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The Separation of Flavins by Paper Electrophoresis and its Application to the Examination of the Flavin Contents of Micro-organisms

By J. L. PEEL

Agricultural Research Council Unit for Microbiology, The University, Sheffield 10

(Received 13 January 1958)

Extracts of *Clostridium kluyveri* are a deep green-brown, but under hydrogen this colour disappears leaving a faint pink colour (Peel & Barker, 1956). Substantially the same observations hold for the rumen organism LC1 of Elsdon, Volcani, Gilchrist & Lewis (1956). It appeared likely that the pigments concerned were flavin derivatives, and the presence of such apparently large amounts in these two strict anaerobes was of particular interest. Since very little data were available on the occurrence of flavins in micro-organisms a survey of a variety of species seemed a worthwhile project.

At the outset, suitable analytical methods were not available. It was considered essential that the principal flavin derivatives should be separated from each other in sufficient quantities for confirmatory tests to be applied and in such a manner as to permit an estimate of the amount of each flavin present in the organisms examined. A separation on the required scale has been accomplished by paper electrophoresis and this separation has been combined with a preliminary extraction and concentration procedure adapted from the methods used by Dimant, Sanadi & Huennekens (1952) in the preparation of flavinadenine dinucleotide. This paper describes the method and the results obtained, preliminary accounts of which have already been given (Peel, 1954, 1955).

The electrophoretic separation of flavins has since been reported from a number of laboratories

(Siliprandi, Siliprandi & Lis, 1954; Cerletti & Siliprandi, 1955; Gaudiano & Cingolani, 1954; Yagi & Matsuoka, 1955; Walaas & Walaas, 1956).

MATERIALS

Flavin derivatives. Riboflavin was obtained from L. Light and Co. (Colnbrook, Bucks); flavin mononucleotide (FMN) was a gift from Sigma Chemical Co.; riboflavinyl glucoside and one sample of flavinadenine dinucleotide (FAD) were gifts from Dr L. G. Whitby. A crude mixture of FMN and FAD was obtained from baker's yeast by following the procedure of Dimant *et al.* (1952) to the stage before partition chromatography. These products were used without further treatment as markers for paper chromatograms. For recovery experiments riboflavin, FMN and FAD were purified by paper electrophoresis as described later. Lumiflavin was prepared from riboflavin as described by Warburg & Christian (1938). Lumichrome (Karrer, Salomon, Schöpp, Schlittler & Fritzsche, 1934) was prepared as follows: a saturated solution of riboflavin in 0.01 M-ammonium formate-formic acid buffer, pH 4.6, was kept for about 7 days in a shallow layer in daylight at room temperature; HCl was then added to a final concentration of about 0.5 M and the mixture extracted with a half volume of CHCl_3 . The extract was evaporated to dryness and the residue dissolved in water for use.

Micro-organisms. Sources of micro-organisms and details of the growth conditions and media employed in obtaining cells for the examination of flavin contents are given in Table 7. In experiments on the extraction procedure the large quantities of the rumen organism LC1 required were obtained as described by Walker (1958).

Hexanoate-oxidizing organisms. The three organisms used were isolated from soil by the enrichment-culture technique. The medium contained 1% (w/v) of sodium hexanoate, 0.1 mg. of riboflavin/100 ml. and the following salts (mg./100 ml.): KH_2PO_4 , 136; Na_2HPO_4 , 213; $(\text{NH}_4)_2\text{SO}_4$, 50; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$, 0.25; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.25; pH 7. Volumes (10 ml.) of medium in 100 ml. Erlenmeyer flasks were inoculated with approx. 2 g. of soil and incubated at 25° or 37°. After 3 days' incubation, the cultures were plated on the above medium solidified by the addition of 2% of washed agar and then incubated at the same temperature as the parent enrichment. After 4 days two types of colony could be distinguished on plates from the culture maintained at 25°. Each type was re-plated twice in succession to obtain pure cultures. The organisms were identified as strains of *Pseudomonas fluorescens* and designated strains CO1 and CO2. The two strains differ in colonial morphology; in addition CO2 grows more slowly than CO1 on all media tested.

Plates from the 37° enrichment showed no growth after 4 days but colonies appeared after 6 days. Only one type of colony was found; this organism was obtained pure by two further platings and identified as *Bacillus sphaericus* var. *rotans*. It was designated strain CO8.

The organisms were maintained on slopes of the medium given in Table 7 with 0.1% of sodium hexanoate as carbon source and solidified with 2% of washed agar.

METHODS

Separation and estimation of flavins

Extraction and concentration of flavins. Micro-organisms (approx. 2 g. dry wt.) were harvested from cultures with the Sharples Super-centrifuge and washed once by resuspending in 100–200 ml. of water and recentrifuging in tared tubes. The weight of cell paste was determined and the cells were resuspended in water to give approx. 200 mg. of wet cells/ml. Duplicate samples of 1 ml. were removed and dried at 110° for 4 hr. to determine the dry weight of organism present. To the remainder of the suspension was added 0.25 vol. of 50% (w/v) trichloroacetic acid, the whole mixed thoroughly and allowed to stand for 5 min. and the insoluble material removed by centrifuging at 9000 *g* for 10 min. In all subsequent operations reagents were chilled to 2° before use and, where possible, vessels were immersed in ice-water. Exposure to light was minimized by working in the dark or in artificial light.

The flavins were next removed from the trichloroacetic acid extract by adsorption on a column of Florisil. The column was of 11 mm. bore and contained 6 g. (12 ml.) of 30/60-mesh Florisil (Floridin Co., Tallahassee, Florida, U.S.A.) bounded at top and bottom by a plug of glass-wool. The Florisil was soaked in 0.1N-HCl for at least 16 hr. before making up the column, after which it was washed with water until the pH of the effluent rose to between 6 and 7. A known volume of trichloroacetic acid extract containing 0.2–1.0 μmole of total flavin was run on to the column as rapidly as possible and washed in with a few millilitres of 2% (v/v) acetic acid. The flow rate was then reduced to 5–10 ml./min. and the column washed successively with 100 ml. of 2% (v/v) acetic acid, 100 ml. of water and 100 ml. of aq. 0.5% (v/v) pyridine. During the first

two washes the flavins remained adsorbed as a yellow band at the top of the column, but moved slowly downwards on application of the 0.5% pyridine. When necessary this last washing was curtailed if the flavins approached the bottom of the column before the whole 100 ml. had been applied.

The flavins were next displaced from the column with aq. 25% (v/v) pyridine. Normally the yellow flavin fraction came off the column in 10–15 ml. In some cases where other pigments obscured the end of the flavin band, 20 ml. was collected after the flavin first began to emerge. Pyridine was removed from the flavin fraction by three extractions with 5–10 vol. of CHCl_3 followed by one extraction with ether. Dissolved ether was removed by aeration and the pH adjusted to 7 with NaOH. The volume and colour intensity of the resulting solution were then measured (stage A), after which the solution was freeze-dried. The solid product was extracted with a known volume of water, usually 1–2 ml., centrifuged and the supernatant used for paper electrophoresis.

A small precipitate was often observed on centrifuging (cf. Dimant *et al.* 1952), the amount varying with the sample of Florisil. It was reduced to 2–3 mg. dry wt. by selecting a suitable batch of adsorbent, by pretreating with HCl and by using 25% pyridine as eluent, which brings the flavins off the column in a smaller volume than the 5% pyridine used by Dimant *et al.* Under these conditions the amount of flavin remaining adsorbed on the precipitate was negligible.

Separation of flavins by paper electrophoresis. The apparatus used was that of Markham & Smith (1952). The flavin concentrate was applied to the paper strip (Whatman no. 3MM, 60 cm. $\times$ 10 cm.) as a transverse streak 8 cm. long and approx. 8 cm. from the middle of the paper. Between 0.1 and 0.2 μmole of total flavin was applied. In drying the solutions applied to the paper the use of hot air was avoided. The paper was next immersed in buffer except for a transverse strip 1 cm. wide containing the flavin streak, which was allowed to moisten by capillarity. Surplus buffer was removed from those parts of the paper which had been immersed by blotting between clean filter paper. The buffer was 0.025M-formic acid-ammonium formate, pH 4.6, and was replaced after each experiment. Higher buffer concentrations resulted in a higher current and hence increased heat output, whereas lower concentrations did not maintain a constant pH along the paper strip throughout electrophoresis. This was tested by spotting drops of indicator along the paper after the experiment.

Electrophoresis was for 2 hr., with an initial potential difference of 1000 v between the electrodes, giving a potential gradient of approx. 20 v/cm. and a current of 10 ma. During the experiment the current rose to 20 ma, the potential difference fell to 900 v and the temperature of the CCl_4 used as coolant rose about 20° above room temperature. The coolant was either replaced between experiments or allowed to cool and dried with pieces of filter paper.

After electrophoresis, the paper was dried at room temperature and the flavins were located by their yellow colour and yellow fluorescence under ultraviolet light. When a permanent record was required, the paper was photographed with panchromatic film and a 3 $\times$ yellow filter (Actina, London). The distance from camera lens to object was 3 ft., the lens aperture *f*3.5, the film Ilford FP3 and the illumination was provided by a strip ultraviolet lamp (Hanovia Ltd., Slough, Bucks) placed 2 ft. from the

object. Under these conditions an exposure of 60 sec. was satisfactory. Legends were written on strips of paper with a saturated solution of 5-aminoacridine as a fluorescent ink.

The mobilities of some flavin derivatives are given in Table 1. Riboflavin, FMN, FAD and lumichrome are readily separated but the conditions do not permit the adequate separation of riboflavin from lumiflavin and riboflavinyl glucoside. This limitation was not important for the present purpose.

After location of the flavin bands, strips containing the individual components were cut out and eluted with water (Dent, 1947) until the eluate was colourless and no fluorescence could be detected on the paper under ultraviolet light: about 0.5 ml. of eluate was required/cm. width of the strip. The volumes and colour intensities of the eluates were measured (stage B), after which they were concentrated by freeze-drying, where necessary, before being used for confirmatory tests.

Recoveries. When a mixture of riboflavin, FMN and FAD was treated with trichloroacetic acid and then subjected to the above procedure, the recovery of total flavin up to stage A was about 80% (Table 2). No loss of flavin was observed between stage A and the extraction of the freeze-dried material. In the routine procedure, the flavins were exposed to trichloroacetic acid for a total time of about 20 min., 5 min. being allowed for manipulations. Electrophoretic analysis showed that, with this treatment, the composition of the flavin mixture after freeze-drying

differed slightly from that of the starting material; the proportion of FAD decreased. The decrease was much more marked after 60 min. exposure to trichloroacetic acid and was due mainly to a breakdown of FAD to FMN (cf. Bessey, Lowry & Love, 1949). Recoveries over the electrophoretic separation were approx. 90% for riboflavin, FMN and FAD, singly or in combination (Table 3).

Spectrophotometric measurements. Flavin solutions were assayed by measuring the intensity of their yellow colour. For standardizing flavin solutions, and in some preliminary experiments, the Unicam SP. 500 instrument was used and the amount of flavin calculated from the optical density at 450 m μ , with the values for molecular-extinction coefficients (ϵ) given by Whitby (1953), i.e. 12.2×10^3 l. mole⁻¹ cm.⁻¹ for riboflavin and FMN; 11.3×10^3 l. mole⁻¹ cm.⁻¹ for FAD. In routine experiments, extinctions were measured in the Hilger-Spekker absorptiometer with the Ilford no. 601 filter (maximum transmission at 430 m μ , transmission at 450 m μ about half of the maximum) and with microcells of 0.5 ml. capacity and 1 cm. light path. This instrument was calibrated separately for riboflavin, FMN and FAD with electrophoretically purified samples of each, standardized with the Unicam SP. 500 instrument.

With the Spekker instrument, the experimentally determined values of ϵ were identical for riboflavin and FMN ($\epsilon_{\text{RBF}} = \epsilon_{\text{FMN}} = 11.0 \times 10^3$ l. mole⁻¹ cm.⁻¹) but the value for FAD was somewhat lower ($\epsilon_{\text{FAD}} = 9.8 \times 10^3$ l. mole⁻¹ cm.⁻¹). For mixtures of these three flavins, the appropriate molecular extinction coefficient (ϵ_M) could be obtained, provided that the molar proportion of FAD (x) was known, from the equation:

$$\epsilon_M = (1 - x) \epsilon_{\text{RBF}} + x \cdot \epsilon_{\text{FAD}}.$$

This relationship was therefore used where extinction measurements on mixtures were used to determine total flavin. Calculation of flavin contents of micro-organisms from extinction measurements is described later under Results.

Confirmatory tests on separated flavin fractions

No tests were done with the riboflavin fractions as these usually contained insufficient flavin. Tests on the FMN and FAD fractions were carried out as follows.

Table 1. *Electrophoretic mobilities of some flavins*

Potential gradient 20v/cm.; duration of experiment 2 hr. Buffer 0.025M-formic acid-ammonium formate, pH 4.6. Other details are given in the text.

Substance	Distance travelled towards anode (cm.)
Riboflavin	-1.6
FMN	10.1
FAD	15.0
Lumiflavin	-1.3
Riboflavinyl glucoside	-1.8
Lumichrome	-0.2

Table 2. *Recovery of flavins during treatment before electrophoresis*

To flavins contained in 20 ml. (Expt. 1) or 13 ml. (Expt. 2) at room temp. (19°), 0.25 vol. of 50% trichloroacetic acid was added and the mixture was applied to the Florisil column after the time indicated. Procedure described in the text was then followed and the composition of the initial and final flavins was determined by electrophoresis. Total flavin was calculated from the extinction at each stage as indicated in the text. In calculating the total flavin at stage A, the proportions of the three flavins were assumed to be the same as after freeze-drying.

	Time of standing with trichloroacetic acid (min.)	Stage of treatment	Total flavin (μ m-moles)	Recovery (%)	Flavin in each fraction (%)		
					Riboflavin	FMN	FAD
Expt. 1	20	Initial flavin	597	—	9	41	50
		Before freeze-drying (stage A)	462	78*	—	—	—
		Extract of freeze-dried material	—	—	11	45	44
Expt. 2	60	Initial flavin	889	—	9	41	50
		Before freeze-drying (stage A)	728	82*	—	—	—
		Extract of freeze-dried material	731	100†	11	52	37

* As percentage of initial flavin.

† As percentage of flavin at stage A.

Table 3. *Recovery of flavins during electrophoresis*

Details of electrophoresis and assay of solutions are given in the text. Initial proportions of the three flavin compounds in the mixture were unknown, but were assumed to be the same as for the recovered flavins in calculating the initial total flavin as indicated in the text.

Test substance	Initial flavin ($\mu\text{m-moles}$)	Recovered flavin ($\mu\text{m-moles}$)	Recovery (%)
Single flavins			
Riboflavin	53	49	93
FMN	59	54	91
FAD	67	61	92
Mixture			
Riboflavin	—	21	—
FMN	—	49	—
FAD	—	92	—
Total	181	162	90

Electrophoresis and chromatography on paper. Electrophoresis was carried out under the same conditions as the original separation but the flavins were applied as spots, and markers were also run on the same strip. The four solvents used for chromatography were: 1, aq. 5% Na_2HPO_4 , single phase, ascending (Carter, 1950); 2, water saturated with isopentanol, single phase, ascending (Whitby, 1952); 3, acetone-isobutyric acid-water (55:15:30 by vol.), single phase, ascending; 4, phenol-butan-1-ol-water (160 g.:30 ml.:100 ml.) lower phase mobile, descending (Dimant *et al.* 1952). Whatman no. 1 filter paper was used throughout and flavins were applied and located as in electrophoresis. Phenol quenches the fluorescence of flavins and had to be removed after using solvent 4. The chromatogram was therefore dried at room temperature, and then washed twice with xylene and once with ether. Riboflavin, FMN and FAD markers were included on all chromatograms.

D-Amino acid oxidase test. Tests for FAD were done with the apoenzyme of D-amino acid oxidase as described by Warburg & Christian (1938). Samples of eluates containing 3–10 $\mu\text{m-moles}$ of flavin were used. The same quantities of FAD increased the uptake of O_2 by 100–300 $\mu\text{l./hr.}$

Behaviour of other flavins. During recent years, a number of new flavin derivatives have been obtained from biological materials and their behaviour in the present procedure must be considered. Riboflavinyl glucoside (Whitby, 1952) has almost the same electrophoretic mobility as riboflavin and would not be distinguished from it, since no confirmatory tests were carried out on the riboflavin fraction. To date it has not been reported in bacteria though Tachibana & Katagiri (1955*a*) found it in the medium after growth of a mutant of *Aspergillus oryzae*. The electrophoretic behaviour of the FAD-X of Huennkens, Sanadi, Dimant & Schepartz (1953), the 'fourth flavin compound' of Yagi (1953) and the riboflavin diphosphate of Tachibana & Katagiri (1955*b*) is unknown. However, FAD-X is known to be inactive in the D-amino acid oxidase test and separates from riboflavin, FMN and FAD in solvent 4 (Dimant *et al.* 1952). Yagi's compound separates in solvent 1 (K. Yagi, personal communication). The behaviour of riboflavin diphosphate in the confirmatory

tests is unknown but in view of its chemical nature the possibility that it would remain undetected in all the tests employed seems remote.

The presence of lyxoflavin in human heart has been reported by Pallares & Garza (1949). This compound differs from riboflavin solely in the configuration of C-4 of the pentose residue and cannot at present be distinguished from riboflavin on a small scale. In what follows, it has been assumed for convenience that the flavins found in micro-organisms are all derivatives of riboflavin, but the possible occurrence of the corresponding lyxoflavin derivatives is not excluded.

Choice of extraction procedure

Extracting agent. Methanol (Kuhn, Wagner-Jauregg & Kaltschmidt, 1934), acetone (Euler & Adler, 1934), Takadiastase (Emmett, Bird, Brown, Pescock & Vandenbelt, 1941), trichloroacetic acid (Bessey *et al.* 1949; Dimant *et al.* 1952) and various forms of heat treatment (Warburg & Christian, 1938; Hodson & Norris, 1939; Yagi, 1951; DeLuca, Weber & Kaplan, 1956) have all been used for the extraction of flavins from biological materials. However, only Yagi (1951) and Bessey *et al.* (1949) have been concerned with the separate estimation of riboflavin, FMN and FAD and have examined the fate of these compounds when subjected to their analytical procedures. These authors worked almost exclusively with animal tissues. Bessey *et al.* extracted homogenized tissues with 10% trichloroacetic acid at 0° for 15 min.; Yagi heated for 2 min. at 80°, homogenized and then heated again at 80° for a further 15 min. Although trichloroacetic acid causes a slow breakdown of FAD at the temperature employed here, it was preferred in the present work because (a) heat treatment may produce artifacts under some conditions: thus Yagi (1953) found that if in his procedure the second heat treatment were modified by heating at 90° for 1 hr. a new flavin was obtained from liver, and the same material could be obtained by heating FAD alone at 95°; (b) heating procedures do not completely remove protein, necessitating further treatment (cf. Warburg & Christian, 1938; Yagi, 1951). Thus extracts of dried cells of the rumen organism LC1 prepared according to DeLuca *et al.* (1956) are green; on adding trichloroacetic acid to a final concentration of 10% a heavy white precipitate is formed and the colour of the supernatant changes to the characteristic yellow of free flavins.

Extraction conditions. Optimum conditions for extraction of flavins by trichloroacetic acid were established for the rumen organism LC1. This organism has a relatively high flavin content and the yellow colour of extracts is probably entirely due to flavin, since (a) no other chromogens were detected in any of the fractions when the flavins of this organism were examined as described above; (b) extracts prepared by various methods are almost completely bleached on adding sodium dithionite, and extracts prepared from dried cells according to Stadtman & Barker (1949) are bleached by H_2 . It was therefore possible to use the colour intensity to follow the extraction of flavins from this organism by trichloroacetic acid.

From the results (Table 4) extraction with 10% trichloroacetic acid for 5 min. at room temperature was selected as the most suitable procedure. These conditions ensure maximum extraction and avoid the slow fall in flavin content of the extract which occurs after longer treatment. No advantage was gained by increasing the

concentration of trichloroacetic acid and at a lower temperature (10°) the extraction was less rapid initially.

Efficiency of extraction. The above experiments establish optimum conditions for extraction with trichloroacetic acid but do not imply that this agent is completely effective either in rendering the cells permeable or in dissociating flavins from flavoproteins. Trichloroacetic acid appears to destroy the permeability barriers of the microbial cell since, when cells of LC1, yeast or *Desulphovibrio* were pre-treated either by acetone-drying or by crushing in a bacterial press, no increase was observed in the total extractable flavin (Table 5). There is therefore no advantage in such pretreatments; in fact it is better to omit them since both the crushing of LC1 and the acetone-drying of *Desulphovibrio* lead to a decrease in extractable flavin. In addition, whereas these treatments are without effect upon

the relative amounts of riboflavin, FMN and FAD extracted from yeast and LC1 (Table 5), acetone-drying of *Desulphovibrio* results in a marked breakdown of FAD to FMN. The analysis of acetone-dried powders in lieu of fresh cells may therefore give erroneous results.

As regards the effectiveness of trichloroacetic acid in dissociating flavins from protein, it was observed with the organism LC1 that whereas whole cells are a deep green-brown, the residue after extraction with the acid is almost pure white, showing that the release of flavin is virtually complete. In applying the method it has been assumed that this holds true for other organisms. In some cases the residues after extraction were not colourless, e.g. with *Micrococcus lysodeikticus* a bright-yellow residue of a hue different from that of flavoproteins was obtained. In such cases the residue was resuspended in water, heated for

Table 4. *Extraction of flavin from cells of LC1 with trichloroacetic acid*

Sufficient trichloroacetic acid to give the required final concentration was added in a volume of 3.3 ml. to samples (6.7 ml.) of a suspension of washed cells of the organism LC1 (approx. 375 mg. of wet cell paste/ml.). After the times indicated, the mixtures were centrifuged for 5 min. at 9000 g and the optical density of the supernatant was determined with the Hilger-Spekker instrument. Trichloroacetic acid (25%) has zero optical density under the conditions used.

Expt.	Concn. of trichloroacetic acid (%)	Temp.	Optical density					
			0 min.	1 min.	5 min.	10 min.	30 min.	60 min.
Expt. 1	2	20°	0.16	—	—	0.15	0.19	0.24
	4	20	0.19	—	—	0.39	0.60	0.58
	8	20	0.58	—	—	0.77	0.67	0.66
	16	20	0.80	—	—	0.79	0.72	0.74
Expt. 2	10	20	—	—	0.62	—	—	—
	15	20	—	—	0.60	—	—	—
	20	20	—	—	0.59	—	—	—
	25	20	—	—	0.63	—	—	—
Expt. 3	10	20	—	0.76	—	0.70	0.69	0.66
	10	10	—	0.63	—	0.71	0.68	0.67

Table 5. *Effect of acetone-drying and crushing of cells on extractable flavins*

A different preparation of washed cell paste (or pressed cake of baker's yeast) was used in each experiment. Where indicated, one-half of the preparation was crushed without abrasive in a bacterial press (Hughes, 1951) or acetone-dried according to Umbreit, Burris & Stauffer (1949); in the latter case, the precipitated material was washed with acetone then ether before drying. Flavins were extracted as described in the text. With the acetone-dried powder a suspension containing 85 mg. dry wt./ml. was used and with crushed cells each millilitre of suspension contained the product from 200 mg. of wet cells. Flavin contents are given in terms of dry wt. of the original cells. Values in parentheses are in terms of dry wt. of acetone-dried powder.

Expt.	Organism	Pretreatment	Total flavin (μ m-moles/g. dry wt.)	Flavin in each fraction (%)			Calculation method for total flavin (see text)
				Riboflavin	FMN	FAD	
1	LC1	None	1330	0	31	69	2
		Acetone-dried	1300	0	29	71	2
2	LC1	None	1470	2	27	71	2
		Crushed	1050	2	27	71	2
3	Baker's yeast	None	—	3	54	43	2
		Acetone-dried	—	1	49	50	2
4	Baker's yeast	None	65	5	44	51	2
		Crushed	63	6	42	52	2
5	<i>Desulphovibrio</i>	None	116	0	21	79	1
		Acetone-dried and stored for 4 days at room temp.	61 (105)	0	44	56	1
6	<i>Desulphovibrio</i>	Acetone-dried and stored for 20 hr. at room temp.	— (122)	1	63	36	1

20 min. at 100° and re-centrifuged. No change was ever apparent in the colour of the residue, though in a few cases a faint-brown colour appeared in the supernatant.

It thus seems probable that the present procedure extracts virtually all the flavin of microbial cells. However, recent studies by Boukine (1955) and by Kearney & Singer (1955) with the succinic dehydrogenase of animal tissues have demonstrated that the flavin component is not completely released by boiling or treatment with cold acid. On treating with proteolytic enzymes, a flavin derivative is released which probably contains FAD linked to a peptide (Singer, Kearney & Massey, 1956). Such derivatives would not be extracted by the procedure usually employed for the extraction of flavins. In assessing the present results it should therefore be remembered that they refer to flavin extractable with trichloroacetic acid.

RESULTS

Qualitative aspects. Electrophoretic fractions in the positions corresponding to FMN and FAD were obtained from all the organisms examined. With most organisms a faint band was also obtained in the riboflavin position. These were the only fractions which were yellow in visible light and fluoresced yellow under ultraviolet light and were therefore presumably the only derivatives of riboflavin. In some cases a faint band was observed which fluoresced pale blue under ultraviolet light and which remained on or near the starting line. This was presumed to be lumichrome formed by the breakdown of some of the flavin during manipulation.

With several organisms other pigments were observed. When the extract of *Desulphovibrio* with trichloroacetic acid was applied to the Florisil column, the band which formed at the top was deep green instead of the usual yellow. During the

wash with 0.5% pyridine, a blue pigment was eluted which turned red on adding excess of alkali and was presumably the prosthetic group of the 'desulphoviridin' of Postgate (1956). *Propionibacterium pentosaceum*, *M. lysodeikticus*, *Pseudomonas* OD 1, *Rhodospirillum rubrum* and *Chlorobium thiosulphatophilum* gave rise to a red-brown band on the Florisil. Much of the contaminating pigment remained in the flavin fraction after elution but separated upon electrophoresis to give a red-brown band, which fluoresced faintly blue under ultraviolet light and migrated towards the cathode with a mobility about double that of riboflavin. A different material having a similar mobility was observed in preliminary experiments with baker's yeast. This material fluoresced orange under ultraviolet light but was not detectable in visible light. It was not observed in all batches of baker's yeast and was not present in the pure culture of *Saccharomyces cerevisiae* grown as described in Table 7.

The identity of the FMN and FAD fractions was confirmed in all cases by the tests described above. The results with LC1 are given in Table 6 as an example. In the chromatographic tests, traces of substances other than the main component were often observed. These were always in positions corresponding to known decomposition products, e.g. traces of FMN and lumichrome were detected in some FAD fractions and were attributed to decomposition during the manipulations after electrophoresis.

Calculation of quantitative results. The recovery experiments (Tables 2 and 3) show that when the procedure adopted for extraction and separation of flavins is applied to known mixtures of riboflavin,

Table 6. *Confirmatory tests on the flavin mononucleotide and flavinadenine dinucleotide fractions of the rumen organism LC1*

Experimental details are given in the text. Rates of oxygen uptake were measured over the period 0-60 min.

1. Chromatography and electrophoresis

Markers	Chromatography R_F values				Electrophoresis distance moved towards anode (cm.)
	Solvent 1	Solvent 2	Solvent 3	Solvent 4	
Riboflavin	0.27	0.38	0.52	0.79	-1.1
FMN	0.49	0.90	0.26	0.09	8.6
FAD	0.35	0.89	0.07	0.15	14.2
Fractions from LC1					
FMN	0.50	0.89	0.26	0.10	8.8
FAD	0.35	0.89	0.07	0.16	14.3

2. D-Amino acid oxidase test

Test substance	Rate of O_2 uptake (μ l./hr.)
None	-5
Authentic FAD (8 μ m-moles)	138
Fractions from LC1	
FMN (7 μ m-moles)	10
FAD (8 μ m-moles)	140

FMN and FAD, substantial losses of flavin occur, although the relative proportions of the three flavins change only slightly. Consequently, in order to obtain a value for the flavin content of the original cells, correction factors have to be introduced and certain assumptions become necessary. The assumptions which have been made are: (a) that the proportions of the three flavins do not change during the manipulations; (b) that after extraction with trichloroacetic acid the flavins are evenly distributed throughout the total volume of cells plus extractant; (c) that the recovery of flavins up to stage A is 80%; (d) that the recovery between stages A and B is 90%. This last assumption is only necessary when interfering pigments are present at stage A.

The amounts of flavin in the individual fractions after electrophoresis were obtained from the measurements at that stage (B). The relative proportions of the three flavins at stage B could thus be found and by assumption (a) this gave the relative proportions in the original cells and at any stage of the manipulations.

The total flavin content of the original cells (F) was calculated from the total flavin at either stage A or stage B (F_A , F_B respectively) according as interfering pigments were absent or present at stage A, the following relationships being used:

$$F = F_B \cdot \frac{100}{80} \cdot \frac{100}{90} \cdot \frac{V_B}{mV_C} \quad \text{Method 1}$$

$$F = F_A \cdot \frac{100}{80} \cdot \frac{V_B}{mV_C} \quad \text{Method 2}$$

where V_B = total volume of cells plus extractant; V_C = volume of extract put on to Florisil column; m = dry wt. of cells extracted.

With Method 1, F_B was obtained by summing the amounts of the individual flavins at stage B. With Method 2, the measurements on the mixture at stage A were used to calculate F_A , with the appropriate value for the molecular extinction coefficient (ϵ_M) obtained as described in the Methods section. For this purpose it was necessary to know the molar proportion of FAD (x), and this was obtained from the measurements at stage B with assumption (a). In a few cases where quantitative data on the three individual flavin fractions were not obtained at stage B, it was assumed that $x = 0.5$. The maximum possible error so introduced in the value for F is 6%.

Both methods 1 and 2 involve assumptions (b) and (c). Method 1 involves in addition assumption (d) and also necessitates quantitative handling between stages A and B which is inconvenient on account of the small volumes used. Method 2 does not have these disadvantages, and although it brings in assumption (a), this is required in any

case to obtain the relative proportions of the three flavins in the original cells. In calculating F therefore method 2 is preferable but can be used only when no interfering pigments are present at stage A. Furthermore, it is possible to dispense with the quantitative handling between stages A and B only if it is known before electrophoresis that no contaminating pigments are present. Fortunately, it was possible with a little experience to decide if this were so from the appearance of the concentrate at stage A and in all cases where such pigments were judged to be absent by this means, none was found on subsequent electrophoresis. Method 2 was therefore used when no contaminating pigments were present, otherwise method 1 was used.

With *Thiobacillus denitrificans*, the value for total flavin could not be obtained as the cells harvested were contaminated with large amounts of sulphur.

Quantitative aspects. With the exception of the hexanoate-oxidizing organisms, which will be considered separately, the micro-organisms examined and results obtained are given in Table 8 in ascending order of flavin content. Details of the organisms and their growth are given in Table 7. There is a wide range of flavin contents, from 30 to 2930 $\mu\text{m-moles/g. dry wt.}$ with *Rhodospirillum rubrum* and *Clostridium kluyveri* respectively. In general, the higher flavin contents were associated with anaerobic growth. For example, anaerobically grown cells of *Saccharomyces cerevisiae* were found to contain about 50% more flavin than aerobically grown cells, in agreement with the earlier observations of Pett (1935) and of Singh, Agarwal & Peterson (1948). The highest amounts of flavin were found in those strict anaerobes producing, as fermentation products, higher volatile fatty acids (HVFA) containing four or more carbon atoms. These organisms all contained more than 450 $\mu\text{m-moles/g. dry wt.}$, whereas the highest value observed with a non-HVFA-producer was 360 $\mu\text{m-moles/g. dry wt.}$ The HVFA-producers are all members of the genus *Clostridium* with one exception, the rumen organism LC1, which though morphologically distinct from the members of this genus resembles them biochemically (Elsden *et al.* 1956). Outstandingly high flavin contents were found in LC1 and *Cl. kluyveri*, which ferment lactate and ethanol respectively to yield mainly HVFA.

The data on the proportion of flavin present in the three fractions show that virtually all the flavin is present in the two known coenzyme forms FMN and FAD. Indeed, except with *Cl. skatologenes* and possibly a few other organisms, the proportion of free riboflavin is so low as to suggest that it might well have arisen by breakdown of FMN

Table 7. *Organisms and growth conditions*

	Organism	Source	Medium	Growth conditions			
				Duration (hr.)	Temp.	Initial gas phase	Other details
1.	<i>Rhodospirillum rubrum</i> S1	NCIB 8255	Malate-citrate-yeast extract-salts medium of Elsdén & Ormerod (1956)	96	25°	None	Full Roux bottles with illumination
2.	<i>Micrococcus lysodeikticus</i>	NCTC 2665	1% Glucose, 0.5% Pronutrin, 2% (v/v) yeast autolysate, 2% agar. pH 7	72	37	Air	Surface growth in Roux bottles
3.	<i>Saccharomyces cerevisiae</i>	Sheffield	2% Glucose, 10% (v/v) yeast autolysate. pH 7	22	25	Air	Penicillin flasks with shaking
4.	<i>Desulphovibrio desulphuricans</i> (Hildenborough)	NCIB 8303	0.35% Sodium lactate, 0.4% Difco yeast extract, 0.2% $MgSO_4 \cdot 7H_2O$, 0.1% Na_2SO_4 , 0.1% NH_4Cl , 0.05% K_2HPO_4 , 0.01% $CaCl_2$, 0.1 mm- $Na_2S \cdot 9H_2O$. pH 7.5	72	30	H_2-CO_2	—
5.	<i>Azotobacter agilis</i>	NCIB 8004	0.5% Mannitol, 0.02% calcium acetate, 0.1% K_2HPO_4 , 0.02% $MgSO_4 \cdot 7H_2O$, 0.0002% Na_2MoO_4 , 0.0001% $FeSO_4 \cdot 7H_2O$, 0.0001% NaCl. pH 7.6	41	25	Air	Penicillin flasks with shaking
6.	<i>Bacillus subtilis</i>	NCTC 3610	2% Glucose, 2% (v/v) yeast autolysate. pH 7	18	25	Air	Penicillin flasks with shaking
7.	<i>Saccharomyces cerevisiae</i>	As no. 3	As no. 3	22	25	Air	Deep culture
8.	<i>Bacillus megaterium</i>	NCIB 8508	2% Glucose, 2% (v/v) yeast autolysate. pH 7	18	25	Air	Penicillin flasks with shaking
9.	<i>Lactobacillus casei</i>	NCTC 8010	2% Pronutrin, 2% glucose, 1% sodium acetate ($2H_2O$), 0.45% KH_2PO_4 , 4% (v/v) yeast autolysate, 0.5% (v/v) of salts B mixture of Barton-Wright (1946). NaOH to pH 7.6	17	37	Air	Deep culture
10.	<i>Pseudomonas</i> OD1	Sheffield	Oxalate-salts medium A of Jayasuriya (1955)	48	25	Air	Penicillin flasks

Under 'Source', Sheffield, NCIB and NCTC refer respectively to organisms maintained in the Microbiology Department, Sheffield University, the National Collection of Industrial Bacteria and the National Collection of Type Cultures. Yeast autolysate was prepared according to Barker & Beck (1942). Pronutrin is a dried commercial casein digest (Herts Pharmaceuticals Ltd., Welwyn) now unobtainable. Penicillin flasks (Jayasuriya, 1955) contained 300 ml. of medium and were subjected to continuous mechanical shaking where indicated. For

'deep culture' Erlenmeyer flasks (3-5 l capacity) contained medium to within a few inches of the top. The two photosynthetic organisms were grown in full Roux bottles closed with rubber bungs. When gas phases other than air were used, culture vessels were designed to allow gas to be bubbled through the culture initially and to allow gas produced during fermentation to escape through a valve. H_2-CO_2 and N_2-CO_2 mixtures both contained 5% (v/v) of CO_2 .

11. <i>Escherichia coli</i> K 4	Sheffield	2% Glucose, 5% (v/v) yeast autolysate, 0.1% Marmite, 1% Pronutrin. pH 7	22	31	Air	Penicillin flasks with shaking
12. <i>Chlorobium thiosulphatophilum</i>	Dr H. Larsen	Thiosulphate-bicarbonate-salts medium of Larsen (1953)	72	30	None	Full Roux bottles with illumination
13. <i>Lactobacillus helveticus</i>	NCTC 4113	Glucose-peptone-growth factors-salts. Riboflavin-free medium of Barton-Wright (1946) with xylose omitted and 10 µg. of riboflavin/100 ml. added	22	37	Air	Deep culture
14. <i>Propionibacterium pentosaceum</i> E21	Dr C. B. van Niel	1.4% Sodium lactate, 10% (v/v) yeast autolysate. pH 7	70	30	N ₂ -CO ₂	—
15. <i>Clostridium welchii</i> SR12	NCTC 6784	2% Pronutrin, 10% (v/v) supernatant from Robertson's meat medium	6	37	H ₂ -CO ₂	—
16. <i>Pseudomonas stutzeri</i>	Dr C. B. van Niel	10% (v/v) Yeast autolysate, 0.5% KNO ₃ . pH 7	17	30	Air	Deep culture
17. <i>Pseudomonas aeruginosa</i>	NCTC 6750	10% (v/v) Yeast autolysate. pH 7	16	25	Air	Penicillin flasks with shaking
18. <i>Clostridium sporogenes</i>	Sheffield	1% Glucose, 1% Pronutrin, 0.5% Marmite, 2% (v/v) supernatant from Robertson's meat medium. pH 7	12	37	H ₂ -CO ₂	—
19. <i>Clostridium scatologenes</i>	Dr R. F. Rosenberger	1% Glucose, 1% Pronutrin, 3% (v/v) yeast autolysate. pH 7	44	37	H ₂ -CO ₂	—
20. <i>Clostridium butyricum</i>	NCIB 6184	2% Glucose, 5% (v/v) yeast autolysate. pH 7	17	37	H ₂ -CO ₂	—
21. Rumen organism LC1	Sheffield NCIB 8927	Lactate-corn-steep liquor medium (Walker, 1958)	16	37	H ₂ -CO ₂	—
22. <i>Clostridium klayveri</i> K1	Dr H. A. Barker	Ethanol-salts medium of Stadtman & Barker (1949) but with biotin plus <i>p</i> -aminobenzoic acid replaced by 0.05% of Difco yeast extract	48	37	H ₂ -CO ₂	—
23. <i>Thiobacillus denitrificans</i>	Sheffield	Thiosulphate-nitrate-salts medium of Baalsrud & Baalsrud (1952) as modified by Trudinger (1956)	96	28	Air	Deep culture
Hexanoate-oxidizing organisms						
24. <i>Pseudomonas</i> CO1	Soil isolates NCIB 8865 NCIB 8866 NCIB 8867	0.2% Na ₂ HPO ₄ , 0.1% KH ₂ PO ₄ , 0.1% (NH ₄) ₂ SO ₄ , 0.1% (v/v) yeast autolysate, 0.04% MgSO ₄ ·7H ₂ O.	(See Table 9			
25. <i>Pseudomonas</i> CO2		Carbon source as indicated in Tables 9 and 10.	(See Table 9			
26. <i>Bacillus sphaereticus</i> var. <i>rotans</i> CO8		pH 7	(See Table 9			
			25	25	Air	Penicillin flasks
			25	37	Air	Penicillin flasks
			37		Air	Penicillin flasks

and FAD in the steps before electrophoresis. The situation is thus similar to that in animal tissues, where Bessey *et al.* (1949) and Yagi (1951) found that in general free riboflavin accounts for less than 3% of the total flavin in normal tissues. The present values for the proportion of FAD should be regarded as minimum figures since there is a small breakdown of FAD to FMN under the conditions of extraction (Table 2), and this has not been corrected for. Nevertheless, it is clear that micro-organisms differ from animal tissues in the proportion of flavin present as FAD. In a variety of animal tissues most of the flavin is FAD, whereas in micro-organisms proportions of FMN and FAD vary from 30% of FAD and 68% of FMN with *Lactobacillus helveticus* to 79% of FAD and 21% of FMN with *Desulphovibrio*. Within the group of HVFA-producers there is much less variation and the proportion of FAD is high.

The data already in the literature on the flavin contents of micro-organisms are fragmentary. Results calculated from the data of other authors are given in parentheses in Table 8: where necessary it has been assumed that wet cells contain 20% of dry matter. In almost all cases the media and growth conditions have not been adequately described and comparison is therefore difficult, but in general there is rough agreement with the present results. An exception is the value of

20 $\mu\text{m-moles/g. dry wt.}$ for *Bacillus subtilis* found by Pett (1935), which is low compared with the present results for *Bacillus* species. Pett used an early method which was relatively crude, being based upon the comparison of the fluorescence of an ethanolic extract with that of a standard solution of riboflavin. It is noteworthy in this respect that Pett failed to detect flavin in *Escherichia coli*, although he reported that his extract showed a blue fluorescence. The present results are therefore probably more reliable. The value of 480 $\mu\text{m-moles/g. dry wt.}$ for total flavin in *Lactobacillus arabinosus* (DeLuca *et al.* 1956) is much higher than values found here for two lactobacilli, and the figure (96%) of DeLuca *et al.* for the proportion of FAD is much higher. These authors also report a remarkable finding with *Cl. kluyveri*. The total flavin content of the organism when grown on synthetic medium (1830 $\mu\text{m-moles/g. dry wt.}$) was comparable with the present result, but when the organism was grown on 'complex medium', the flavin content was about 8% of this value. The significance of this result is difficult to assess as the authors give no details of growth conditions or the medium used. Bornstein & Barker (1948) found that *Cl. kluyveri* required, as carbon source for growth, ethanol plus acetate, propionate or butyrate and no other compounds were found which would replace these.

Table 8. *Flavin contents of some micro-organisms*

Numbers preceding names of organisms refer to positions in Table 7.

Organism	Total flavin ($\mu\text{m-moles/g. dry wt.}$)	Flavin in each fraction (%)			FAD content ($\mu\text{m-moles/g. dry wt.}$)	Calculation method
		Riboflavin	FMN	FAD		
1. <i>Rhodospirillum rubrum</i>	30	7	38	55	16	1
2. <i>Micrococcus lysodeikticus</i>	40	0	28	72	27	1
3. <i>Saccharomyces cerevisiae</i> (aerobic)	100*	0	47	53	52	2
4. <i>Desulphovibrio desulphuricans</i>	116	0	21	79	92	1
5. <i>Azotobacter agile</i>	125	0	47	53	67	2
6. <i>Bacillus subtilis</i>	125 (20)†	0	41	59	74	2
7. <i>Saccharomyces cerevisiae</i> (anaerobic)	135*	—	—	—	—	2
8. <i>Bacillus megaterium</i>	160	0	43	57	92	2
9. <i>Lactobacillus casei</i>	180	0	55	45	81	2
10. <i>Pseudomonas</i> OD1	200	8	41	51	104	1
11. <i>Escherichia coli</i>	245 (346)‡	0	67	33	81 (80)§	2
12. <i>Chlorobium thiosulphatophilum</i>	265	4	46	50	132	1
13. <i>Lactobacillus helveticus</i>	270	2	68	30	81	2
14. <i>Propionibacterium pentosaceum</i>	275	7	49	44	121	1
15. <i>Clostridium welchii</i>	290	5	40	55	160	2
16. <i>Pseudomonas stutzeri</i>	310	6	34	60	185	2
17. <i>Pseudomonas aeruginosa</i>	360	2	38	60	217	2
18. <i>Clostridium sporogenes</i>	460	3	25	72	330	2
19. <i>Clostridium scatologenes</i>	480	13	33	54	260	2
20. <i>Clostridium butyricum</i>	580 (490)§	2	31	67	390	2
21. Rumen organism LC1	1480	2	25	73	1080	2
22. <i>Clostridium kluyveri</i>	2930 (1830)§	2	25	73	2140	2
23. <i>Thiobacillus denitrificans</i>	—	0	45	55	—	—

* Values between 96 and 245 $\mu\text{m-moles/g. dry wt.}$ have been reported by various authors (Ingram, 1955). DeLuca *et al.* (1956) found an FAD content of 82 $\mu\text{m-moles/g. dry wt.}$

† Pett (1935).

‡ Yagi & Mitsuhashi (1951).

§ DeLuca *et al.* (1956).

In the present work cultures were harvested in the late log phase of growth, as judged roughly either by the rate of gas evolution where this occurred or by the visual appearance of the culture. With several organisms more than one culture was examined and in all such cases the quantitative results showed good agreement (e.g. the data for LC1 in Tables 5 and 8). However, the comparison of aerobically and anaerobically grown *Sacch. cerevisiae* and the effect of hexanoate on certain organisms (see below) show that growth conditions may have a marked effect upon flavin content and this may account for some of the discrepancies between the results of different authors.

Hexanoate-oxidizing organisms. In view of the association of a high flavin content with the fermentative production of HVFA, it appeared of interest to examine the flavin contents of organisms oxidizing such acids. Accordingly, the three hexanoate-oxidizers, *Pseudomonas* strains CO 1 and CO 2 and *Bacillus sphaericus* CO 8, were isolated and studied.

When grown with hexanoate as carbon source, the two *Pseudomonas* strains contained approximately the same amount of flavin as *Ps. stutzeri* and *Ps. aeruginosa* but appreciably less than the HVFA-producers (Table 9). *B. sphaericus* grown on hexanoate also contained less than the HVFA-producers, but about three times as much as the other *Bacillus* species examined. When other carbon sources were used a marked response to growth on hexanoate was revealed. The wet cell pastes of glucose-grown cells of *Pseudomonas* CO 1 and CO 2 were much paler than hexanoate-grown cells, which are a light-buff colour, and the flavin content was less than one-half of that of hexanoate-grown cells. *B. sphaericus* does not utilize glucose as carbon source but similar effects were observed with acetate-grown cells.

In hexanoate-grown cells of all three organisms a high proportion of the total flavin was FAD. The proportion of FAD in cells grown on other carbon sources was determined with only one organism, namely glucose-grown *Pseudomonas* CO 1, and was much lower than with hexanoate-grown cells.

These results could be completely correlated with the rate of oxygen consumption of washed cells in the presence of hexanoate after growth on the different carbon sources. With all three organisms, the oxidation of hexanoate by washed cells appears to be dependent upon its presence during growth (Table 10). The results should, however, be interpreted with caution. The organisms grow more slowly on hexanoate than on other carbon sources and hexanoate-grown cells were therefore harvested after longer periods of growth. It is notable that with glucose-grown cells of the two

Pseudomonas strains not only the rate of hexanoate oxidation, but also the rate with glucose and the endogenous rate are less than with hexanoate-grown cells. Similar remarks apply to acetate-grown cells of *B. sphaericus*. The apparent adaptation to hexanoate oxidation might therefore merely be one manifestation of a generally lower metabolic activity of cells grown on substrates other than hexanoate. However, with all three organisms the response of hexanoate oxidation was considerably greater than the response of glucose or acetate oxidation. It therefore seems reasonable to conclude that the ability to oxidize hexanoate and the formation of flavin, particularly FAD, are adaptive, though whether this is due to induced synthesis or the selection of mutants remains an open question.

Application of the method to other materials. No difficulties were encountered when the method was applied to guinea-pig liver, which was first homogenized for 3 min. with 4 ml. of 12.5% trichloroacetic acid/g. of wet liver and then extracted in the usual way. The following results were obtained: total flavin, 352 μ m-moles/g. dry wt., distributed as riboflavin 0%, FMN 29%, FAD 71%. Values for rat liver calculated from the data of Yagi (1951), assuming the same dry wt. content (24%) as that found for guinea-pig liver, are: total flavin, 453 μ m-moles/g. dry wt., distributed as riboflavin 1%, FMN 9%, FAD 90%.

Three plant materials, cabbage leaves, tomato fruits and beetroot, were also tested and were first frozen at -20° to disrupt the cells and then minced before extraction. The method failed with beetroot because the red pigment was still present in the material taken for electrophoresis and spread over the whole of the paper during the attempted separation. Cabbage and tomato both gave rise to a red-brown pigment which separated upon electrophoresis, as was observed with some of the bacteria. The amount of flavin in these two plants was small and the flavin bands were very streaky and incompletely separated, presumably owing to the presence of relatively large amounts of interfering substances. It was possible to identify FMN and FAD in the appropriate bands but the method in its present form appears unsuitable for quantitative work with plant materials.

DISCUSSION

The methods proposed to date for the estimation of the individual flavins in biological materials are of two general types. The method of Yagi (1951) and that described here involve a preliminary separation of the individual flavins by chromatography or electrophoresis, followed by the separate determination of each. In the second type the principal

Table 9. *Flavin contents of hexanoate-oxidizing organisms*

Numbers preceding names of organisms refer to Table 7, where details of growth are given.

Organism	Carbon source for growth	Duration of growth (hr.)	Total flavin (μ m-moles/g. dry wt.)	Flavin in each fraction (%)			FAD content (μ m-moles/g. dry wt.)	Calculation method
				Riboflavin	FMN	FAD		
24. <i>Pseudomonas fluorescens</i> CO1	0.1% Sodium hexanoate 1% Glucose	66 48	330 158	0 2	29 54	71 44	228 69	2 2
25. <i>Pseudomonas fluorescens</i> CO2	0.1% Sodium hexanoate 1% Glucose	120 45	387 108	0 —	25 —	75 —	256 —	2 2
26. <i>Bacillus sphaericus</i> CO8	0.1% Sodium hexanoate 1% Sodium acetate	120 90	432 128	0 —	31 —	69 —	291 —	2 2

Table 10. *Oxidative properties of hexanoate-oxidizing organisms*

Washed cell suspensions and determinations of dry weight were made as for examination of flavin contents. Q_{O_2} (μ l. of O_2 uptake/mg. dry wt. of cells/hr.) was measured with Warburg manometers containing (in a total reaction volume of 2 ml.) 1 ml. of 0.1M-potassium phosphate, pH 6.8, washed cells as indicated and substrate. Substrates, 10 μ moles of sodium acetate, 10 μ moles of sodium

hexanoate or 5.6 μ moles of glucose, originally in 0.1 ml. in the side arm, were added at zero time; 0.2 ml. of 10% KOH was in the centre well. Rates of O_2 uptake were measured over the period 0-20 min. except with acetate-grown *B. sphaericus* CO8 and glucose-grown CO1, where they were measured over the periods 0-30 and 0-60 min. respectively.

Organism	Carbon source during growth	Q_{O_2}				Corrected Q_{O_2}			Wt. of organism/manometer (mg. dry wt.)
		No substrate (a)	With hexanoate (b)	With glucose (c)	With acetate (d)	With hexanoate (b-a)	With glucose (c-a)	With acetate (d-a)	
<i>Pseudomonas</i> CO 1	Hexanoate	10.2	59.8	26.6	—	49.6	16.4	—	14.4
	Glucose	5.6	6.7	12.7	—	1.1	7.1	—	13.4
<i>Pseudomonas</i> CO 2	Hexanoate	10.3	67.8	49.7	—	57.5	39.4	—	4.7
	Glucose	1.5	2.7	29.2	—	1.2	27.7	—	7.8
<i>Bacillus sphaericus</i> CO 8	Hexanoate	62.5	102.5	—	92.3	40.0	—	29.8	9.6
	Acetate	14.3	22.0	—	37.1	7.7	—	22.8	10.8

flavins are not separated from each other but are estimated by differential fluorimetry (Bessey *et al.* 1949) or by a combination of differential fluorimetry with the enzymic assay of FAD (DeLuca *et al.* 1956). Both types of method are open to criticism and it is questionable whether any method is precise. Those based on separation procedures, although ensuring greater specificity and freedom from interference by contaminating materials, involve assumptions about the losses in the stages before the actual assay. On the other hand, methods based on differential fluorimetry and enzymic assay entail fewer preliminary manipulations but presume the identity of the materials being assayed and give less control over interfering substances.

Since little information was available about the flavins of micro-organisms, the present rather laborious procedure was adopted as it enabled the identity of the individual flavins to be established with reasonable certainty. The method is not strictly quantitative and the influence of factors such as age of culture on flavin content has not been investigated. The emphasis has been on a semi-quantitative survey of a number of different physiological types, and for these reasons the deductions drawn from the quantitative data are limited to a few general conclusions.

The principal outcome of the present experiments is the demonstration that FMN and FAD occur in all the micro-organisms examined, and that quantitatively these are the most important flavins. The correlation between a high FAD content and the production of HVFA is also of interest. It suggests that FAD is involved in the metabolism of these acids, and this view is supported by the observations with the hexanoate-oxidizing organisms; not only the flavin content but also the capacity to oxidize hexanoate is greatly increased by growth on this substrate.

There exists at first sight an anomaly in the results obtained with the hexanoate-oxidizers. Even after growth on hexanoate there is much less flavin in these organisms than in either *Cl. kluyveri* or LC1, the two most active HVFA-formers. This difficulty can be resolved on closer consideration. The energy-yielding mechanisms of these last two organisms are not fully understood, but it is evident that the fermentation of lactate by LC1 and of ethanol by *Cl. kluyveri* cannot yield more than a few molecules of adenosine triphosphate (ATP) for each molecule of hexanoate or butyrate produced. By contrast, the complete oxidation of hexanoate by the currently accepted route (Mahler, 1953; Lynen, 1955) would yield some 50 molecules of ATP. The oxidation of a given amount of hexanoate would therefore be expected to support the synthesis of far more cell

material than the formation of the same amount by fermentation. In this way, the low flavin contents of the hexanoate-oxidizers relative to the HVFA-producers can be reconciled with the view that flavins are concerned in the metabolism of HVFA *in vivo*.

Work on systems *in vitro* has already established the enzymic role of flavins in fatty acid metabolism. A number of distinct acyl dehydrogenases have been isolated from pig liver and all contain FAD (Crane, Hauge & Beinert, 1955). One of these enzymes, the butyryl dehydrogenase, is specific for the coenzyme A thioesters of the C₄-C₈ fatty acids and is a green cuproflavoprotein (Mahler, 1954).

In a stimulating review, Kaplan (1955) has suggested that, in anaerobes, phosphorylation to yield ATP might be coupled with electron transfer from diphosphopyridine nucleotide to flavoprotein or between two flavoproteins of different oxidation-reduction potentials. Such a system may well operate in HVFA-producers with butyryl dehydrogenase as the electron acceptor (Barker, 1956), and the present work emphasizes the possible importance of such reactions in strict anaerobes.

The present survey has been confined to the intracellular flavins of *Sacch. cerevisiae* and various species of bacteria. Under certain conditions several micro-organisms excrete flavins into the medium (Robinson, 1951) and outstanding in this respect is *Eremothecium ashbyi*. When grown aerobically on glucose-peptone medium both the mycelium of this organism and the medium contain several times more flavin than *Cl. kluyveri* (Yagi, Matsuoka, Kuyama & Tada, 1956). There appears to be no correlation in this case between a high flavin content and the metabolism of HVFA. However, the fact that so much flavin, mostly riboflavin, is excreted into the medium and that crystals of riboflavin have been observed in the mycelium, suggests that in such organisms the synthesis of flavin is not controlled by the requirement of the cell for flavoproteins.

SUMMARY

1. The separation of riboflavin, flavin mononucleotide (FMN) and flavinadenine dinucleotide (FAD) by paper electrophoresis in 0.025 M-ammonium formate-formic acid buffer, pH 4.6, is described. Recoveries of flavin from the paper are about 90%.

2. The use of 10% trichloroacetic acid for 5 min. at room temperature has been selected as the most convenient procedure for extracting flavins from microbial cells. Flavins so extracted are obtained in a form suitable for electrophoresis by fractionation with a Florisil column giving 80% recovery.

3. These methods have been combined in an examination of the flavins present in a range of micro-organisms. FMN and FAD have been demonstrated in all organisms examined. Very little, if any, free riboflavin is present and no other flavin derivatives were detected.

4. Flavin contents varied from 30 to 2930 μm -moles/g. dry wt. with *Rhodospirillum rubrum* and *Clostridium kluyveri* respectively. High flavin contents are associated with the fermentative formation of butyrate and higher volatile fatty acids.

5. With three hexanoate-oxidizing organisms isolated from soil, the oxidation of hexanoate is adaptive and is associated with a higher flavin content.

6. The proportions of flavin present as FMN and FAD vary widely from 21 % of FMN and 79 % of FAD with *Desulphovibrio* to 68 % of FMN and 30 % of FAD with *Lactobacillus helveticus*. Those organisms oxidizing hexanoate or producing higher volatile fatty acids contain a high proportion of FAD.

I am grateful to numerous workers for cultures of micro-organisms and advice on their growth. In addition I wish to thank Drs S. R. Elsdén, G. Weber, L. G. Whitby and J. R. Postgate for valuable discussions and gifts of materials, Mrs M. Fox for technical assistance and the Floridin Co., The Distillers Co. and Sigma Chemical Co. for gifts of Florisil, corn-steep liquor and FMN respectively. This work was supported in part by a grant from the Rockefeller Foundation.

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