67	0.40	0.32	0.48	0.70
P, act. from 25 μ l. TCA extract (cts./min.)	2080	1019	554	751	970
P, sp. act. (cts./min./ μ g.)	3105	2545	1730	1566	1385
Ratio: $\frac{\text{sp. act. of hexose-P + triose-P}}{\text{sp. act. of inorganic-P}}$	1.01	0.905	0.580	0.600	0.570

* Less than 0.1 μ g. P, an amount too small to be estimated accurately.

† This activity is due to the trailing of radioactive ATP (see text pp. 252–253).

Sp. act. (cts./min.) = specific activity (counts/min.).

volume. Most of this was placed on paper and developed with solvent C. Again (Table 2) very little radioactivity was found in the FAD areas; such radioactivity as was found did not exceed that of controls to which no FAD was added. This trace of activity was probably due to trailing. The presence of FAD in these areas was confirmed both by observation of fluorescence under ultraviolet light and by manometric assay with D-amino acid oxidase. Other controls showed that the phenol extraction procedure did not destroy or interfere with the enzymic assay.

Experiments without added flavin-adenine dinucleotide. In view of the inhibitory effect of added FAD on the oxygen uptake of liver preparations, further experiments were carried out on liver suspensions containing only the naturally occurring flavin nucleotides. More concentrated suspensions

and larger volumes had to be used; 24–150 ml. containing 5–30 g. liver were incubated aerobically in Erlenmeyer flasks. ATP was added to increase the accuracy of adenosine phosphate radioactivity measurements. Details of an experiment are shown in Table 3. As before, considerable radioactivity was found in the adenosine phosphates and in the hexose and triose phosphates area. After phenol extraction and separation with solvent C, only traces of radioactivity were found in the FAD area, not exceeding those obtained in the unincubated control. The activity was therefore probably due to trailing of phosphate.

In a similar experiment on pigeon liver, the relative specific radioactivities of total adenosine phosphates, hexose and triose phosphates, and FAD were 0.503, 0.436 and 0.027, respectively, taking the activity of phosphate as 1.00.

Table 3. *Incorporation of [³²P] phosphate into preformed FAD and into adenosine phosphates in rat liver*

(Rat-liver suspension 1 in 5: 0.0025 M-MgCl₂; 0.025 M α-oxoglutarate; 0.001 M ATP; 0.002 M phosphate; 0.077 M-KCl; 100 μC [³²P] phosphate. 24 ml. final volume. O₂. 30°. The reaction was stopped by 2.5 ml. 30% (w/v) TCA and cooling at 0°.)

Period of incubation at 30° (min.)	0	10
FAD found in 24 ml. (μg.)	93	122
Sp. act. of total adenosine phosphates (cts./min./μg. P)	13	891
Sp. act. of inorganic phosphate	3722	1624
Ratio: $\frac{\text{sp. act. of total adenosine phosphates}}{\text{sp. act. of inorganic phosphate}}$	.	0.550
Sp. act. of 'hexose + triose phosphates' (cts./min./μg. P)	47	537
Ratio: $\frac{\text{sp. act. of 'hexose + triose phosphates'}}{\text{sp. act. of inorganic phosphate}}$	.	0.331
Phenol-extracted flavins from 12 ml. TCA extracts, separated by solvent C		
FAD area		
Extra O ₂ uptake of 1/12 of extract with D-amino acid oxidase (30 min. 40°) (μl.)	97.5	99
P (μg.) (one-quarter of phenol-extracted flavins)	0.49	0.45
Radioactivity (cts./min.) (one-quarter of phenol-extracted flavins)	93	63
Sp. act. (cts./min./μg. P)	.	140
Ratio: $\frac{\text{sp. act. of FAD area}}{\text{sp. act. of inorganic phosphate}}$	.	<0.086

Effect of solvents on distribution of radioactivity in chromatograms. These findings are in contrast with those of Hummel & Lindberg who reported a relatively high radioactivity of FAD after incubation under similar conditions. With their technique, i.e. with the use of a phenol-containing solvent for the chromatographic separation (solvent B) of flavins extracted with phenol from saturated ammonium sulphate solutions, it was confirmed that the FAD area was radioactive. But this technique cannot be regarded as reliable. As Hummel & Lindberg point out, the FAD area in their chromatograms was grossly contaminated by other phosphates: 'the minute amounts of flavin present would contribute only immeasurably to the total phosphorus'. If this unknown phosphorus were only slightly radioactive, the value calculated for the radioactivity of FAD would be invalid.

It was found that combination of the extraction of flavin from the TCA extracts with phenol and the use of a phenol-containing solvent for the development of the chromatogram caused radioactivity in the FAD area.

The contamination of the FAD area by other phosphates is illustrated by the following experiment with solvent B. In an area calculated to contain 0.15 μg. FAD phosphorus, an amount of 4.13 μg. phosphorus was actually found. The ATP area which showed no absorption under ultraviolet light contained 2.71 μg. phosphorus. When pure solutions of radioactive phosphate, with or without FAD or FMN, or radioactive ATP, were taken through the full extraction procedure, radioactivity was found in the phenol extract from both incubated and unincubated liver suspensions, in spite of

applying the procedure of Hummel & Lindberg of washing with KH₂PO₄. With different mixtures, the following proportions of the original radioactivity were found in the phenol extracts: unincubated liver suspension, 0.02–0.03%; incubated suspensions, 0.07–0.18%; pure solutions of phosphate with up to 30 μg. FAD, 0.02–0.03%; pure solutions of phosphate with 95 μg. FAD, 0.225%; pure solutions of radioactive ATP, 0.06%. The fact that ATP is more readily extracted than phosphate might partly account for the higher activity found in extracts of incubated, as compared with unincubated, suspensions. Radioactive sugar phosphates may also contribute to the difference.

Further data on the effect of the solvents on the distribution of radioactivity were obtained in the following experiment. Phenol-extracted flavins of incubated guinea pig liver suspensions (30 g. liver in 150 ml. medium containing [³²P] phosphate) were dissolved in 0.8 ml. water. Samples of 25 μl. were chromatographed with solvent B (pH about 3.7), solvent C (pH about 3.1), and solvent D (pH about 6.2). The distribution of radioactivity of the papers is shown in Table 4. The results obtained for solvent B are similar to those obtained by Hummel & Lindberg in that the FAD area showed radioactivity but no appreciable radioactivity was found in the FAD areas when solvents C and D were used. The appearance of radioactivity is clearly not due simply to the pH of solvent B. It varied, however, with the amount of material on the paper. The radioactivity of the FAD area rose tenfold when the amount of FAD was quadrupled. Data shown in Table 5 demonstrate that the FAD area also becomes radioactive when aqueous solutions of

Table 4. *Radioactivities of FAD areas on papers developed with various solvents*

(30 g. guinea pig liver suspended in 150 ml. medium were incubated with 100 μ C [32 P] phosphate for 10 min. at 20°. Flavins were phenol-extracted by the method of Hummel & Lindberg (1949) and made up to a final volume of 0.8 ml. with water. pH was measured with a glass electrode in the undiluted solvent.)

Solvent	Approx. pH of solvent	Vol. of extract run on paper (μ l.)	Radioactivity (counts/min.)			Total radioactivity in FAD area (%)
			ATP + phosphate area	FAD area	Rest of paper	
B	3.70	25	63	96	25	52.2
C	3.08	25	189	1	0	0.5
D	6.18	25	170	17	8	8.7
B	3.70	10	28	17	30	22.7
B	3.70	40	103	190	13	62.1

Table 5. *Distribution of radioactivity from phenol extracts of unincubated material after separation on paper with solvents B and C*

(Material extracted in Expt. 1: 95 μ C. FAD, 20 μ C [32 P] phosphate, 3% neutralized TCA. Total volume, 10 ml.; in Expt. 2: 6.5 g. rat liver suspended in 25 ml. 0.9% (w/v) KCl; 75 μ C [32 P] phosphate; 0.5 ml. 0.1 M-MgCl₂; 3 ml. 30% (w/v) TCA. Filtered, and filtrate neutralized to pH 6.0.)

Experiment	Final vol. of phenol extract (μ l.)	Vol. of phenol extract separated (μ l.)	Solvent used	Total counts/min. on papers	Distribution of total radioactivity on papers (%)		
					ATP + phosphate area	FAD area	Rest of paper
1	700	25	B	142	38.0	21.8	40.2
	700	70	B	380	16.6	54.2	29.2
	700	25	C	135	98.4	0.8	0.8
	700	70	C	390	90.6	0.9	8.5
2	200	5	B	45	93.4	2.2	4.4
	200	10	B	88	30.8	23.9	45.3
	200	15	B	141	27.6	32.6	39.8

phosphate and FAD without animal tissue are taken through the phenol extraction procedure described by Hummel & Lindberg, and chromatographed with solvent B. The amount of radioactivity varied with the FAD concentration and the ammonium sulphate content of the phenol extract, but this point was not further investigated. Under the same conditions, pure FMN preparations did not give an FMN area which was radioactive.

No radioactivity occurs in the FAD area when liver suspensions are deproteinized with TCA and a sample of the filtrate is developed with solvent B.

The present experiments were only concerned with the participation of free flavins, or readily dissociating flavoproteins, in oxidative phosphorylation. A possibility, left open, is that a flavoprotein participates which does not readily dissociate, either under physiological conditions or during deproteinization.

DISCUSSION

Whilst the experimental observations of Hummel & Lindberg—radioactivity in the FAD area of chromatograms of liver suspension incubated with

32 P phosphate—were confirmed, their interpretation of the results must be modified. The experiments reported here show that the phenol extract of a mixture of pure solutions of FAD with radioactive phosphate or ATP gives FAD spots on paper chromatograms which contain appreciable amounts of radioactive material if the developing solvent contains phenol. The phosphate content of the FAD spot can be more than ten times greater than the amount of FAD phosphorus. It is noteworthy that the phosphate does not cover the paper evenly, but is concentrated in the FAD area. Zetterström & Ljunggren (1951) have already reported, quoting unpublished experiments by Ernster, Lindberg & Zetterström, that the radioactive phosphate in the FAD area 'is considerably more acid-labile than the phosphate in the FAD molecule itself. Hence, the flavin-adenine dinucleotide isolated is probably a larger molecule than FAD'. Whilst it is not impossible that a phosphorylated FAD occurs in tissues, at least two facts argue against it, namely, that the amount of 'labile' phosphate has no constant stoichiometric relation to the FAD phosphate and can be up to ten times greater than that of FAD phosphate, and that it is not found

when chromatogram solvents are used that are no more acid than the phenol solvent. These findings indicate that the radioactive phosphate in the FAD area is a contamination, rather than part of a chemical entity.

SUMMARY

1. Paper chromatographic procedures for the separation of flavin nucleotides from other phosphates are described.

2. The incorporation of [^{32}P] phosphate into flavin mononucleotide and flavin-adenine dinucleotide (FAD) in liver suspensions is negligible under conditions where ATP rapidly becomes radioactive. It is concluded that the phosphate of free flavin nucleotides plays no direct part in oxidative phosphorylation.

3. Evidence is presented which indicates that the contrary findings of Hummel & Lindberg (who reported a rapid incorporation of [^{32}P] phosphate into FAD) can be explained by incomplete separation of FAD from other phosphates by certain techniques. Contamination of FAD with other phosphates occurs when FAD is extracted with

phenol from aqueous, saturated, ammonium sulphate solutions and phenol-containing solvents are used for the development of paper chromatograms.

4. FAD (10^{-5} – 10^{-4}M) inhibits the oxygen uptake of liver suspensions.

We wish to thank Prof. H. A. Krebs, F.R.S., for his help and advice. This work was aided by a grant from the Rockefeller Foundation.

REFERENCES

- Bartley, W. (1953). *Biochem. J.* **54**, 677.
 Crammer, J. C. (1948). *Nature, Lond.*, **161**, 349.
 Eggleston, L. V. & Hems, R. (1952). *Biochem. J.* **52**, 156.
 Ennor, A. H. & Rosenberg, H. (1952). *Biochem. J.* **52**, 591.
 Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
 Hems, R. & Bartley, W. (1953). *Biochem. J.* **55**, 434.
 Hummel, J. P. & Lindberg, O. (1949). *J. biol. Chem.* **180**, 1.
 Krebs, H. A. & Hems, R. (1953). *Biochim. biophys. Acta*, **12**, 172.
 Krebs, H. A., Ruffo, A., Johnson, M., Eggleston, L. V. & Hems, R. (1953). *Biochem. J.* **54**, 107.
 Warburg, O. & Christian, W. (1938). *Biochem. Z.* **298**, 150.
 Zetterström, R. & Ljunggren, M. (1951). *Acta chem. scand.* **5**, 291.

The Carbohydrates of *Agave vera cruz* Mill.

2. DISTRIBUTION IN THE STEM AND POLE

BY M. SRINIVASAN AND I. S. BHATIA

Division of Quality Control, Central Food Technological Research Institute, Mysore

(Received 26 June 1953)

The carbohydrate gradient in the stem of *Agave vera cruz* was previously reported upon by Srinivasan & Bhatia (1953). It was then indicated that where the carbohydrate content was high and uniform throughout the stem, such a stem belonged to a fairly aged plant. To confirm these indications, a plant which had poled was selected for study, since it is known that such a plant is mature. Data relating to the make-up and distribution of carbohydrates in the stem and the pole are presented here. Evidence of identification of the simple sugars present is given by Bhatia, Satyanarayana & Srinivasan (1953*a*). The present study was of added interest from the point of view of the production of fructose from this

source, an aspect investigated by us and for which it was desirable to have information regarding the site of maximum fructosan concentration in the plant.

EXPERIMENTAL

Materials. A plant which had infloresced and partly bulbeled was chosen and sectioned into stem and pole (inflorescence axis). These were arbitrarily further subsectioned as shown in the diagram (Fig. 1). The material from each section was grated and juice was pressed out. The total time taken for these operations was about 90 min. (normally, no significant enzymic degradation of polysaccharides takes place in this material within this period). The juice was then clarified as described previously (Srinivasan & Bhatia, 1953) and used for analysis.

Fig. 1. Stem and pole of *Agave vera cruz* drawn to scale (1 cm. = 10 in.). Shaded sections taken for experiment.