CIRCULAR PAPER CHROMATOGRAPHY

Part X. Separation, Identification and Quantitative Estimation of Riboflavin and Flavin Compounds

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Summary

A method for the separation, identification and quantitative determination of riboflavin and its derivatives (flavin mononucleotide, flavin-adenine dinucleotide, lumiflavin and lumichrome) by circular paper chromatography is described. A number of solvent combinations have been tried in an attempt to obtain the best separation of the flavins, and *n*-butanol-acetic acid-water in the proportion 40 : 10 : 50 was found to give the best resolution both for qualitative characterisation as well as quantitative evaluation. Quantitative determination of the individual flavins was made by cutting out the bands from the chromatograms (after identification under ultra-violet light), eluting them with water and measuring the fluorescence in a fluorimeter. Spectrophotometric analysis was also carried out as a check-up and the recovery was found to be satisfactory. Some applications of the method for the study of riboflavin metabolism in a mutant yeast BY2, are briefly described. The advantages and possible applications of the technique are discussed.

The metabolic significance of riboflavin which occurs in the free form, as riboflavin-5'-phosphate (flavin mononucleotide, FMN) and as flavin-adenine dinucleotide (FAD), one or the other of the latter two compounds serving as a component of 'flavoprotein' enzymes, is well recognised. The chemical, biological and microbiological methods so far developed for the quantitative determination (individually) of these flavins which are of biological interest, and their common degradation products, lumiflavin and lumichrome are found to be unsatisfactory in the case of mixtures of all these compounds. Paper chromatography has been applied by a number of workers to separate flavins in a mixture. Crammer (1948) has attempted to separate the biologically active flavins by using unidimensional strip chromatography. Hais and Peckákova (1949) applied paper chromatography to detect the degradation products of riboflavin exposed to hydrogen peroxide and found that in addition to lumiflavin and lumichrome, three other fluorescent spots could be observed on the chromatograms. Similar attempts to separate flavins in a mixture have been made by others (Yagi, 1951; Dimant,

Sanadi and Huennekens, 1952), using paper chromatographic methods. In some instances, paper chromatographic methods were used as adjuncts to ion-exchange chromatography employed for the isolation of nucleotides (Whitby, 1952, 1953). However, in no case was a complete resolution of all the flavins achieved, nor the quantitative determination of the separated constituents accomplished.

Circular paper chromatography (Giri and Rao, 1952) has been successfully applied in this laboratory and elsewhere for the separation, and in some instances quantitative determination of a variety of biologically important materials, such as amino acids (Giri, Radhakrishnan and Vaidyanathan, 1952), organic acids (Giri, Krishnamurthy and Rao, 1953, Airan *et al.*, 1953), purines and pyrimidines and their breakdown products (Giri, Krishnaswamy, Kalyankar and Rao, 1953), carbohydrates (Giri and Nigam, 1954), vitamin B_1 and riboflavin (Giri and Balakrishnan, 1955) and inorganic analysis (Airan, 1952), etc. In this paper, a circular paper chromatographic method for the separation, identification and estimation of all the flavins normally encountered, and some applications of the method to problems in riboflavin metabolism are described.

Experimental

Materials

Riboflavin was a commerical preparation obtained from Hoffmann La-Roche.

Flavin mononucleotide (FMN) was obtained from the Nutritional Biochemicals Corporation, Inc. (U.S.A.). On chromatography, it showed the presence of riboflavin as an impurity. Spectrophotometrically, its purity was found to be 82%

Flavin-adenine dinucleotide (FAD) was obtained as a gift sample through the courtesy of the California Foundation for Biochemical Research (USA).

Lumiflavin was prepared from riboflavin according to the method of Warburg and Christian (1932). A solution of riboflavin (pH 9·0) was irradiated by exposing it to bright sunlight for 30 minutes and the lumiflavin formed was repeatedly extracted with acidified chloroform. After evaporating off the chloroform, the residue was treated with a small volume of water (acidified with acetic acid) and lumiflavin was obtained in a crystalline form after keeping.

Lumichrome was also prepared from riboflavin by irradiation at neutral pH, according to the method of Karrer *et al.* (1934). The material was obtained in a crystalline form by treating the residue obtained after evaporation, with 90% ethanol and repeating the treatment a number of times.

Flavin solutions of known concentration were prepared and stored in cold n a dark place. Exposure of the solutions to bright light was avoided as far as possible.

Solvents.—All the solvents used for irrigating the chromatograms were distilled before use.

Filter-paper.—For small-scale separation, identification and estimation, Whatman No. 1 filter circles (22–24 cm. diameter) were used. For semi-preparative purposes, Whatman No. 3 filter circles of the same dimension were used. Prewashing of filter-papers for the fluorometric analysis of the flavins was not found to be essential, whereas for spectrophotometric determinations, washing the filter-paper was a necessary prerequisite.

Procedure.—The chromatographic procedure was essentially the same as described by Giri and Rao (1952). Mixed chromatograms with mixtures of the flavins as well as individual components applied side by side were run for determining the R_f values. Spotting and irrigation of the chromatograms were carried out in the absence of bright light or in semi-darkness to avoid decomposition of the flavins.

Identification of the compounds.—After irrigation with the solvent, the chromatograms were dried in air. The bands corresponding to the flavins were located under an ultra-violet lamp with Wood's filter (Philips HPW-125 W. TYP 57202 E/70). The compounds were identified by reference to the bands obtained from a known sample run side by side.

Bands due to riboflavin and FMN showed bright yellow, and the band due to FAD showed pale yellow fluorescence. Lumiflavin gave greenish yellow fluorescence, while that due to lumichrome was bright sky-blue. Occasionally, when sufficient care was not taken to maintain dark or semi-dark surroundings, decomposition of the flavins occurred. In fact, the instability of FAD below pH 3, at elevated temperatures and in the presence of light, can be confirmed by paper chromatography. Unless the chromatograms are run in near darkness, usually a band of lumichrome will appear on the chromatogram.

Photographs.—The photographs of the chromatograms were taken in the ultra-violet light using a filter of saturated sodium nitrite solution.

Fluorimetry.—The fluorescence of the flavins was determined in a Klett-fluorimeter using appropriate filters (Primary B_2 and orange filters).

Spectrophotometry.—Absorption spectra were determined in a Beckmann Spectrophotometer, model DU. All determinations were carried out in quartz cuvettes of 0·996 cm. thickness in solutions at pH 7·0, unless otherwise stated. Concentrations of flavin solutions were determined from light absorption measurements at 260, 375 and 450 mμ. From the usual equation, $\log I_0/I = \epsilon\, cd$, the molar extinction coefficients of the different flavins at these wavelengths were used to calculate the concentrations of the flavins. The following assumptions were made for spectrophotometric analysis.

Purity of the substances, on a dry weight basis, was also obtained by the above considerations. Spectrophotometric estimations were always made by measuring the optical density of the solutions at more than one wavelength as a check-up.

TABLE I

Molecular extinction coefficients of different flavin compounds

Compound	Mol. wt.	(l mole^{-1}cm.$^{-1}$ × 10^{-3})			Reference
		260 mμ	375 mμ	450 mμ	
Riboflavin ..	376·4 ($C_{17}H_{20}N_4O_6$)	25·7	9·8	11·3	Warburg and Christian, 1938
FMN ..	456·4 ($C_{17}H_{21}N_4O_9P$)	27·1	10·4	12·2	Whitby, 1953
FAD ..	785·6 ($C_{27}H_{28}N_9O_{15}P_2$)$^-$	37·0	9·0	11·3	Warburg and Christian, 1938

RESULTS

Separation of flavins in different solvent systems

A number of solvent combinations were tried to accomplish the best separation of all the flavin compounds. For determination of R_f values in different solvent systems, identical conditions were maintained regarding the size of the filter-paper, quantity of the individual flavins applied, length of run on the paper and chromatographic equipment. The R_f values of the flavins in different solvent systems are given in Table II.

Of the various solvents tried, *n*-butanol-acetic acid-water in the proportion 4 : 1 : 5 was found to give the best results with good separation and sharp definition without tailing which is usually encountered in unidimensional strip chromatography (Crammer, 1948). The compactness with which the bands separate makes it highly suitable for cutting out for elution prior to quantitative determination. With the same solvent, R_f values of the flavins increased when Whatman No. 3 filter-paper was used, though their relative positions on the chromatograms remained the same. With the other solvents, though the separations of the individual flavins were satisfactory, the bands lacked compactness and sharpness and were diffused. When water saturated with iso amyl alcohol was used as the solvent, the order in which the flavins separated was entirely reversed. However, the bands were found to be very diffused. Thus none of these solvents was found to be useful for application to quantitative determination. Only solvent No. I was used both for qualitative identification as well as quantitative estimation. For semi-preparative purposes, Whatman No. 3 filter-paper was used with the same solvent.

Quantitative estimation of flavins after separation by circular paper chromatography

For this purpose, accurately measured volumes of the solution containing flavin compounds of known concentration were applied on paper from a calibrated

TABLE II

R_f values of flavin compounds with different solvent systems

Compounds	R_f values					Nature of fluorescence
	Solvents					
	I *n*-butanol, acetic acid, water (4 : 1 : 5) (Crammer, 1948)		II *n*-butanol, *n*-propanol, water (2 : 2 : 1)	III *n*-butanol, formic acid, water (77 : 10 : 13)	IV Water saturated with isoamyl alcohol	
	Whatman No. 1	Whatman No. 3	Whatman No. 1			
Riboflavin ..	0·45	0·55	0·21	0·16	0·52	Bright yellow
FMN ..	0·29	0·40	0·06	0·04	0·89	Bright yellow
FAD ..	0·17	0·25	0	0	0·92	Pale yellow
Lumiflavin ..	0·52	0·64	0·36	0·30	0·30	Greenish yellow
Lumichrome ..	0·69	0·75	0·61	0·51	0·14	Bright sky-blue

micro-pipette. The chromatograms (Whatman No. 1, 24 cm. diameter) were run as usual and dried. The bands corresponding to the different flavins were marked with pencil under ultra-violet light. Strips of paper each containing one flavin were cut out and eluted with 5 ml. of glass distilled water in test-tubes (15 × 1 cm.) covered with dark paper, for 6 hours, by which time the elution was found to be complete. The paper segments were then drained off carefully with a glass rod taking care to avoid dispersion of cellulose in the eluting fluid. Occasionally, a larger volume of water was used for elution as a check on recovery. The fluorescence of the eluate was determined in a Klett-fluorimeter, as described earlier, the fluid collected under the same conditions after extraction of a similar piece of filter-paper from the same circle being used as a blank. The amount of flavin was estimated by comparing the fluorescence with that of a known solution of the pure compound. For determining the recovery, the same quantities of the flavins which were subjected to chromatography were made up to 5 ml. with glass-distilled water and the fluorescence of the two were compared.

The recoveries were also determined by spectrophotometric analysis of the eluates from paper strips. For spectrophotometric estimations, a number of strips were pooled together to give sufficient concentrations of the flavin for giving measurable optical densities. The results are presented in Table III (*a*) and (*b*).

TABLE III

Recovery of flavin mixtures separated by circular paper chromatography

(*a*) *Fluorimetric analysis*

Substance	Amount applied (μg.)	Amount recovered (μg.)	Recovery %
Riboflavin	0·75	0·75	100·0
	1·50	1·43	95·3
	3·00	3·10	103·3
	6·00	5·92	98·6
FMN	0·80	0·80	100·0
	1·60	1·46	91·2
	4·00	4·10	102·5
	5·60	5·55	99·1
FAD	0·90	0·82	91·1
	4·50	3·98	88·4
	7·20	6·92	96·1
Lumiflavin	0·50	0·49	98·0
	2·50	2·39	95·6
	5·00	4·88	97·6
Lumichrome	0·50	0·46	92·0
	2·50	2·42	96·8
	5·00	4·79	95·8

(*b*) *Spectrophotometirc analysis*

Substance	Amount chromotographed (μg.)	Amount recovered (μg.)	Recovery %
Riboflavin	12·0	13·0	108·6
	24·0	25·3	105·4
FMN	8·0	8·4	105·0
	16·0	17·0	106·2
FAD	9·0	9·3	103·3
	18·0	18·6	103·3

The results show that the recovery of riboflavin, FMN and FAD, as well as other flavins is satisfactory both when determined fluorimetrically and spectrophotometrically.

Some applications of the method to the study of riboflavin metabolism in a mutant yeast BY2

The method was applied to the study of some aspects of flavin metabolism in a riboflavin excreting mutant yeast BY2. The physical and chemical factors governing flavinogenesis in this organism have been investigated previously (Mitra, 1952; Giri and Krishnaswamy, 1954).

(i) *Identification of the flavins excreted by BY2.*—The mutant yeast BY2 has been shown to excrete large amounts of riboflavin into the culture medium, the quantity excreted increasing with the age of culture. In earlier studies, the total flavin content of the culture medium was estimated as riboflavin. On paper chromatographic analysis (Whatman No. 3), the culture medium (after concentrating 100 ml. to 1 ml.) was found to contain two other fluorescent bands below the position of riboflavin with R_f values 0·40 and 0·25. They were identified as FMN and FAD respectively by the following tests.

A number of circular filter-papers (Whatman No. 3, 24 cm. diameter) on which the concentrated culture medium was spotted were developed in a pack (Giri, 1954, 1955), using solvent No. I. After development, the flavins were marked under ultra-violet light and the portions containing the flavins were cut out and eluted with the minimum amount of glass-distilled water. The eluate of the band corresponding to R_f value of 0·25 had absorption maxima at 450, 375 and 263 mμ which was characteristic of FAD. The eluate was evaporated to dryness in a desiccator and the dry residue was hydrolysed with a small volume of 6N-HCl for 2 hours at 125° C. in a sealed tube. The hydrolysate was found to give the characteristic colour test for 4-amino-5-imidazole carboxamidine (AICA), with

Folin's reagent as described by Giri *et al.* (1953). Since AICA is a degradation product of adenine and adenine containing compounds, this test proved the existence of the adenine moiety in the flavin corresponding to FAD. The eluate of the other band corresponding to R_f value 0·40 gave absorption maxima at 445, 373 and 266 mμ which is characteristic of FMN. Further, when this was hydrolysed with N–H_2SO_4 at 100° C. for 4 hours, cooled and neutralised to pH 7·0, the spectrum of the final solution was found to be identical with the spectrum of riboflavin. Paper chromatography showed that riboflavin was the only flavin present in the final solution. In the following table, the quantities of the flavins present in the culture medium on different days of incubation are given.

TABLE IV

Concentrations of riboflavin, FMN and FAD excreted by BY2 into the culture medium on different days of incubation

[The organism was grown in a synthetic medium in Roux flasks. The cultural conditions, methods of harvesting the culture medium for flavin analysis, etc., were the same as described in an earlier paper (Giri and Krishnaswamy, 1954). For the estimation of FMN and FAD which occurred in low concentrations, the culture media were concentrated a hundred-fold.]

	Age of culture in days				
	1	2	3	4	8
Riboflavin, mg./100 ml. medium	0·0560	0·1160	2·800	3·690	4·170
FMN, μg./100 ml. medium	16·0	21·0	21·0	38·6	39·2
FAD, μg./100 ml. medium	12·3	19·6	22·8	31·6	32·7

(ii) *Quantitative studies on the enzymes, flavokinase and nucleotide pyrophosphatase in BY2.*—Experimental evidence is available to show that riboflavin undergoes phosphorylation and the phosphorylated derivative (FMN) is further converted to the biologically active flavin-adenine dinucleotide by two separate processes catalysed by enzymes designated as 'flavokinase' and 'nucleotide pyrophosphatase' respectively (Kearney and Englard, 1951; Kornberg, 1955). The circular paper chromatographic method was used to conduct a preliminary assay of these two reactions in BY2.

(*a*) *Flavokinase.*—A 24-hour old growth of the organism, after washing with 0·9% saline, was made up in a known volume of M/5 phosphate buffer, pH 8·2 and this intact cell suspension was used as the enzyme. The reaction mixture consisted of 0·2 ml. riboflavin solution ($1·0\times10^{-4}$ M), 0·2 ml. ATP ($1·0\times10^{-3}$ M), 0·1 ml. $MgSO_4$ solution ($3·0\times10^{-4}$ M), 1·0 ml. M/5 phosphate buffer pH 8·2

and 1·0 ml. enzyme preparation (6·2 mg. N.), total volume 2·5 ml. The mixture was incubated for a period of 2 hours at 30° C. At known intervals 0·2 ml. aliquots were withdrawn from this mixture, 0·1 ml. of 17·5% trichloroacetic acid was added. After boiling for 10 minutes (to hydrolyse the traces of FAD formed), it was centrifuged at 3,500 r.p.m. for 10 minutes and equal volumes (50 μl) of the supernatant were chromatographically analysed for FMN, as described earlier. The amounts of FMN formed at different intervals are given in Table V.

TABLE V

FMN formation from riboflavin by cell-suspensions of BY2

Time in minutes ..	30	60	90	120
FMN formed, mμM/2·5 ml. reaction mixture	24·8	44·2	81·0	98·5

(*b*) *Nucleotide pyrophosphatase.*—The cells, after harvesting, were dried by treating with a large volume of cold acetone and dried in air on a Buchner funnel. This dry powder (10 g.) was autolysed with 30 ml. of water for 24 hours at room temperature. This was centrifuged and the supernatant was dialysed against water at 3–4° C. for 6 hours. The dialysate was used directly as the enzyme without further purification. The reaction mixture consisted of 0·1 ml. of $MgCl_2$ solution (0·15 M), 0·2 ml. of ATP solution (0·02 M), 0·2 ml. of FMN solution (2×10^{-4}M), 1·0 ml. M/5 phosphate buffer, pH 7·5, 1·0 ml. of enzyme preparation, total volume 2·5 ml. The mixture was incubated at 37° C. and at definite intervals 0·2 ml. aliquots were withdrawn, immersed in boiling water for 3 minutes, cooled and centrifuged. Equal volumes (50 μl) of the supernatant were chromatographically assayed for FAD, as described earlier.

TABLE VI

FAD formation from FMN by an enzyme preparation from BY2

Time in minutes	5	10	30	60
FAD synthesised, mμM/ 2·5 ml. reaction mixture	29·2	62·3	118·6	242·0

(iii) *Formation of flavins* in vitro *by a cell-free preparation of BY2 with α-ketoglutarate and 4-amino 5-imidazole carboxamide.*—It was observed that in transamination reaction mixtures in which α-ketoglutarate and AICA were incubated with dialysed cell-free enzyme preparations from BY2 (Giri and Krishnaswamy, unpublished), intense fluorescence developed with increase in time (48–96 hours). The fluorescence was similar to that given by the flavins. On circular paper chromatograms, bands corresponding to riboflavin, FMN and FAD appeared, when the reaction mixture was subjected to chromatography (Fig. 2).

The reaction mixture contained of 1·0 ml. of M/15 α-ketoglutarate solution, 1·0 ml. of M/15 AICA solution, 2·0 ml. of M/5 phosphate buffer, pH 7·2, and 2·0 ml. enzyme solution in a total volume of 6·0 ml. After incubating at room temperature for 72 hours the mixture was immersed in boiling water for 5 minutes, cooled and centrifuged. The supernatant was concentrated to a small volume (0·5 ml.) and spotted on a number of Whatman No. 3 filter circles and developed as usual with solvent No. I. The characteristic tests for identifying the bands were carried out and the data are presented in Table VII.

TABLE VII

Identification of the fluorescent substances formed by a cell-free preparation of BY2

R_f values of the fluorescent bands	Absorption maxima of the eluates	Result of hydrolysis or other treatment
0·56	445, 373 and 269 mμ	On alkaline irradiation of the eluate, a band corresponding to lumiflavin (R_f-0·64) was the only one observed
0·40	445, 373 and 266 mμ	On hydrolysis with N-H_2SO_4 at 100° C. for 4 hours, riboflavin was the only flavin detected as the breakdown product
0·25	450, 375 and 263 mμ	Gave a positive carboxamidine test on acid hydrolysis indicating the presence of adenine moiety

The enzyme preparations were found to be totally free from pentoses (negative orcinol test). It is hoped that the method would prove to be of considerable use in evaluating the complexities of this interesting reaction in greater detail.

(iv) *Breakdown products of riboflavin on exposure to hydrogen peroxide.*—Riboflavin has generally been reported to be stable to oxidising agents and its photolysis has always been considered to be a complex process (Rudy and Zechmeister, 1939). Lumichrome (6:7 dimethyl alloxazine) and lumiflavin (6:7:9 trimethyl isoalloxazine) are the only two compounds isolated as the final products of photolysis of riboflavin. Hais and Peckákova (1949) observed that after exposing riboflavin to hydrogen peroxide, the spots of riboflavin and lumichrome were accompanied by three more spots, two with blue (R_f 0·08 and 0·22) and one with orange fluorescence (0·16). However, by applying the circular paper chromatographic method, it was found that a number of fluorescent

bands appeared on the chromatograms in addition to those observed by the above authors (Fig. 3). The results are presented in Table VIII.

TABLE VIII

Breakdown of riboflavin on exposure to hydrogen peroxide

[To 10 ml. of riboflavin solution (0·5 mg./ml.) at pH 9·0, 3 ml. of 30% H_2O_2 (Merck) was added. After 3 hours exposure, aliquots were chromatographically developed]

R_f value of the band	Nature of fluorescence
0·85	Blue
0·75	Bright sky-blue
0·66	Blue
0·55	Yellow—riboflavin
0·42	Faint band—blue
0·38	Orange
0·35	Faint band—blue
0·27	Deep blue (Thiochrome or quinine type of fluorescence)
0·24	Do.

DISCUSSION

The chromatographic methods reported in literature for the separation of flavins are not altogether satisfactory nor can they be utilised for reliable quantitative determinations. In some instances simultaneous separation of the several riboflavin derivatives is not achieved, in several others the separation of the individual components is not sharp, while in most cases the R_f values of the compounds seem to vary considerably. The circular paper chromatographic method described in this paper not only permits a sharp and well-defined separation of riboflavin and its derivatives but also serves as a simple method for quantitative estimation of flavin compounds in micro quantities. It was observed by running unidimensional paper strip chromatograms simultaneously that a higher resolving power is achieved by the circular chromatographic method than by the vertical strip method (Figs. 1 and 4). In the strip method, spots were obtained which were invariably diffused and the tailing phenomenon was a common feature.

Satisfactory recovery of the constituents could be obtained by this method as evidenced by the results presented and the method could be used with advantage

in the study of enzyme reactions where the quantitative assay of riboflavin and its nucleotides formed are to be made.

A singular advantage of the circular technique is that it affords a semi-preparative or preparative method for dealing with fairly large quantities of substances (Giri, 1954 and 1955). As shown in the results, it can be employed for obtaining sufficiently concentrated eluates of the unknown flavin compounds to determine the absorption spectra and to carry out hydrolysis or other forms of chemical treatment to identify the compounds. In a similar way, the technique might be exploited in tracer work also. The method can be employed for detection and estimation of riboflavin in foodstuffs and other natural materials since very minute quantities of this vitamin or its derivatives can be detected by this method. Further work is in progress on the comparative merits and limitations of the chromatographic method as compared to the chemical and microbiological methods.

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EXPLANATION OF PLATES

FIG. 1. Circular paper chromatogram showing the positions of lumichrome (LC), lumiflavin (LF), riboflavin (RF), flavin-mononucleotide (FMN) and flavin-adenine dinucleotide (FAD) and their separation in a mixture (M). [Whatman No. 3 filter-paper, 24 cm. diameter; Solvent: *n*-butanol-acetic acid-water (4 : 1 : 5).]

FIG. 2. Circular paper chromatogram showing the formation of flavins *in vitro* by cell-free preparations from a riboflavin-excreting yeast BY2. The outermost ring corresponds to riboflavin while the lower two correspond to FMN and FAD respectively.

[Whatman No. 3 filter-paper, 24 cm. diameter; Solvent: *n*-butanol-acetic acid-water (4 : 1 : 5); 0·1 ml. reaction mixture spotted at the centre.]

FIG. 3. Circular paper chromatogram of the breakdown products of riboflavin exposed to hydrogen peroxide. 1. Riboflavin solution exposed to hydrogen peroxide for 3 hours (20 μl spotted). 0 & 0′—Riboflavin solution spotted immediately after adding hydrogen peroxide (5 and 10 μl respectively).

M—Mixture of lumichrome, lumiflavin, riboflavin, FMN and FAD. [Whatman No. 3 filter-paper, 24 cm. diameter; Solvent: *n*-butanol-acetic acid-water (4 : 1 : 5).]

FIG. 4. Unidimensional strip chromatogram (ascending) of riboflavin exposed to hydrogen peroxide (same solution used in Fig. 3).

[Whatman No. 3 filter-paper; Solvent, *n*-butanol-acetic acid-water (4 : 1 : 5); Length of run, 22 cm. ; 5μl spotted.]

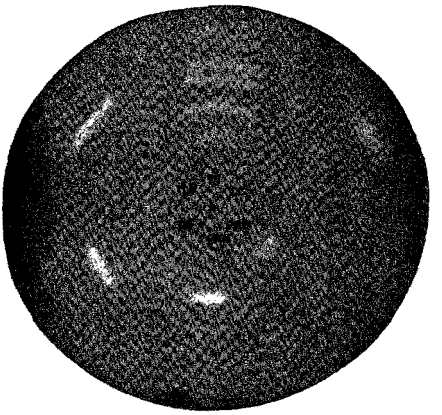

FIG. 1

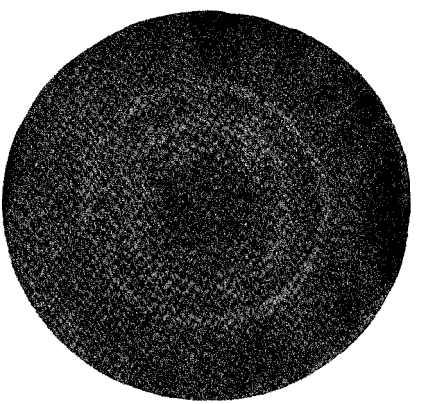

FIG. 2

FIG. 3

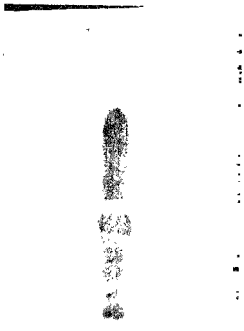

FIG. 4