THE SEPARATION AND ESTIMATION OF
Riboflavin and its derivatives

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ABSTRACT

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An attempt has been made to separate riboflavin and its derivatives by paper electrophoresis. This has proved fruitless, however, mainly because of evaporation from the paper during the electrophoretic procedure.

A more complete separation has been effected using two-dimensional ascending chromatography but this method was found to be incapable of yielding quantitative recoveries of flavins. A further separation by one-dimensional ascending paper chromatography, using a 5% sodium hydrogen phosphate solution as solvent, was observed. This separation yielded a nearly quantitative recovery of flavins and derivatives when the concentrations of applied mixtures were below one microgram for each compound applied.

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INTRODUCTION

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Riboflavin. or Vitamin B_2, is one of the best known and most important of the vitamins needed in our diet. The lack of it tends to inhibit the action of certain enzymes including amino acid oxidase, xanthine oxidase, diaphorase, and cytochrome reductase, all of which are indispensable in biological oxidations. The inability of such enzymes to catalyze these oxidations in riboflavin deficient systems is due to the lack of the two important co-enzyme derivatives of riboflavin, flavin mononucleotide and flavin adenine dinucleotide, referred to as FMN and FAD respectively.

Chemical methods of analysis for these flavins have been devised. These have not been completely satisfactory. Errors arise because there are analogous products--usually photo-degradation products of riboflavin--which are reported as riboflavin in the results of analyses, but which do not function as it does in the tissue.

Two of these are lumiflavin and lumichrome. They are produced from the derivatives FMN and FAD, as well as from riboflavin, by the action of light on alkaline and acid solutions, respectively. The chemical analysis for riboflavin involves the measurement of its fluorescence in ultra-violet light. Both

lumiflavin and lumichrome fluoresce at the same wavelength as riboflavin. Thus the presence of either or both of these products in a sample will introduce errors in the determination of the concentration of the vitamin itself. However, this seems to have been overlooked in the reporting of riboflavin levels heretofore.

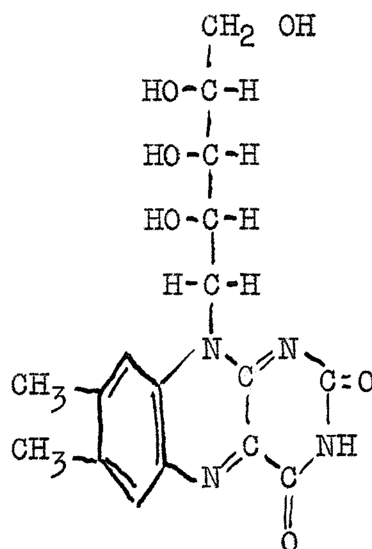
The object of this research, then, was to find a rapid quantitative method for the separation and estimation of each of riboflavin and its more common derivatives, FMN, FAD, lumiflavin and lumichrome, preferably in both plant and animal tissues.

Two methods which seemed to offer more promise for the initial separation were paper electrophoresis and paper chromatography. Several investigations have reported that the former procedure effects a quantitative separation of artificial mixtures of these substances. This seemed to provide the best starting point in our own study, though our ultimate aim was to develop a method which could be applied to natural sources of the vitamin and its derivatives.

REVIEW OF THE LITERATURE

REVIEW OF THE LITERATURE

In 1933, one of the constituents of the Vitamin B_2 complex was identified as lactochrome, a naturally occurring yellow pigment (1) first extracted from milk in 1876 (2) and obtained in crude form from the same source in 1925 (3). This yellow pigment, which has a characteristic green fluorescence, was obtained in crystalline form from both egg white (1) and milk (1, 4). Subsequent chemical studies by Karrer and Kuhn and their co-workers limited the structural possibilities to the isomeric 6,7-dimethyl-9-(tetrahydroxy-amyl)-isoalloxazines. By preparation of the possible isomers, the structure was finally established by Karrer and co-workers (5, 6) and Kuhn and cO-workers (7, 8) who independently synthesized the vitamin in 1935. The vitamin which has since been termed riboflavin has the following structure:



While the purification and proof of structure of riboflavin were being accomplished, simultaneous investigations showed the vitamin to be associated with certain enzymes. In 1932, Warburg and Christian (9) isolated a co-enzyme essential for the functioning of the yellow enzyme. This co-enzyme was found to be related to Vitamin B_2 (10, 11) and in 1936 its identity with synthetic riboflavin-1,5-phosphoric acid was demonstrated (12). Since the flavin phosphate is composed of a base (dimethyl iso-alloxazine), a sugar alcohol (ribotol), and phosphate, it may be termed a nucleotide (flavin mononucleotide, FMN).

Another co-enzyme containing riboflavin was discovered in 1936 by Das (13) as a dialyzable co-enzyme of an amino acid oxidase, and was later isolated by Straub (14) and by Warburg and Christian (15, 16). The latter investigators demonstrated that the co-enzyme contained a flavin and adenine in the form of a dinucleotide. This co-enzyme, which is considered a combination of adenylic acid and riboflavin-1,5-phosphate by a pyrophosphate bond, is more versatile in its action than FMN which cannot replace the dinucleotide for many apoenzymes (17). This new flavin nucleotide was named flavin adenine dinucleotide, FAD.

The structure of riboflavin was elucidated through the study of photo-decomposition products. Two of these

compounds were found to be lumiflavin, first isolated by Warburg and Christian from an alkaline irradiated solution of riboflavin (9), and lumichrome, isolated from acid or neutral irradiated solutions (18). Both of these products were found to have no vitamin activity within the body.

The quantitative determination of Vitamin B_2 in various plant and animal tissues has been estimated by several authors. Three general methods have been found to be most satisfactory: fluorimetric, microbiological, and animal.

Animal assays were developed prior to the fluorometric and microbiological methods. The rat assay (19) was the original standard for animal assays and this method is still used today, though the values obtained are somewhat higher than expected due to the presence of additional amounts of other B vitamins in the food analyzed (20).

The original microbiological method, based on the growth of Lactobacillus casei in a given period of time, was due to Snell and Strong. The production of lactic acid, due to the growth of the bacteria, was measured by titration with standard base and the riboflavin present thus estimated by reference to a pre-determined curve obtained with known amounts of riboflavin. This method has been slightly altered by Roberts and Snell (22), who have used a different basal medium for the growth of the bacteria. However, the modern

microbiological estimation is essentially the same as the original one of Snell and Strong.

The fluorometric method of estimation is the most rapid and inexpensive, but the least widely applicable. It requires a fluorometer and a constant voltage supply. The original fluorometric assays were those of Hodson and Norris (23) who based their method on the phenomena that a) Riboflavin fluoresced under ultra-violet light. b) It was not destroyed by mild oxidation or reduction. c) It could be reduced with sodium hydrosulfite and reoxidized in air. d) It was not reduced by stannous chloride.

Their method has been improved upon by several authors. However, its basic principles are still maintained by the official A.O.A.C. procedure (24).

These available methods for the determination of the individual flavins of biological interest (FMN, FAD, and riboflavin), and of their most common breakdown products (lumiflavin and lumichrome) have proved unsatisfactory when applied to a mixture of these compounds since one does not determine the concentration of one compound only, but rather the sum of concentrations of several of them.

Attempts to separate different flavins in a synthetic mixture have been made by previous authors (25, 26, 27, 28, 29, 30) using paper chromatography. They did not obtain,

however, a complete resolution of all flavins, nor could they determine quantitatively the separated compounds. Siliprandi, Siliprandi, and Lis (31) have reported the separation of riboflavin, FMN, and FAD by paper electrophoresis, and Cerletti and Siliprandi (32) have reported a method for the quantitative separation and estimation of riboflavin and of its derivatives, also by paper electrophoresis.

It was our belief, since the latter authors had obtained the quantitative recovery of such large amounts of flavins and derivatives, that, perhaps by electrophoresis and/or chromatography, a similar method might be obtained by which smaller amounts of flavins and derivatives might be quantitatively separated and recovered from material sources, as well as from synthetic mixtures.

MATERIALS

MATERIALS

The study which was undertaken here required five major compounds; these were riboflavin, FMN, FAD, lumiflavin and lumichrome.

Riboflavin, FMN, and FAD were obtained from the Nutritional Biochemical Company. The purity of these compounds was checked by ascending paper chromatography of each flavin individually using the bottom layer formed by mixing 4 parts n-butanol, 1 part acetic acid, and 5 parts water together (25). The Rf value of these flavins, which is the ratio of the distance travelled by one component to that travelled by the solvent, was then determined and compared to that originally reported for them. These values checked and since only one discrete spot was formed for each component when chromatographed, each compound was assumed to be pure.

Lumiflavin was prepared by the method of Warburg and Christian (9). Fifty milligrams of riboflavin were dissolved in one liter of 0.5 N NaOH solution and the solution irradiated for 48 hours in a shallow porcelain dish, using a 1000 Watt Mazda lamp. The solution was extracted with chloroform three times and the extract allowed to evaporate to about 5 ml. This extract was placed in a refrigerator for 24 hours at which time a yellow crystalline precipitate was found to have formed. The precipitate was filtered off and redis-

solved in 2 N acetic acid, and the acid solution warmed to 50° C., filtered and kept at 10° C. for 48 hours. At the end of this time the solution was examined and yellow crystals were again found to have formed which were separated and dried as lumiflavin. The purity of this compound was checked by ascending paper chromatography as in the case of the purchased flavins. Only one spot was found to remain after chromatography, and the Rf value obtained also matched. It was thus established that the compound formed was pure lumiflavin.

Lumichrome was prepared by the method of Karrer, Salomon, Schopp and Schlittler (18). One hundred milligrams of riboflavin were dissolved in one liter of 25% methanol and the neutral solution irradiated for 48 hours using a 1000 Watt Mazda lamp. This solution was slowly evaporated to a volume of about 25 ml and stored in a refrigerator at 10° C. for 24 hours at which time a pale yellow precipitate had formed. This precipitate was dissolved in a small amount of methanol, warmed to 50° C. and filtered. The filtrate was then evaporated to a volume of about 5 ml and cooled at 10° C. After 24 hours a yellow solid had formed which was filtered off and stored as lumichrome. The quality of the compound formed was tested by paper chromatography as in the other cases. This was quite important since some lumiflavin

is usually formed during the irradiation of the neutral solution. However, only one spot was formed after chromatography which had a sky-blue fluorescence under ultra-violet light and had a Rf value corresponding to that for lumichrome in this particular solvent.

For all methods, both qualitative and quantitative, the compounds riboflavin, FMN, and FAD were dissolved in a 50% solution of ethanol and water, while lumiflavin was dissolved in methanol and lumichrome in 50% methanol and water. This facilitated drying of the compounds when applied on filter paper, Munktell no. 20 being used in all cases.

METHODS AND RESULTS

METHODS AND RESULTS

Paper Electrophoresis

This was carried out on Munktell 20 paper strips 6 x 50 cm. in a Reco Model E-800-2 electrophoresis unit. A general picture of this apparatus is shown in Figure 1.

Mixtures of the five compounds studied were applied in one spot to a strip of the paper at a distance of 10 cm. from the anode. The paper was then moistened with sodium acetate buffer, pH 5.1, ionic strength = 0.05. A potential of 380 volts was applied for various lengths of time, giving a current of 3.6 milli-amperes/cm. width of the paper.

The particular design of this apparatus allowed two methods of separation for our study, horizontal open strip electrophoresis, which was purely qualitative, and free hanging strip paper electrophoresis, which could be utilized in quantitative separations.

Horizontal open strip technique. With this method, the paper actually lay in contact with the platform surface, so that a slight sample loss took place. A water cooling system, running just under the platform surface, limited evaporation from the paper due to heating effects taking place while the apparatus was in use. This method is illustrated in Figure 2.

After electrophoresis had taken place for a designated

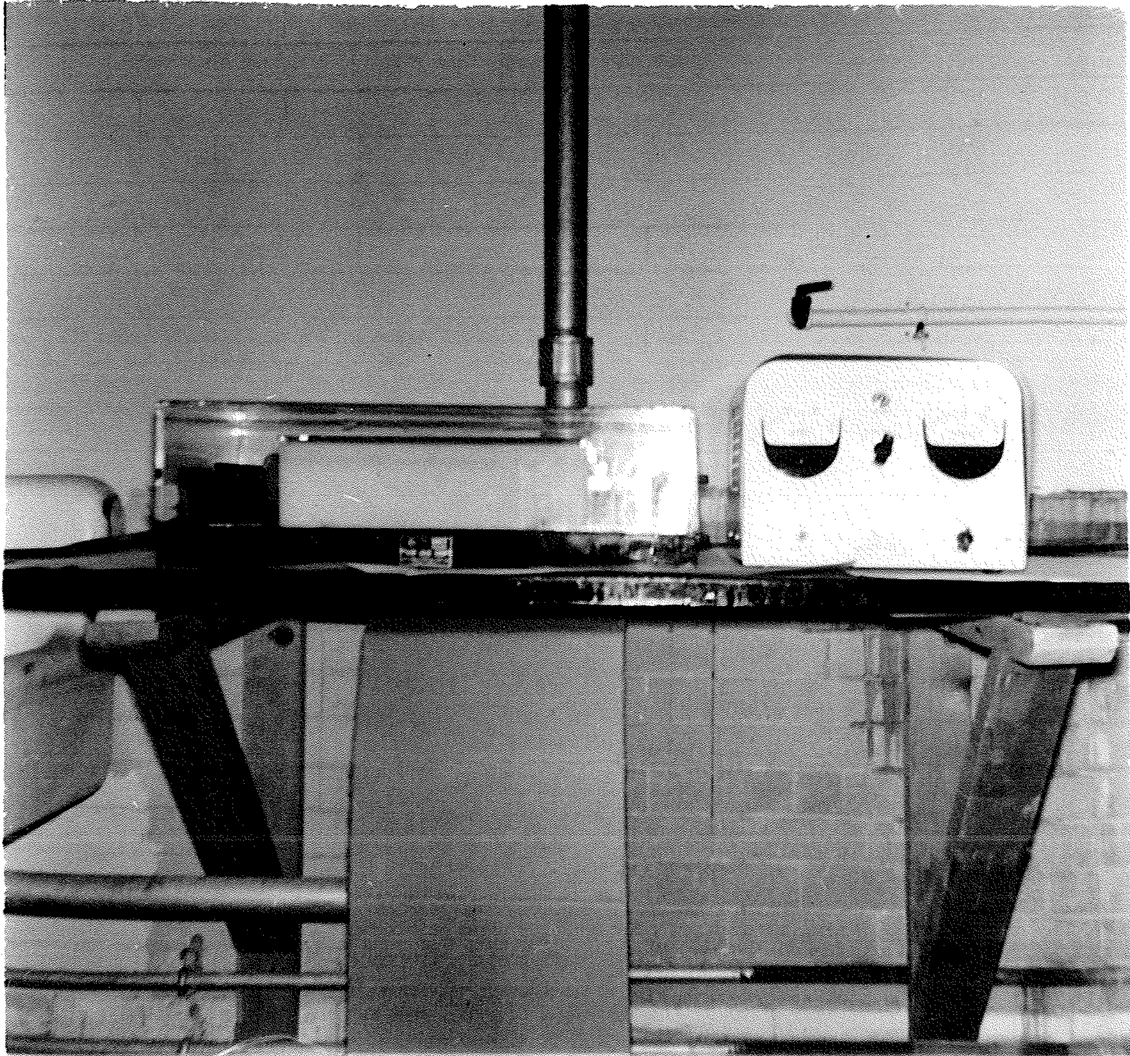


Figure 1.

Paper electrophoresis apparatus (general)

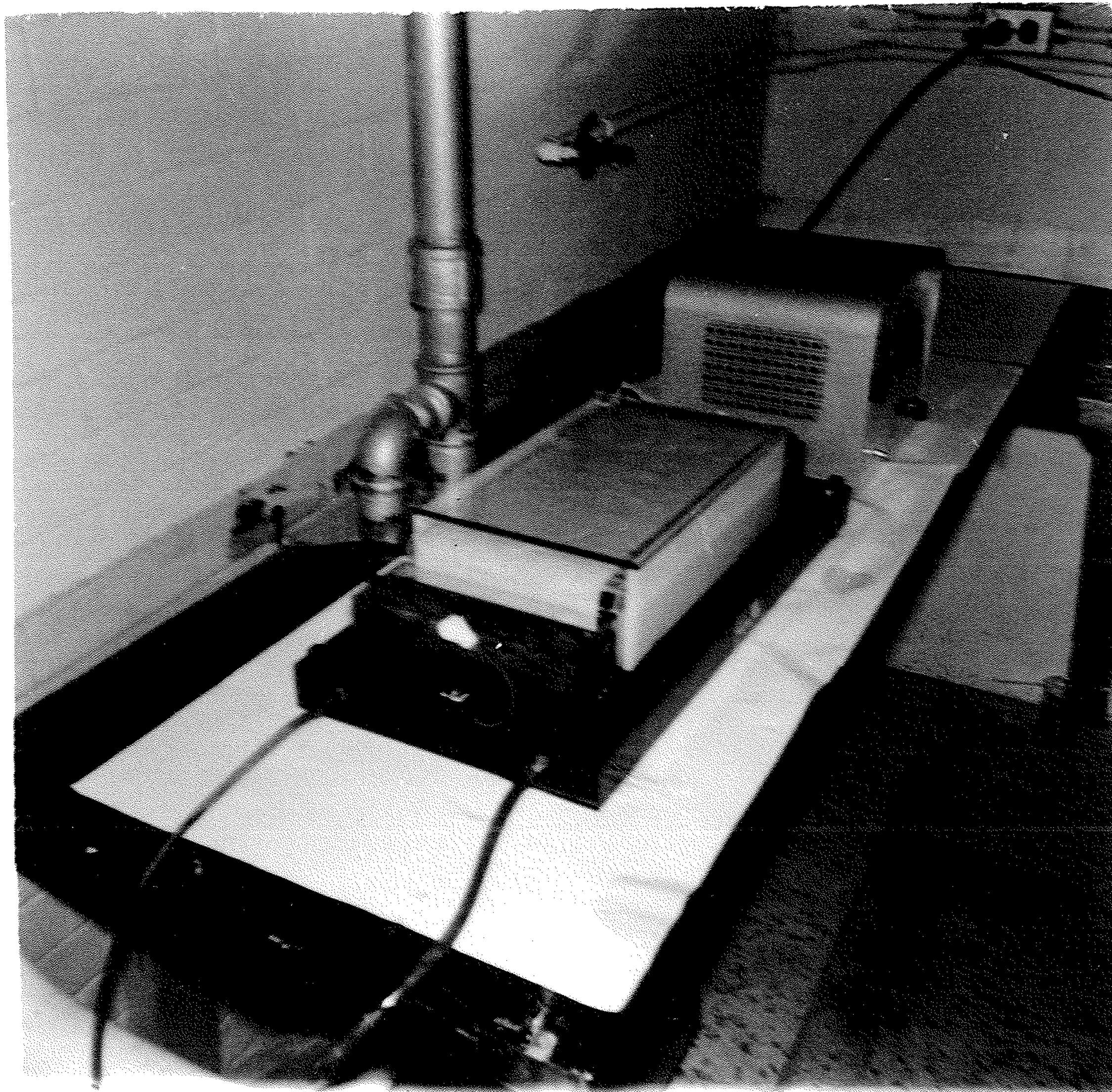


Figure 2.

Paper electrophoresis apparatus (horizontal)

time, the ends of each strip used were cut off in order to prevent the spread of the liquid from the buffer solution into the paper, and the paper was dried in a stream of hot air. The spots were located with a Mineralight short-wave ultra-violet lamp, a filter Sl 2537 (Ultraviolet Products Inc., South Pasadena, California) being used, and their identification was carried out by reference to the spots obtained from known compounds used in parallel runs.

Spots due to riboflavin and FMN showed a bright yellow, and the spot due to FAD showed pale yellow. The spot due to lumiflavin showed an intense greenish yellow, and that due to lumichrome a bright, sky-blue fluorescence.

The qualitative separation of the flavins over various periods of time is shown in Figure 4. FAD and FMN migrated toward the anode, FAD being twice as fast as FMN; riboflavin, lumiflavin, and lumichrome migrated in decreasing order of mobility towards the cathode. Figure 4 shows five strips run for varying times under the above given conditions. Strips a and b were run for four hours and all compounds were only beginning to separate; the strips numbered d and e were run for six hours and all five components were almost completely separated. However, FAD almost moved off the paper for these two strips, and, in strip c, which was allowed to run

for eight hours, FAD moved completely off the strip though all other components were separated.

Because of these results, it was decided to move the starting point for the separation from 10 to 15 cm. from the anode. This only yielded separation of FMN and FAD towards the anode, riboflavin, lumiflavin, and lumichrome barely separating at all, even after 24 hours. Excessive evaporation from the paper took place during this interval.

This method of electrophoresis was thus abandoned, since no one set of conditions could be utilized which would afford favourable separation of all five components.

Free hanging strip technique. The hanging paper method was next attempted. For this separation, the only change from the horizontal separation involved the raising of the center of the paper one inch above the platform surface so that, except at the ends where the paper dipped into the buffer dishes, it did not touch the platform. With this method, then, a quantitative estimation could be performed, providing, of course, that a set of conditions allowing complete separation of all five components could be found. The solution was again applied 10 cm. from the anode, and electrophoresis allowed to proceed for lengths of time which, experience showed, effected separation of the components. Figure 3 shows the apparatus being used in this way.

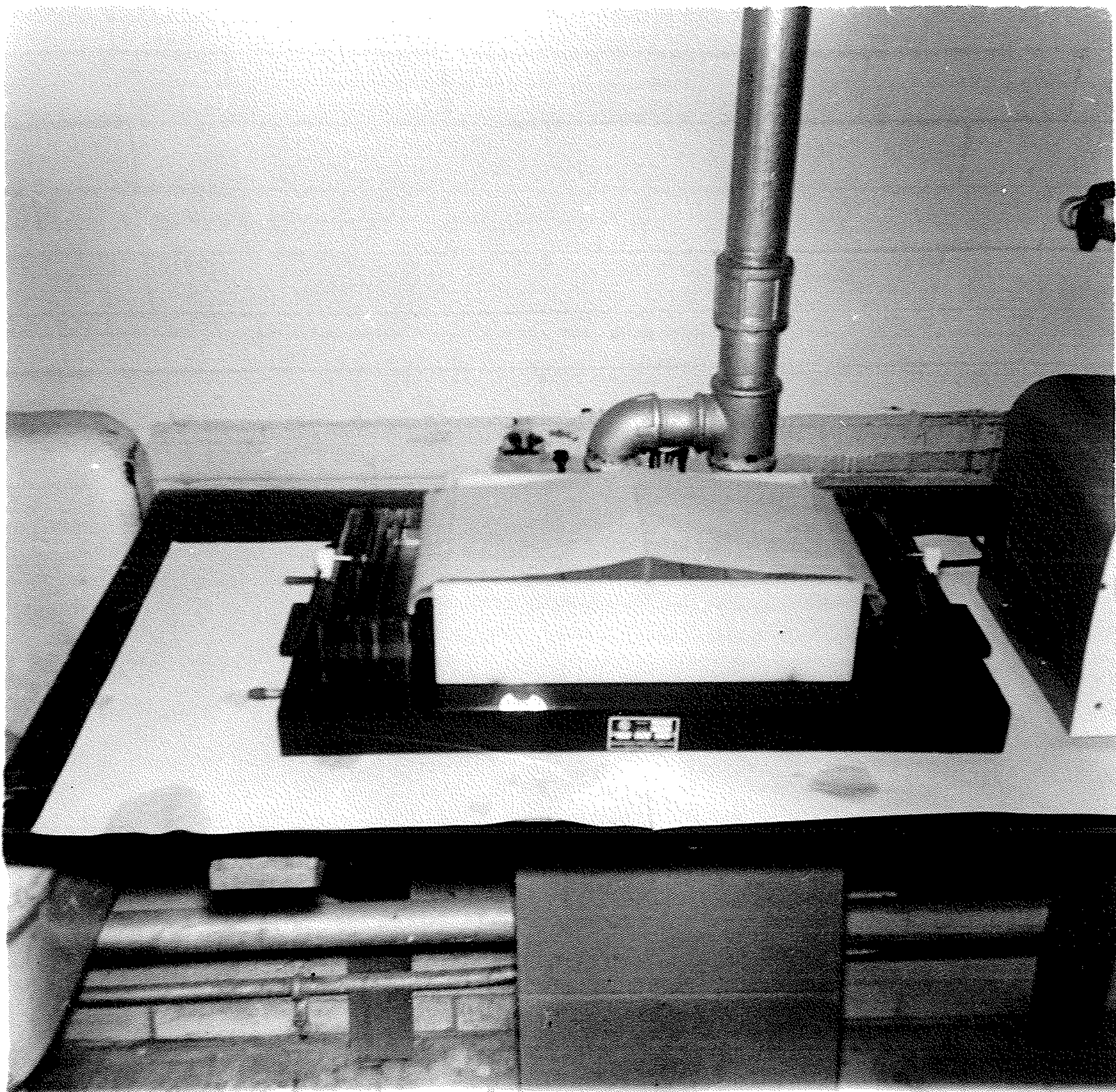


Figure 3.

Paper electrophoresis apparatus (hanging strip)

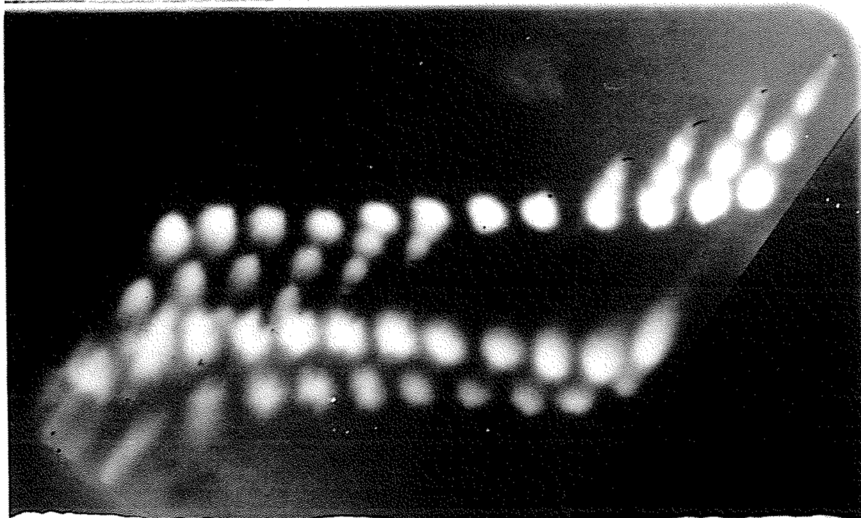


Figure 3.

Electrophoresis of flavins (hanging strip)

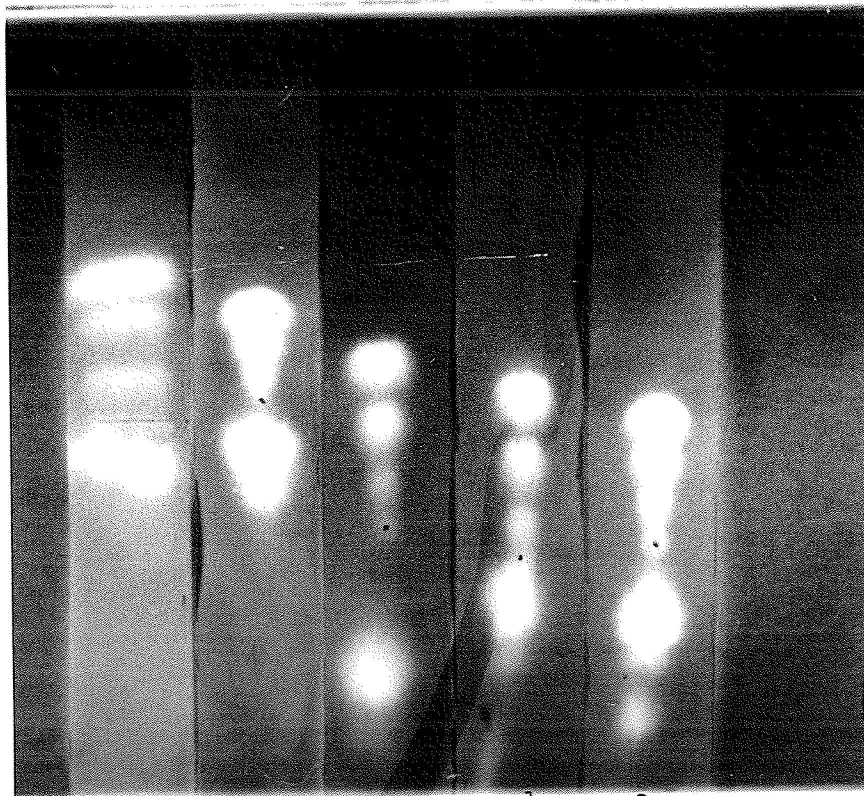


Figure 4.

Electrophoresis of flavins (horizontal)

In every run attempted from this position, the results were exactly the same as those shown by the horizontal method. Similarly, when a new position 15 cm. from the anode was attempted, the separation effected was the same as that described for the horizontal method at 15 cm. from the anode. Excessive evaporation again resulted, especially since the cooling system was not effective for this technique.

Because of the unusual results obtained, it was decided to perform an experiment in the hope of finding some point of application on the paper for which a quantitative separation could best be obtained. To this end, a sheet of filter paper 15 x 50 cm. was ruled in a diagonal position from end to end and mixtures applied one inch apart along this diagonal. Thus a series of 12 spots was obtained. The paper was then carefully moistened with buffer solution, and hanging paper electrophoresis allowed to take place for 6 hours using the same conditions as for horizontal electrophoresis. This yielded a current of 3.6 milliamperes/cm. width of the paper. Evaporation from the paper was again noted, and for this reason, some smudges in the final separated spots took place. The results are shown in Figure 5. All mixtures applied near the cathode moved towards the center of the paper, with FMN and FAD moving most rapidly. For solutions applied near the anode, FMN and FAD remained station-

ary while the other three components moved toward the cathode. For solutions applied at the center of the paper, near the apex, only FMN and FAD moved toward the anode, the other three substances remaining as one spot.

The large amount of evaporation taking place during these experiments led us to believe that perhaps the applied potential was too great. Thus, another series of experiments under exactly the same conditions, except for the applied voltage, was devised. Potentials of from 100 to 300 volts were applied to various mixtures of the compounds in use, for times varying from 4 to 72 hours. However, a clear cut separation did not occur at voltages below 200 volts, and any separation at higher voltages yielded results analogous to those previously mentioned.

Because of these results, separation by paper electrophoresis of any type seemed to be impossible, so it was decided to attempt the separation using the alternate method of paper chromatography.

Two reasons were apparent for the failure to produce a quantitative electrophoretic separation--one chemical and one physical.

Chemically, why should lumiflavin and lumichrome have migrated in the first place? Neither contained a potential ionic component such as phosphorus, as did the other three

compounds studied, nor did they form zwitterions in an electrolytic solution. True, these molecules could have moved due to the phenomenon of endosmosis, a passive migration of uncharged molecules or amphoteric materials in an electric field due to flow of electrolyte. Still, such migration of this type is usually quite pronounced and some separation should have occurred if endosmosis had actually occurred.

Secondly, and more important, was the problem of evaporation. If large amounts of evaporation had taken place at the center of the paper, as they probably did, then, to maintain equilibrium, a stream of buffer from both buffer dishes towards the center of the paper must have been continually taking place. This streaming of buffer should then have counteracted any electrophoretic migration towards cathode or anode, and, in fact, probably instituted a chromatographic migration towards the center of the paper.

These two explanations are best illustrated in Figure 5. Here, all solutions applied at the center of the paper, except FMN and FAD, have not separated since they were opposed on both sides by buffer flow towards them, and since they were probably not affected by electrical charge. FAD and FMN opposed this buffer flow, since they have become highly charged, and moved towards the anode. Riboflavin remained nearly stationary, probably because it

did not take a very great charge when electrically stimulated. When mixtures were applied near the cathode, all movement was towards the anode, with FAD and FMN moving fastest, and riboflavin, lumiflavin and lumichrome moving in that order from the applied spot.

FAD and FMN probably moved at the greatest rate because they were aided by both electrophoretic and chromatographic migration. At the anode, however, the movement of the buffer solution towards the center of the paper was so great that it completely opposed any migration of FMN and FAD in the reverse direction. In fact, it sent the other components moving rapidly towards the cathode.

Thus, the phenomena of chromatography due to evaporation, and non-migration of lumiflavin and lumichrome due to lack of both particle charge and endosmosis, would seem to explain why no separation by electrophoresis could be used in this study.

<u>Paper Chromatography</u>

Two methods of paper chromatography were attempted in this research: ascending and descending. In all cases, mixtures of flavins and derivatives were applied 4 cm. from the top or bottom of a sheet of Munktell 20 paper of length 48 cm. using a micro-pipette. The spot was dried with a fan and the paper strip or sheet, as the experiment demanded, was

chromatographed using a pre-selected solvent. After the ascending or descending front had moved to within 10 cm. of the end of the strip, the strip was removed and dried in a stream of hot air. The spots were then identified by the same procedure as for electrophoresis, using an ultra-violet lamp and parallel runs with known mixtures as references.

Descending paper chromatography. This method was first attempted because in most cases it usually resulted in a quick separation of mixtures. For our purposes, however, the results were unfavourable. No matter which of several solvents was used, descending chromatography always ran much too quickly, and so streaking of all compounds present always occurred. This method was then of little value, since a clear cut separation was a necessity for our purpose.

Ascending paper chromatography. This later method, on the other hand, proved much more satisfactory since the advancing solvent front moved more slowly in comparison with that for descending chromatography and the resulting chromatograms were found to be streak-free. It was this second method, then, with some slight variations, which was used for the remainder of this research.

Solvent Choice

N-butanol-acetic acid-water (4:1:5). The first solvent chosen was that composed of four parts n-butanol, one part acetic acid, and five parts of water (25). These components were shaken together and the resulting solution allowed to stand for twenty-four hours. At the end of this time two layers had formed. The bottom layer was used as the developing solvent for this chromatography. A portion of this layer was placed in a chromatography tank to a depth of one half of an inch and a small beaker containing a portion of the upper layer was also placed in the tank to maintain equilibrium. The strip of paper being chromatographed was then placed in the tank and attached by a glass rod to clamps along its top, so that the bottom of the strip just dipped into the developing solvent. Chromatography was allowed to take place for 18 hours at which time the solvent front had usually advanced to within 10 cm. of the top of the strip. The strip was then removed, dried, and examined under ultra-violet light. The results are shown in Figure 6.

Lumichrome is the fastest moving compound in this solvent, with lumiflavin moving just behind it. Riboflavin moves at a medial rate, while FMN and FAD barely move at all. The Rf values for these compounds in this solvent are:

Figure 6.

Separation of flavins and derivatives

(one-dimensional ascending chromatography)

(solvent: n-butanol-acetic acid-water (4:1:5)

(lumichrome not shown)

lumichrome	0.67
lumiflavin	0.46
riboflavin	0.33
FAD	0.02
FMN	0.01

The Rf values reported above denote the problem which confronted us using this solvent. FMN and FAD had such low Rf values that they did not completely separate. Several re-runs were made with the same sheet in the hope that a more definite separation might be made, but this only succeeded in making the other separated components more diffuse in the quality of the individual spots formed.

Water saturated 2,4-lutidine. Since FMN and FAD could not be separated using the first solvent, it was decided to attempt the separation with a different solvent which would separate these two compounds. Water saturated 2,4-lutidine was chosen for this purpose (33). The only deviation from the method used for the previous solvent was the time lapse allowed for each run. Water saturated 2,4-lutidine is extremely slow running, and after 30 hours the solvent front had barely moved more than 30 cm. At the end of this time the paper was removed, dried, and examined with an ultraviolet lamp. The results are shown in Figure 7.

The order of movement in increasing mobility along

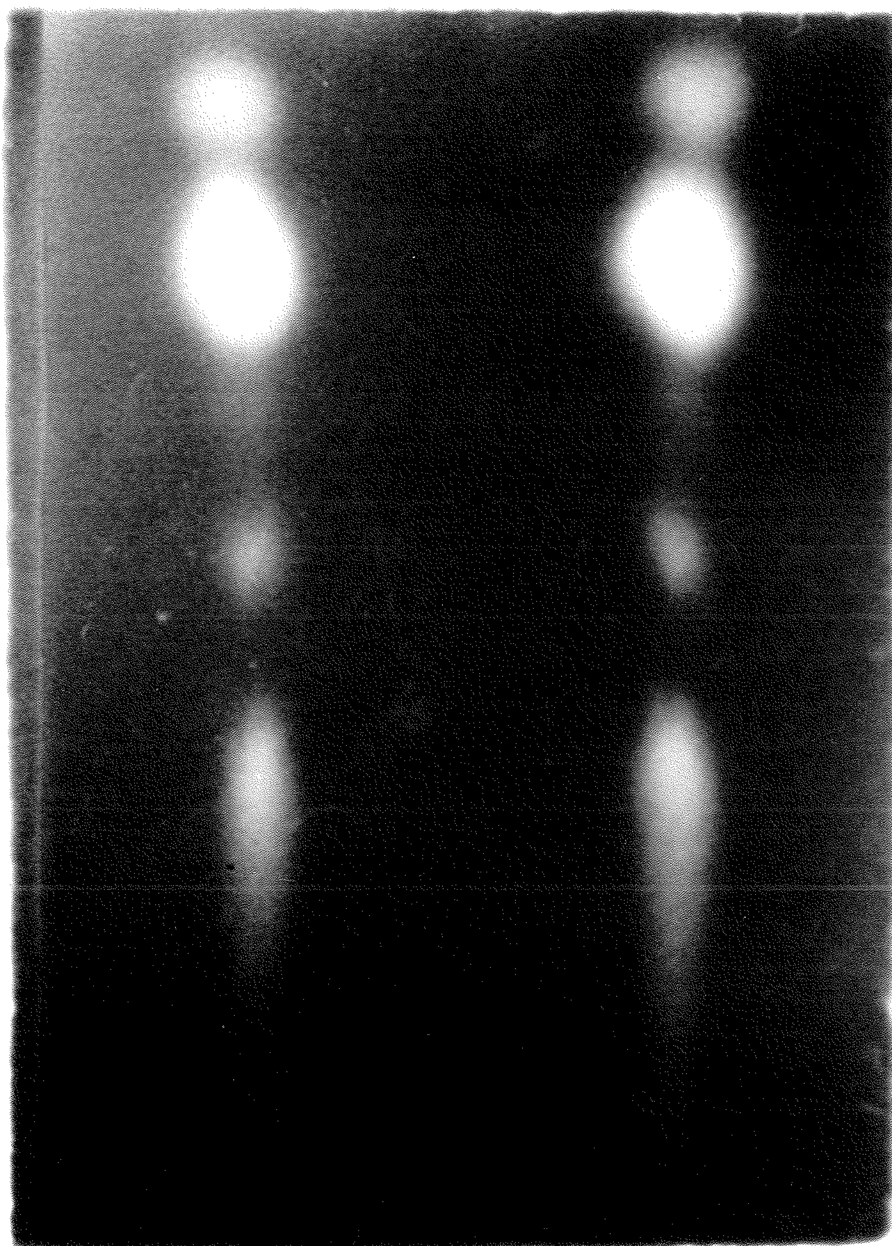


Figure 7.

Separation of flavins and derivatives

(one-dimensional ascending chromatography)

(solvent: water saturated 2,4-lutidine)

(lumichrome not shown)

with the Rf values for the components in this solvent are as follows:

FMN	0.410.41
FAD	0.55
lumiflavin	0.82
riboflavin	0.85
lumichrome	0.92

A slight streaking of FMN is also shown by Figure 7 but this was not uncommon and did not interfere with any other components in the mixture. Lumichrome cannot be seen here because it moves so quickly that it appears in the ascending front which also fluoresces under ultra-violet light and thus masks the presence of the lumichrome. Our problem, in this case, was the separation of riboflavin and lumiflavin which appear close together when chromatographed in this solvent. Their quantitative estimation was extremely difficult because of their slight overlap towards each other. Also, the appearance of lumichrome in the solvent front caused some consternation because of the difficulty in eluting a fluorescent material in a fluorescent solvent front.

Thus, no matter which solvent was chosen neither tended to give a clear cut separation of all five components. However, since FMN and FAD were separable using the latter solvent, and riboflavin and lumiflavin were separable using the former solvent, it seemed likely that it would be

possible to separate all five components by two dimensional ascending chromatography.

Two Dimensional Ascending Chromatography

Mixtures of the five compounds were deposited at a point 4 cm. from the left hand edge of a sheet of Munktell 20 paper 48 x 48 cm. and 4 cm. from the bottom of the same sheet. The paper was subjected to ascending chromatography using the water saturated 2,4-lutidine for 30 hours at which time it was removed and dried. The paper was then turned at an angle of 90° to the left so that all components previously travelling up the left edge of the paper would lie along the bottom of the paper. The sheet was then chromatographed for 24 hours using the n-butanol, acetic acid, water solvent, removed from the tank and dried in a stream of hot air. The resulting chromatogram was then examined under ultra-violet light. The results are shown in Figure 8. Each component has completely separated, with lumichrome lying towards the top right center of the sheet, lumiflavin in the top left center of the sheet, riboflavin to the left of lumiflavin, and FAD lying above FMN along the left side of the sheet.

Because the separation was so complete, it was decided to attempt a quantitative separation and estimation of all five components by this technique. To this end, a program was devised by which synthetic mixtures of the five sub-

Start

Figure 8.

Separation of flavins and derivatives

(two-dimensional ascending chromatography)

(solvent 1. water-saturated lutidine)

(solvent 2. n-butanol, acetic acid, water)

stances would be separated by two dimensional chromatography and the amount of each component determined. The percent recovery could then be calculated. This estimation was based on a fluorimetric measurement of the eluate obtained from the individual spot.

Unfortunately, any quantitative estimation by this method was subject to gross errors since the fluorescence of 2,4-lutidine was much greater than that of any of our standards used. That is, a total fluorescence of approximately 60% could contain anywhere from 0.1 to 1.0 micrograms of material being analyzed which might give a normal fluorescence of from 2.0 to 35.0% in any other solvent. Thus, in determining how much fluorescence is due to the substance being analyzed large errors would probably occur in the determination of substances of minute concentration (below 0.5 micrograms). Several attempts were made to solve this problem but none proved suitable so this procedure was also abandoned.

Five Per Cent Sodium Hydrogen Phosphate Solution

A third attempt at this five component separation was made using a 5% sodium hydrogen phosphate solution. A separation had been previously reported (30) using this solvent but no quantitative estimations were ever given. Strips of Munktell 20 paper 10 x 48 cm. were spotted 4 cm. from one

end and the paper placed in a chromatographic tank containing the phosphate solvent. The solvent was found to be very fast running and after 12 hours had nearly reached the top of the strip. The paper was then removed, dried, and examined with an ultra-violet lamp. The results obtained are shown in Figures 9a and 9b.

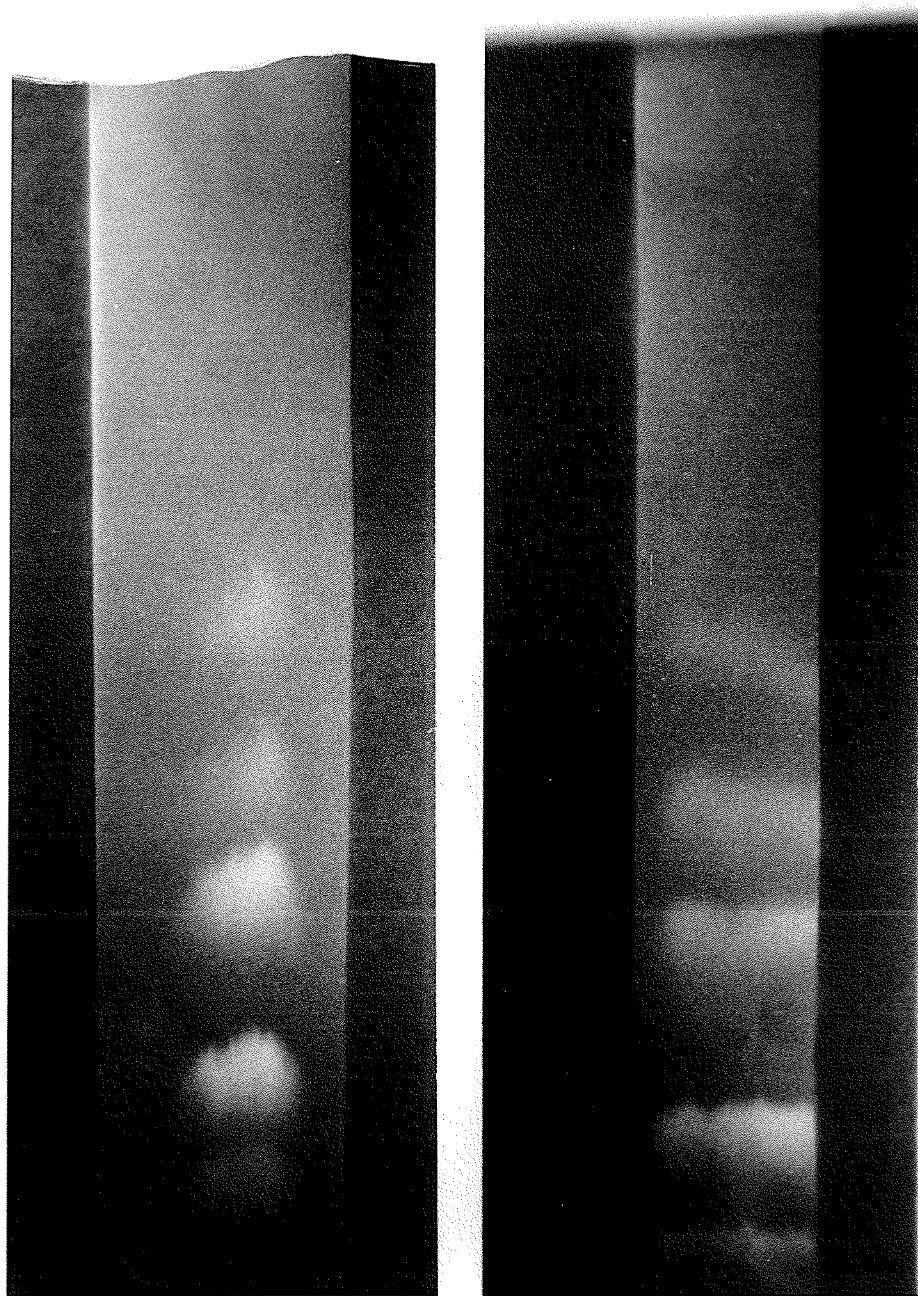
All five components have again separated completely with the following order of descending mobility including Rf values:

FMN	0.54
FAD	0.40
riboflavin	0.30
lumiflavin	0.18
lumichrome	0.07

Since in this case the separation was so clear cut, it seemed likely, then, that this method could probably yield quantitative separations.

Quantitative Considerations

For the quantitative separation and estimation of mixtures of the substances studied, the following procedure was used. Sheets of Munktell 20 paper, 48 x 48 cm., were marked out 8, 24, and 40 cm. from one edge and 4 cm. from the bottom. Known amounts of mixtures were then spotted on each marked out point using a "Prazision" micropipette. The



(a) Figure 9. (b)

Separation of flavins and derivatives

(one-dimensional ascending chromatography)

(solvent: 5% sodium hydrogen phosphate solution)

spots were then dried, and the sheets chromatographed for 12 hours in a darkroom at 37° C. using 5% Na_2HPO_4 solution as the mobile solvent. At the end of this time the sheets were removed and dried in a stream of warm air for 30 minutes. The separated components were then marked out under ultraviolet light and eluted into photofluorometer cuvettes by the method of Brimley and Barrett (34) using a 10% acetic acid solution for all elutions except that of lumichrome. For the elution of the latter a 50% methanol-water solution was used since lumichrome only fluoresced in solution in either methanol or chloroform.

After 24 hours of elution the cuvettes were removed and the solutions diluted to a volume of 8 cc. The fluorescence of each solution was then measured using a Coleman Model 12B Photofluorimeter which had been standardized with a dilute fluorescein solution before and during each analysis. About 10 milligrams of sodium hydrosulfite were then added to each cuvette to reduce the flavin or flavin derivative to its non-fluorescent form and the fluorescence of the solution again measured. This latter measurement thus took into account the fluorescence of any foreign materials present and when subtracted from the first measurement gave the true fluorescence due to the component being eluted. This fluorescence was then compared with that due to standards which had pre-

viously been chromatographed and eluted.

The amount of each component recovered was thus determined and from this the percentage recovered was also made. The results for typical runs ranging from mixtures of 0.3 to 1.0 micrograms each component are given in Table I. It is to be noted that mixtures below 0.3 micrograms were not analyzed since the fluorescence of these components at such low concentrations becomes exceedingly small and thus any errors made in fluorimetric readings would have been extraordinarily large.

TABLE I

RECOVERY OF RIBOFLAVIN AND ITS DERIVATIVES USING FIVE PER CENT SODIUM PHOSPHATE SOLUTION

Micrograms Applied	Percent Recovery				
	Riboflavin	FMN	FAD	Lumiflavin	Lumichrome
0.3	80.8	91.0	100.0	100.0	118.0
0.4	87.5	87.5	100.0	95.0	112.0
0.5	95.0	100.0	95.0	98.0	110.0
0.6	110.0	90.0	100.0	91.0	116.0
0.7	110.0	110.0	107.0	107.0	142.0
0.8	93.7	71.2	93.7	81.2	120.0
0.9	88.8	63.3	88.8	88.8	120.0
1.0	75.0	66.0	95.0	78.0	95.0

Each mixture applied contained the same concentration of each component.

DISCUSSION

DISCUSSION

The method used for the electrophoretic separation attempted here has shown itself to be unsatisfactory for the separation of mixtures of flavins and derivatives. The results obtained are not in complete agreement with those of previous workers (31, 32, 35). Yagi, and Siliprandi et al, have shown that FMN, FAD, and riboflavin can be separated by paper electrophoresis, and our work has confirmed this, especially in the latter case in which all physical and chemical conditions for the separation were duplicated. The results of Cerletti and Siliprandi could not be equated, however, and an explanation for this failure has already been given. The separation of these mixtures would, perhaps, be better accomplished using the method of Gordon, Keil and Sebasta (36) who made use of electrophoresis in a silica gel. This method would, of course, eliminate the problem of evaporation which plagued us during our experiments; similarly, the phenomenon of endosmosis in silica gel might proceed with more vigor so that the separation obtained by Cerletti and Siliprandi could be duplicated. Nevertheless, until the problems of evaporation and endosmosis are overcome, it seems feasible that no five component flavin separation by paper electrophoresis will be accomplished, at least not in western

Canada where the dry climate enhances evaporation.

Paper chromatography, on the other hand, has shown some success. It is true that neither the solvents made from a mixture of n-butanol, acetic acid, and water, nor that obtained from water saturated 2,4-lutidine were independently capable of clearly separating all five components in a mixture applied as one spot. However, the fact that both solvents could together separate the mixture by two dimensional paper chromatography is in itself gratifying. It is regretted that no quantitative recoveries of flavins could be made because of the fluorescence of the water saturated 2,4-lutidine front. Still, the fact that a two dimensional separation could be obtained leads us to believe that, by the careful choice of other solvents which do not fluoresce and which do not completely separate such mixtures by themselves, a quantitative two dimensional separation could be obtained.

The quantitative separation obtained using 5% Na_2HPO_4 solution was indeed fortunate, though not completely unexpected, since during electrophoretic procedures the sodium acetate buffer had shown tendencies to separate applied mixtures by chromatography. This phenomenon has already been fully explained with reference to the problems of evaporation.

The percent recoveries given in Table I indicate that the final procedure used in this study was nearly quantitative. Unfortunately, the recovery of FMN at higher concentrations was not complete. No particular reason can be given for this deviation from the excellent recoveries made at the lower levels. However, the high recoveries of lumichrome during the course of the analyses appear to indicate the probability of breakdown of FMN into lumichrome. True, the other important flavins might also split into lumichrome, but probably the breakdown of FMN proceeds at a faster rate than that of the other flavins. This is offered as a theory which has yet to be proved. It should also be noted that, at the higher concentrations used, the recoveries of the important flavins were poorest. This again, is probably due to breakdown of these flavins since it would seem logical that at higher concentrations a greater amount of degradation would take place.

However, the results as a whole are satisfactory, with over 60% recovery in all cases, and over 80% recovery below 0.8 mg.

Thus a nearly quantitative method for the separation and estimation of synthetic mixtures of riboflavin and its derivatives has been found. It is suggested that the next step be to attempt the quantitative recovery of flavins in tissue by this method but that will be left to some future date.

SUMMARY AND CONCLUSIONS

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The separation of riboflavin and derivatives by paper electrophoresis could not be obtained and reasons for this failure are given.

A qualitative method of separating riboflavin and its derivatives by two dimensional paper chromatography has been reported.

A nearly quantitative separation of riboflavin and its derivatives has been obtained by one dimensional ascending paper chromatography using 5% Na_2HPO_4 as a solvent.

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