with water to 10 cc and turbidity values determined with the Evelyn photoelectric colorimeter. Results are given in Table I. In no case did kynurenine or hydroxyanthranilic acid, either singly or combined, in the presence or absence of sub-optimal quantities of niacin, enhance growth. Ornithine, glutamine and arginine, which increase nicotinamide production by some strains of *E. coli*, have also been shown to be ineffective in substituting for the niacin requirement of *L. arabinosus*,[3] the organism most commonly used for assay of this vitamin.

Summary. Kynurenine and hydroxyanthranilic acid, which appear to be intermediates in the conversion of tryptophan to nicotinic acid by *Neurospora*, neither replace nicotinic acid nor enhance the growth response to sub-optimal amounts of nicotinic acid for *L. arabinosus, Leuc. mesenteroides, S. faecalis, Proteus vulgaris,* or *Torula cremoris*. These substances do not, therefore, interfere in microbiological assays for niacin which employ these test organisms.

Appreciation is expressed to Professor Ch. Weizmann whose kindness made this study possible.

16358

Paper Partition Chromatographic Analysis and Microbial Growth Factors: The Vitamin B_6 Group.

WALTER A. WINSTEN AND EDWARD EIGEN. (Introduced by A. E. Sobel.)

From Food Research Laboratories, Inc., Long Island City, N.Y.

Microbial growth factors often exist in nature in more than one chemically defined form. In the past, differential assays with several microörganisms have been used to demonstrate this multiplicity.

The present report deals with an alternate procedure for recognizing the presence of different forms of a growth factor. The method involves the combined use of paper partition chromatography and a microbial indicator and represents, as far as the writers are aware, a new methodological principle for the recognition and resolution of different chemical forms of a growth factor.

As will be seen, the method to be described is the counterpart of the technique used in the differention of the several forms of penicillin by Goodall and Levi[1] and independently by Winsten and Spark[2] except that in dealing with growth factors, zones of microbial growth serve to delineate the different members of a complex instead of zones of inhibition, as in the case of the antibiotics. The use of zones of microbial growth to assay vitamins was suggested by the work of Mager and Aschner.[3]

In the present method, the underlying principle involves the separation on a paper strip chromatogram of the different chemical forms of a growth factor. After the separation has been achieved, the positions of the different forms on the paper strip are revealed by the use of a microbial indicator, that is, a microörganism capable of utilizing at least one and usually several forms of the factor for growth. The positions of the various chemical entities on the chromatogram serve to characterize and identify the particular substances present. This is a consequence of the fact that the distance each substance moves is related to its specific partition coefficient for the solvents employed in developing the chromatogram.

In what follows, the application of this method to the resolution and identification of the members of the B_6 group will be described. This particular complex of growth factors was

[1] Goodall, R. R., and Levi, A. A., *Nature*, 1946, **158**, 675.

[2] Winsten, W. A., and Spark, A. H., *Science*, 1947, **106**, 192.

[3] Mager, J., and Aschner, M., *J. Bact.*, 1947, **53**, 283.

studied first since its component parts are well known, principally due to the work of Snell.[4] The present report is intended to demonstrate the potentialities of the technique employed and to describe some results obtained by its use.

Method. In applying the method to the vitamin B_6 group, a 0.007 ml drop of a solution (pH adjusted to 5.0) containing 1-5 μg per ml of each of the various members of the group is applied near the head of a paper strip chromatogram (Whatman No. 4 paper strips 1″ by 16″ are used). The chromatogram is then developed for about 6 hours with wet butanol in a humid atmosphere in the manner first devised for amino acids by Consden, Gordon and Martin.[5] After drying for 15 minutes at 65°C, the paper strip chromatogram with the various members of the vitamin B_6 complex now occupying definite positions along the strip, is laid on an agar plate, seeded with *Saccharomyces carlsbergensis,* strain 4228, an organism which exhibits a growth response to all three known forms of the vitamin. The nutrient agar[6] contains all factors necessary for the growth of the organism with the exception of the vitamin B_6 group. In making the agar plate a bottom layer of the nutrient agar is made by pouring 300 ml of the agar medium on a plate 11″ by 18″. This is allowed to harden. A 200 ml portion of the nutrient agar cooled to 48-50° C is seeded with 10 ml of sterile physiological saline to which has been added a loopful of a 24-hour culture of the yeast. The seeded agar is then poured on the hardened underlayer and allowed to cool. (Using such a plate, as many as 8 strip chromatograms resulting from eight separate analyses may be laid parallel to each other on the nutrient agar at one time). The paper strip chromatogram is allowed to soak for 5 minutes on the surface of the moist agar in order to transfer the various B_6 members from the strip to the agar plate. The strip is then removed leaving its imprint, however, on the agar surface. The agar plate is then incubated overnight at 27-30°C. After incubation well defined elliptical zones of growth of the organism are seen at various intervals along the locus of the chromatogram marking the loci of the various members of the vitamin B_6 group. The area of each zone of growth is a measure of the amount of the particular substance causing the growth. While it has proved possible to obtain a dose response curve for each substance by plotting area of growth against weight of substance per ml, the greatest utility of the method lies in its ability to resolve and identify the various members in complex mixtures of the different forms of vitamin B_6. Visual inspection of the size of the zones of growth gives a graphic picture of the relative amounts of the various forms present in a mixture.

The different members of a complex are identified by their relative positions along the length of a chromatogram. Each substance has its characteristic R_F value which is defined according to Consden *et al.*[5] as the ratio of the distance a substance has moved down the chromatogram to the distance the developing solvent has traveled. This is conveniently measured directly on the agar plate if one imprints on the agar the spot where the original drop was applied as well as the boundary on the strip to which the solvent front has moved. This is readily done when the paper strip is still in place on the agar surface. The R_F value is then equal to the distance of the center of a zone of growth from the point of application of the sample being analyzed, divided by the distance the solvent has moved measured from the point of application of the drop.

Results. The above technique was first applied to the resolution of the members of the vitamin B_6 group in a synthetic mixture.

On chromatographing a 0.007 ml sample of a mixture containing 5 micrograms each per ml of pyridoxal, pyridoxine and pyridoxamine with wet butanol, the characterizing R_F values for the component factors were found to be: pyridoxamine 0.18, pyridoxal

[4] Snell, E. E., *J. Am. Chem. Soc.*, 1944, **66**, 2082.

[5] Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, 1944, **38**, 224.

[6] Atkin, L., Schultz, A. S., Williams, S. L., and Frey, L. M., *Ind. Eng. Chem., Anal. Ed.*, 1943, **15**, 141.

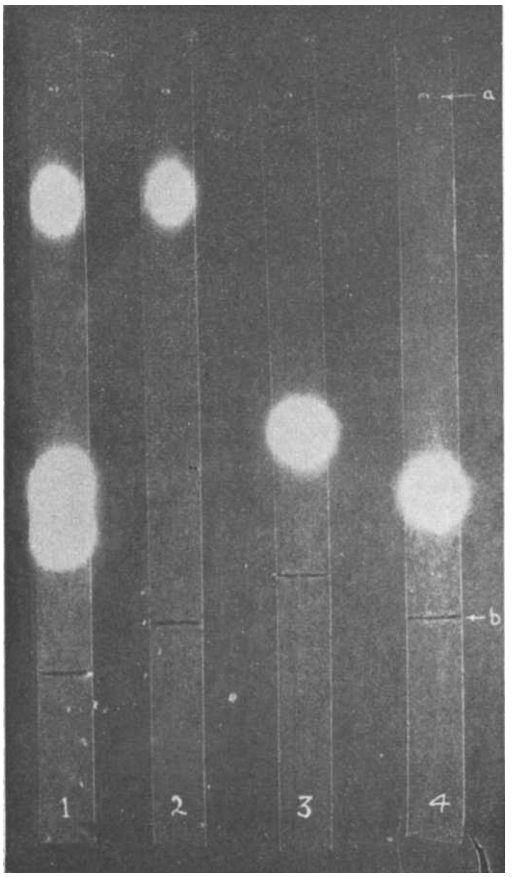

FIG. 1.
Results of paper partition chromatography applied to synthetic members of the B_6 group. The photograph represents a picture of a typical agar plate and shows the zones of growth of *S. carlsbergensis*, Strain No. 4228, along the loci of the paper strip chromatograms. The symbol (a) refers to the locus of point of application of the sample. The symbol (b) refers to the boundary to which the solvent had moved. The strips represent the result of chromatographing a mixture of pyridoxal, pyridoxamine, and pyridoxine (strip 1), pyridoxamine alone (strip 2), and pyridoxal alone (strip 3), and pyridoxine alone (strip 4).

0.68, pyridoxine 0.75. That is, pyridoxamine moves least rapidly on the chromatogram. Fig. 1 shows the results of such an experiment.

The R_F values were found to vary 10 to 20% from day to day. However, on any given day replicate chromatograms gave self-consistent R_F values. The relative positions on the chromatograms of the different forms of B_6 remained the same.

In order to simulate what might occur on chromatographing an extract of the vitamin B_6 group obtained by the usual acid hydrolysis of natural products, a study was then made of the influence of a mixture of amino acids on the R_F values of the B_6 group members since various amino acids might be expected to be present in such extracts. Accordingly, the various forms of B_6 dissolved in a solution containing 5 per cent casein hydrolysate adjusted to pH 5, were chromatographed as above.

The presence of the amino acids caused a diminution in the R_F value of pyridoxamine from 0.18 to 0.06; that for pyridoxine decreased from 0.75 to 0.64.

Since the presence of amino acids in solutions being analyzed for the B_6 group alters the R_F values, these are not sufficient to identify the B_6 components unequivocally in an unknown mixture. It has been found expedient to identify members of the B_6 group in an extract of natural origin by obtaining a second chromatogram of the sample to which a known form of B_6 has been added to serve as a marker. The relative position occupied by the various B_6 members to that of the known form of B_6 added as a marker, serve to identify the former.

The results obtained on chromatographing a solution of pyridoxal and casein hydrolysate amino acids indicated that pyridoxal had reacted with certain constituents of the hydrolysate. Two zones of growth were observed. One was identified as pyridoxamine. On standing for several days at icebox temperature under aseptic conditions the pyridoxamine zone increased in size indicating that almost half the original pyridoxal was converted to pyridoxamine. Snell and Rannefeld[7] have shown that on heating pyridoxal in solutions of amino acids, it is converted to pyridoxamine. However, the ease with which this reaction occurs, even at low temperatures, has not been fully appreciated. This finding throws some doubt on the validity of the usual type of microbiological assay for pyridoxal where the extract also contains amino acids, since these might convert an unknown frac-

[7] Snell, E. E., and Rannefeld, A. H., *J. Biol. Chem.*, 1945, **157**, 475.

tion of pyridoxal to pyridoxamine.

The second zone observed on chromatographing pyridoxal in casein hydrolysate solution, namely that due to pyridoxal itself, was found to be more diffuse and cover a larger area along the locus of the chromatogram. This lengthening of the zone was interpreted as being due to a reversible reaction of pyridoxal and certain amino acids, a fact suspected by Snell but not heretofore easily demonstrated.

In using the analytical technique under discussion it has been possible to demonstrate a heretofore unrecognized reaction of a reversible type between *L*-cysteine and pyridoxal. This reaction which is non-enzymatic, occurs merely on incubating together a concentrated solution of cysteine and pyridoxal. The reaction is to be distinguished from that occurring between pyridoxal and other amino acids such as glutamic acid to yield pyridoxamine. In addition to a small but variable amount of pyridoxamine which does form during the reaction, there is an additional substance formed which moves more slowly on the chromatogram than does pyridoxamine. A typical experiment in which pyridoxal and cysteine were allowed to react was conducted as follows: 179 mg of pyridoxal hydrochloride and 157 mg of cysteine hydrochloride were dissolved in one ml of water. After standing overnight at 37°C the solution was diluted to 5 ml and 0.16 ml of pyridine was added to neutralize the acidity. The solution was then allowed to stand 2 days at room temperature. After appropriate dilution to yield solutions containing the nominal amount of 360 μg per ml and 36 μg per ml of pyridoxal hydrochloride based on the original amount of pyridoxal hydrochloride used, and adjustment to pH 5.0, 0.007 ml samples were taken for chromatographic analysis. The results of this analysis appear in Fig. 2. An examination of the replicate chromatograms 3 and 4 obtained for the more concentrated sample, reveals two zones of growth connected by a band of growth. The lower zone is due to the faster moving unchanged pyridoxal. The upper zone is due to a new substance which moves on the chromatogram at a rate less than that of pyridoxamine itself as established in a separate experiment. The new zone of growth probably represents an adduct formed from cysteine and pyridoxal and is either a new form of vitamin B_6 or a substance which readily regenerates the original pyridoxal for use by the microörganism. The reaction to yield the substance in question is evidently reversible. Such reversibility would explain the band of growth connecting the two zones since, as the solvent used to develop the chromatogram moves the pyridoxal down the column, its removal from the original point of application of the sample will reverse

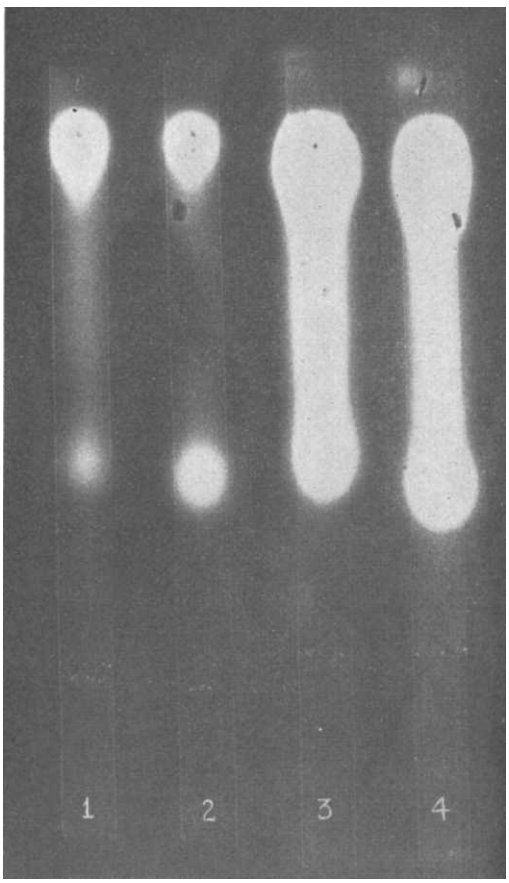

FIG. 2.
Results of paper chromatographic analysis of a reaction mixture of *l*-cysteine and pyridoxal. Duplicate strips 1 and 2 represent the analyses of a 0.007 ml sample of a solution containing the equivalent of 36 micrograms of pyridoxal hydrochloride per ml. Duplicate strips 3 and 4 are those obtained for a solution containing the equivalent of 360 μg per ml of pyridoxal hydrochloride.

the equilibrium reaction of cysteine and pyridoxal regenerating the latter; this would then make its appearance known by causing a tail of growth on the main pyridoxal zone as observed experimentally.

Examination of the replicate chromatograms 1 and 2 (Fig. 2) of the more dilute sample again reveals 2 zones of growth, one due to a new substance moving more slowly than pyridoxamine, and the other moving like pyridoxal itself. There is little trace seen of the band of growth connecting the zones as observed in analyzing the more concentrated sample. This result suggests the formation of a second more stable derivative of pyridoxal and cysteine whose existence is not so readily disturbed by dilution and which moves at about the same rate as the unstable unknown substance whose existence was noted in the more concentrated sample. As a consequence, for the more concentrated sample only one composite zone is seen at the top of the chromatogram and may be due as indicated above to two substances.

The reaction of pyridoxal and cysteine may take place in a manner similar to that of other simple aldehydes and aldoses with cysteine as demonstrated by Schubert.[8] Thus a simple adduct may form between the cysteine sulfhydryl group and pyridoxal. This reaction might be expected to be quite reversible. The adduct may then cyclize to give a thiazolidine ring derivative of vitamin B_6. The latter compound would probably be more stable to hydrolysis than the 1st adduct mentioned. This compound might represent the slow moving substance observed on chromatographing the more dilute sample.

The compounds formed from pyridoxal and cysteine for which evidence has been presented above, may represent new forms of vitamin B_6 of physiological interest hitherto unrecognized because of their unstable character.

It might have been quite difficult to demonstrate such a reaction between cysteine and pyridoxal with the usual form of microbiological assay.

A further result of practical interest is the finding that many preparations of synthetic nature such as pyridoxal itself and pyridoxal phosphate are impure and contain foreign substances which may themselves be active as growth substances replacing B_6. Thus the present technique provides a delicate test of purity for standard preparations of growth factors used in biological and biochemical investigations.

Studies are now being conducted on natural products using this method. These will be reported at a later date.

The potential usefulness of the general technique involving a combination of paper partition chromatography and microbiological indicators should be of value in studies on the differentiation of other growth factors such as folic acid which occur in nature in more than one chemically defined form. This consideration also prompted the present report.

Summary. 1. A new technique of resolving and identifying the members of the B_6 family of growth factors is described. It involves the use of paper partition chromatography coupled with a microbial indicator.

2. Using this method it has been demonstrated that pyridoxal undergoes a non-enzymatic transamination reaction even in the cold. Pyridoxal also appears to react reversibly with other amino acids.

3. The reaction between cysteine and pyridoxal differs from that with other amino acids in that one can demonstrate the formation of at least one adduct which moves more slowly on the chromatogram than does pyridoxamine. This reaction may produce a new form of vitamin B_6 which may also have a transient existence in nature.

4. The technique described should prove of value as a means of establishing the purity of the various forms of vitamin B_6 used in biological and biochemical studies.

5. The general technique employing paper partition chromatography and microbial indicators should be of value in resolving other multiform vitamins into their constituent parts.

[8] Schubert, M., *J. Biol. Chem.*, 1939, **130**, 601.