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Studies on the Streptomycin Complex Using Paper Partition Chromatography

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Recent studies on the nature of the antibiotic streptomycin have demonstrated that crude preparations of this substance contain at least two antibiotic entities. One of these is streptomycin itself, now known to be composed of the disaccharide streptobiosamine linked glucosidically to a diguanidine base, streptidine.[1] The other is streptomycin B or as it is now called, mannosidostreptomycin.[2,3,4]

In demonstrating the existence of mannosidostreptomycin, Fried and Titus[2] made use of the Craig counter-current distribution technique. Evidence was also offered by these authors for the existence of a third form of the antibiotic.[5]

Since paper partition chromatography has proved to be an effective tool in resolving complex mixtures of the penicillins as shown by Goodall and Levi,[6] and by Winsten and Spark,[7] it was felt desirable to adapt this technique to the streptomycin problem. The present report concerns the development of a paper partition chromatographic method for the separation and identification of the antibiotic entities comprising the streptomycin complex.

Recently, Horne and Pollard[8] have described a method for the identification of streptomycin using paper-strip chromatograms. However, the technique as described by these authors differs from that here reported inasmuch as it does not serve to separate the various forms of streptomycin from one another.

The method as developed by the present writers is carried out in a manner analogous to that used in the separation of the penicillins.[6,7] Briefly it consists of separating the various forms of streptomycin on a paper-strip chromatogram, using a suitable developing solvent as the mobile phase and water adsorbed on paper as the stationary phase.

After developing the chromatogram the solvent is removed and the dried chromatogram is then laid on the surface of nutrient agar previously seeded with specific bacteria whose growth is inhibited by the antibiotics in question. The moist agar leaches the antibiotics from the strip chromatogram, which is then lifted from the surface of the agar. After allowing a suitable incubation period for growth of the bacteria, examination of the agar plate reveals zones of inhibition of growth along the locus of the strip chromatogram. The positions of the zones of inhibition serve to characterize the various antibiotics present in the samples analyzed. The size of the zones is a measure of the amounts of the various antibiotics present in the original mixture.

In applying this procedure to the problem of the separation and identification of streptomycin-like antibiotics, great difficulty was experienced in finding a suitable solvent.

In the light of the success of Titus and Fried[5] in separating the streptomycins at least partially, using as a solvent a solution of wet *n*-butanol containing a suitable concentration of *p*-toluenesulfonic acid, this solvent was tried first. Upon using the solvent as the developing liquid, it was found that it did indeed move the antibiotics in a crude streptomycin preparation down a paper strip chromatogram. However, no separation was achieved of the various antibiotic entities suspected of being present in the preparation. It was believed that this failure resulted from an interaction of the antibiotics with the paper support. That is, it was considered possible that the paper was not behaving merely as an inert support for the stationary water phase.

After many trials, solvent systems were found which were capable of resolving mixtures of streptomycin-like antibiotics. A discussion of these systems and the results obtained with them, form the body of this report.

Experimental

In applying the method to the streptomycin complex, a 0.007–0.010 ml. drop of a solution of the antibiotic is applied near the head of a paper strip chromatogram (Whatman No. 4 paper strips 1.5″ by 16″ are used). (The *p*H of the antibiotic solutions to be chromatographed is not critical since the *p*H of the strip chromatogram is governed by the *p*H of the developing solvent used. Solutions of the preparations studied had *p*H values in the range 6–7 and were used without further adjustment.

The concentration of antibiotics in the solutions tested varied from about 300 units per ml. up to as high as 12,000 units per ml. The more concentrated solutions were used in seeking for the presence of streptomycin-like antibiotics present in trace quantities).

The chromatogram is then developed at room temperature for a period of time which depends on the particular solvent employed as the mobile phase, in the manner first devised for amino acids by Consden, Gordon and Martin.[9]

The solvent used is then removed by successive washes of the chromatogram in ether, conveniently contained in liter cylinders. Three washes are employed and the strip chromatogram is then air-dried.

After drying, the paper strip chromatogram with the various members of the streptomycin complex now occupying definite positions along the strip, is laid on an agar plate, seeded with *Staphylococcus aureus*, American Type Culture Collection No. 9996, a strain often used for the

(1) F. A. Kuehl, Jr., E. H. Flynn, N. G. Brink and K. Folkers, THIS JOURNAL, **68**, 2679 (1946).
(2) J. Fried and E. Titus, *J. Biol. Chem.*, **168**, 391 (1947).
(3) J. Fried and H. E. Stavely, THIS JOURNAL, **69**, 1549 (1947).
(4) S. A. Waksman, *Science*, **107**, 233 (1948).
(5) E. Titus and J. Fried, *J. Biol. Chem.*, **168**, 393 (1947).
(6) R. R. Goodall and A. A. Levi, *Nature*, **158**, 675 (1946).
(7) W. A. Winsten and A. H. Spark, *Science*, **106**, 192 (1947).
(8) R. E. Horne and A. L. Pollard, *J. Bact.*, **55**, 231 (1948).
(9) R. Consden, A. H. Gordon and A. J. P. Martin, *Biochem. J.*, **38**, 224 (1944).

assay of streptomycin. The streptomycin assay agar used may be obtained from the Difco Laboratories in dehydrated form. The plate employed to contain the agar consists of a galvanized iron frame 18" long and 11" wide with $^3/_4$" high walls. The edge of the frame is $^3/_4$" wide. A sheet of window plate is placed in the iron frame and held there by waterproof adhesive tape 1" wide. The plate is first sterilized by washing with ethyl alcohol; 300 ml. of streptomycin assay agar is then poured uniformly over the plate and allowed to harden; 10 ml. of a 16–24 hour culture of *S. aureus* (grown on FDA nutrient broth[10]) is then added to 200 ml. of the assay agar which has first been melted and cooled to 50°. The culture and agar are mixed thoroughly and then poured evenly over the base layer and allowed to harden. (Using such a plate, as many as six strip chromatograms may be laid parallel to one another on the agar at one time.)

After allowing the paper strip chromatogram to soak for five minutes on the surface of the moist agar, the strip is removed. The agar plate is then incubated for five to twenty-four hours at 37°. After incubation zones of inhibition of growth, which may or may not be connected, are seen along the locus of the strip chromatogram. These zones mark the positions of the particular substances causing the inhibition of microbial growth. The areas of the zones of inhibition are a measure of the amounts of the various antibiotics present. While it is possible to obtain a dose response curve relating area of zone of inhibition to amount of sample tested, in the present report the method has been used solely as a means of qualitatively identifying the members of the streptomycin complex in a variety of preparations.

The solvents used as components of the various developing liquids, namely, *n*-butanol, 2,4,6-collidine, 2,4-lutidine and piperidine, were of reagent grade and not further purified. The first three solvents mentioned were each saturated with water at room temperature and filtered free of water droplets. *p*-Toluenesulfonic acid monohydrate was purchased from the Eastman Kodak Company.

The streptomycin preparations employed in the present study were obtained through the courtesy of Dr. Max Tishler of Merck and Co. and Dr. O. Wintersteiner of the Squibb Institute for Medical Research. These preparations varied widely in purity.

A sample of the FDA working standard for streptomycin also was studied. No information was available as to the origin of the various preparations or on the methods used to obtain them. The preparations have been numbered from I to VIII. The available information on these preparations is summarized in the following table:

Preparation	Antibiotic activity in terms of micrograms of streptomycin base per mg.	Remarks
I	400	A crude, partly purified preparation containing principally streptomycin
II	740	Highly purified calcium chloride double salt of streptomycin
III	300	Contains a high proportion of streptomycin B
IV	160	Residue from purification of streptomycin
V	800	FDA working standard
VI	223	Possibly like IV
VII	163	Possibly like IV
VIII	123	Possibly like IV

The preparations were dissolved to give as high as 3% (W/V) solutions. The actual concentrations used are given in the legends to the figures.

(10) Federal Register, April 4, 1947; 12 F. R. 2215, 2217–2226.

In order to identify a given member of the streptomycin complex, its position on a chromatogram was compared with that of a known streptomycin.

Results and Discussion

Collidine–Piperidine as the Mobile Phase.—A partial separation of the constituent antibiotics in the streptomycin complex was obtained on a paper chromatogram using wet collidine as a developing solvent, in an atmosphere resulting from the addition of 3% ammonium hydroxide to the bottom of the humidifying chamber in which the chromatograms were developed. However, this system proved to be very inefficient. The results suggested that it would be worth while to investigate the use of the stronger base, piperidine, added directly to the wet collidine. It was thought that if the guanidonium groups of the streptomycin could be converted even in small degree to the free base form, the solubility of the streptomycin in collidine might be increased thus resulting in more rapid development of the chromatogram. It was realized that above *p*H 11, the stability of streptomycin is diminished. However, it was nevertheless deemed worth while to raise the *p*H of the aqueous stationary phase on the paper chromatogram.

To wet collidine was added 2, 4, 6 or 10% (V/V) of piperidine and the resulting solvents were used as mobile phases in developing a series of chromatograms. No advantage was obtained by raising the piperidine content above 2%. Using wet collidine–2% piperidine, the development of the chromatogram was still very slow (although superior to the use of the collidine–ammonia combination). It was necessary to chromatograph for two to three weeks to obtain any separation. However, while exceedingly slow, the solvent proved to be quite selective.

Results of chromatographing samples of several preparations are given in Fig. 1. In examining this and subsequent figures it is important to note that the sizes of the several zones of inhibition on a given chromatogram do not necessarily indicate the relative amounts of the various antibiotics present in the sample taken for analysis. The reason for this lies in the fact that in using relatively concentrated solutions of a given preparation in order to show the presence of any antibiotics present in trace amounts, one may be dealing with the plateau of the dose-response curve for the antibiotic or antibiotics present in large concentration, while for an antibiotic present in small amount, the dose–response would fall on the ascending portion of that antibiotics' dose–response curve. It is also true that the shape of the dose–response curve may differ for the various antibiotics present.

Examination of strip 2 (Fig. 1) which represents the results of chromatographing a highly purified streptomycin preparation (preparation II) shows a large zone of inhibition which locates the position of authentic streptomycin, the major component

present. The "tail" of inhibition trailing this zone shows signs of splitting which was more apparent in the original plate than in the reproduction in Fig. 1.

Strips 3 and 4 were obtained as a result of chromatographing preparation III which was stated to contain a large proportion of mannosidostreptomycin (streptomycin B). Examination of these strips reveals in addition to the streptomycin zone (the lowest one), a second large zone directly over that of streptomycin. This second zone, due to an antibiotic moving more slowly on the chromatogram than streptomycin, is considered to be due to streptomycin B in the light of the information supplied with this preparation.

Immediately above the streptomycin B zone are two small zones which, as will be discussed later, are either artefacts or may be attributed to two antibiotics other than streptomycin and streptomycin B. One of these may also be present in the highly purified preparation II (Fig. 1, strip 2).

Strip 1, which represents the results obtained for preparation I, indicates (starting with the most rapidly moving antibiotic) the presence of streptomycin, streptomycin B and two trace zones of inhibition which may be artefacts or be due to two other antibiotics.

Finally, strips 5 and 6, obtained for preparation IV, indicate, in addition to streptomycin, streptomycin B and the possible existence of two other antibiotics (the top zone has a doublet character), the presence of at least one antibiotic which moves even more rapidly than authentic streptomycin on the chromatogram and forms a poorly shaped zone of inhibition directly ahead of the streptomycin zone. The absence of this zone in chromatograms of the other preparations lends credence to the existence of such an antibiotic and tends to discount the possibility that it results from an artefact. This more rapidly moving antibiotic may represent the third form of streptomycin suggested by Titus and Fried.[5]

The data obtained with collidine–2% piperidine as the mobile phase for a three-week period has been interpreted with caution especially in the case of the trace zones of inhibition found, for example, in strips 3 and 4 of Fig. 1. Such trace zones which might indeed be due to antibiotics originally present in the preparations examined, might also have been due to an artefact in the method itself or may have resulted from subtle structural changes induced in the antibiotics originally present, by their long exposure in a solution of alkaline *p*H. This possibility will be dealt with again later in this paper.[11]

(11) It is to be noted that in using the solvent collidine–2% piperidine a small undetermined amount of *n*-butanol vapor was present in the chromatography chamber from a previous run. The effect of this in aiding the separation noted in Fig. 1 was not subsequently determined due to the very slow action of the solvent in question in bringing about the required separation of antibiotics. It was considered more fruitful to obtain solvents which would allow for greater speed of development while preserving the selective action of the collidine–piperidine combination.

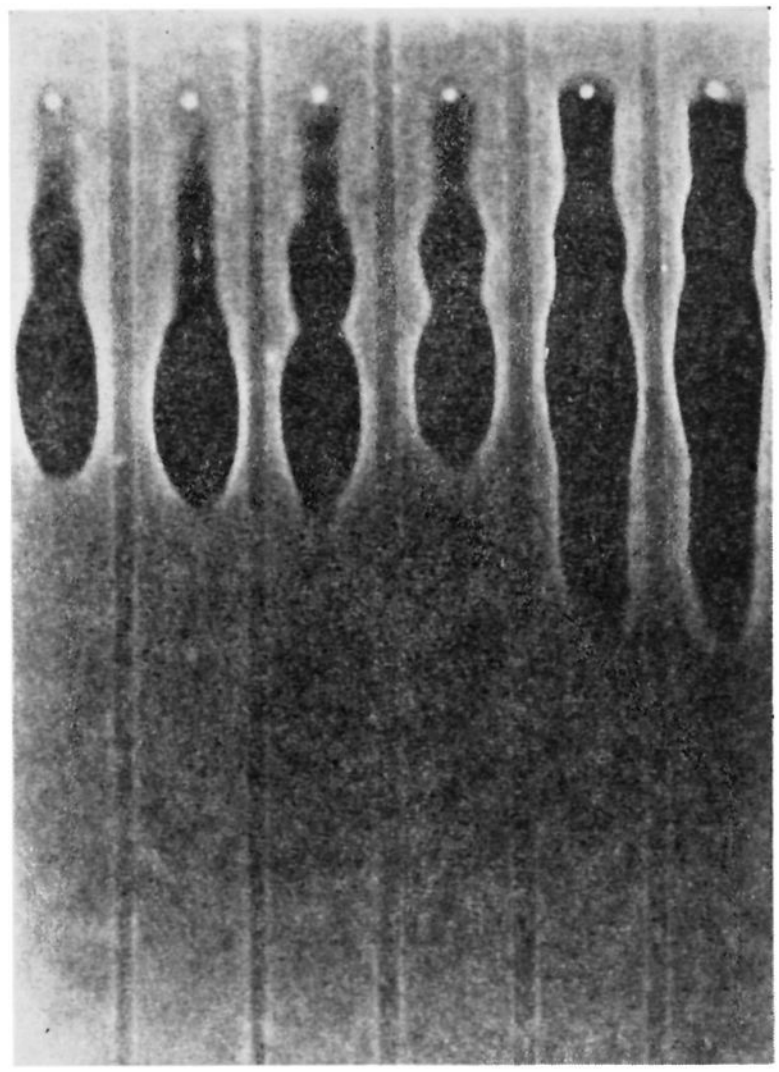

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Fig. 1.—Chromatography using collidine–2% piperidine as the developing liquid at room temperature; period of development three weeks: strip 1, preparation I (0.9% solution); strip 2, preparation II (0.45% solution); strips 3 and 4 (duplicates), preparation III (1.2% solution); strips 5 and 6, duplicates, preparation IV (3% solution). The white spots near the top of each chromatogram are indentations in the agar, indicating the point of application of the 0.007-ml. samples of antibiotic solutions.

Butanol–Piperidine (Containing Dissolved *p*-Toluenesulfonic Acid) as the Mobile Phase.—The most useful solvent so far obtained which combines speed of development with a high degree of selectivity is a combination of wet butanol, piperidine and *p*-toluenesulfonic acid. It will be recalled that the use of a butanol–*p*-toluenesulfonic acid combination failed to separate the various antibiotic entities. However, by using the *p*-toluenesulfonic acid and an excess of piperidine, both dissolved in wet butanol, an effective combination was obtained. The amounts of piperidine and *p*-toluenesulfonic acid dissolved in butanol could be varied in wide limits. In fact even where *p*-toluenesulfonic acid was present in excess of the amount equivalent to the piperidine, a separation of streptomycin from streptomycin B could be achieved, despite the resultant acid *p*H of the aqueous phase on the strip. It was observed that the more alkaline the mixture used, as determined by shaking a volume of sol-

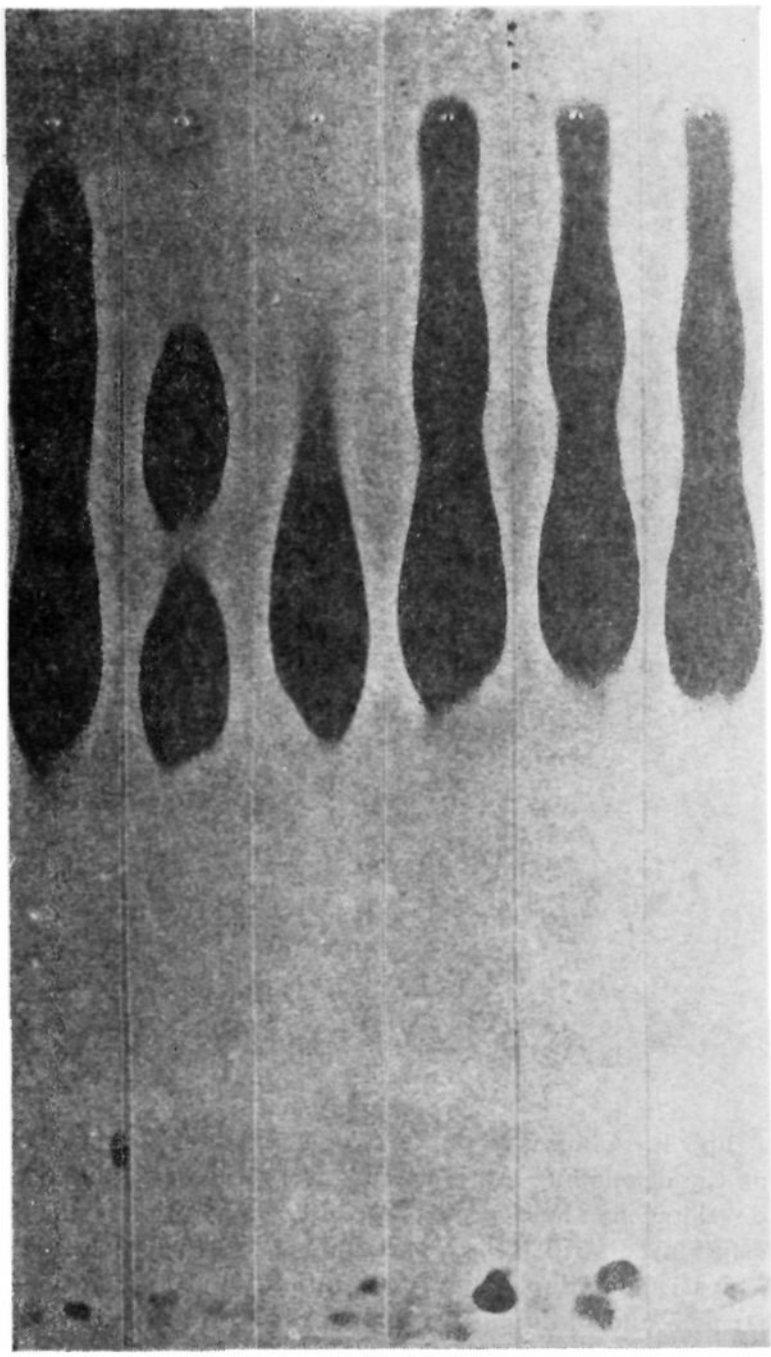

Fig. 2.—Chromatography using butanol–2% piperidine–2% *p*-toluenesulfonic acid monohydrate as the developing liquid at room temperature; period of development twenty hours: strip 1, preparation IV (3.0% solution); strip 2, preparation III (1.2% solution); strip 3, preparation V (0.8% solution); strip 4, preparation VI (3.0% solution); strip 5, preparation VII (3.0% solution); strip 6, preparation VIII (3.0% solution).

vent with an equal volume of water and measuring the resulting *p*H of the aqueous phase, the more specific the solvent appeared to be in separating the various antibiotics of the streptomycin complex.

A developing solution consisting of 98 volumes of wet *n*-butanol, 2 volumes of piperidine and 2 g. of *p*-toluenesulfonic acid monohydrate has given as good results as any other combination tried. Its "equilibrium *p*H" as determined above is 10.7. The acid can also be used equally well at a 3% level with little loss in selectivity. On raising the concentration still higher, selectivity begins to be lost. This can be circumvented by raising the piperidine concentration.

In Figure 2 are shown the results of using the solvent butanol–2% piperidine–2% *p*-toluenesulfonic acid monohydrate.

Strip 3 is the chromatogram of preparation V (the FDA working standard) and locates the position of streptomycin itself.

Strip 2, which represents results obtained for preparation III, shows the zone of inhibition due to streptomycin B above the streptomycin zone.

Strip 1, for preparation IV, indicates the presence of connected zones of inhibition. The lowest zone is due to streptomycin; that of the zone abutting on it is due to streptomycin B. This last, however, exhibits an odd shape at its top suggesting the presence of a slower moving antibiotic which trails streptomycin B closely. (In passing, it is to be noted that using a 1.0% solution of preparation IV a complete separation of the streptomycin and streptomycin B zones was obtained, similar to that for preparation III Strip 2. It is inferred that the antibiotic trailing streptomycin B has been "diluted out." Similar findings on the separation of streptomycin B from authentic streptomycin are obtained for the other antibiotic preparations noted in Fig. 2 if one dilutes them to an appropriate concentration. These experiments on the more dilute samples will not be detailed for the sake of brevity.)

Strips 4, 5 and 6 pertaining to preparations VI, VII and VIII, respectively, are all similar and discussion of strip 4 will cover the others. The bulbous connected zone of inhibition in strip 4 has the following component parts: That due to streptomycin (the most rapidly moving antibiotic), that due to streptomycin B (the next most rapid), and that due to some antibiotic(s) which hardly moves from the original spot of application of the antibiotic solution to the paper. This last zone is evidently due to an antibiotic which moves more slowly than that suspected of trailing streptomycin B in strip 1.

The experiment illustrated in Fig. 2 was repeated and the chromatograms were allowed to develop for forty-four hours in order to separate more completely (if possible) the two new antibiotics suspected of being present in strips 1, 4, 5 and 6. The results of this second experiment are given in Fig. 3.

Strip 1 in Fig. 3 shows the separation of the antibiotic which trails streptomycin B down the chromatogram as suspected from strip 1 of Fig. 2. Strips 2 (preparation III) and 3 (preparation V) require no comment. Strip 4 (preparation VI) shows a light zone of inhibition which has barely moved away from the site of application of the sample. It is connected to the streptomycin B zone by a diffuse tracing of inhibition which may indicate a non-specific artefact. Between the upper main zone due to streptomycin B and that due to streptomycin (on strip 4) there is a connect-

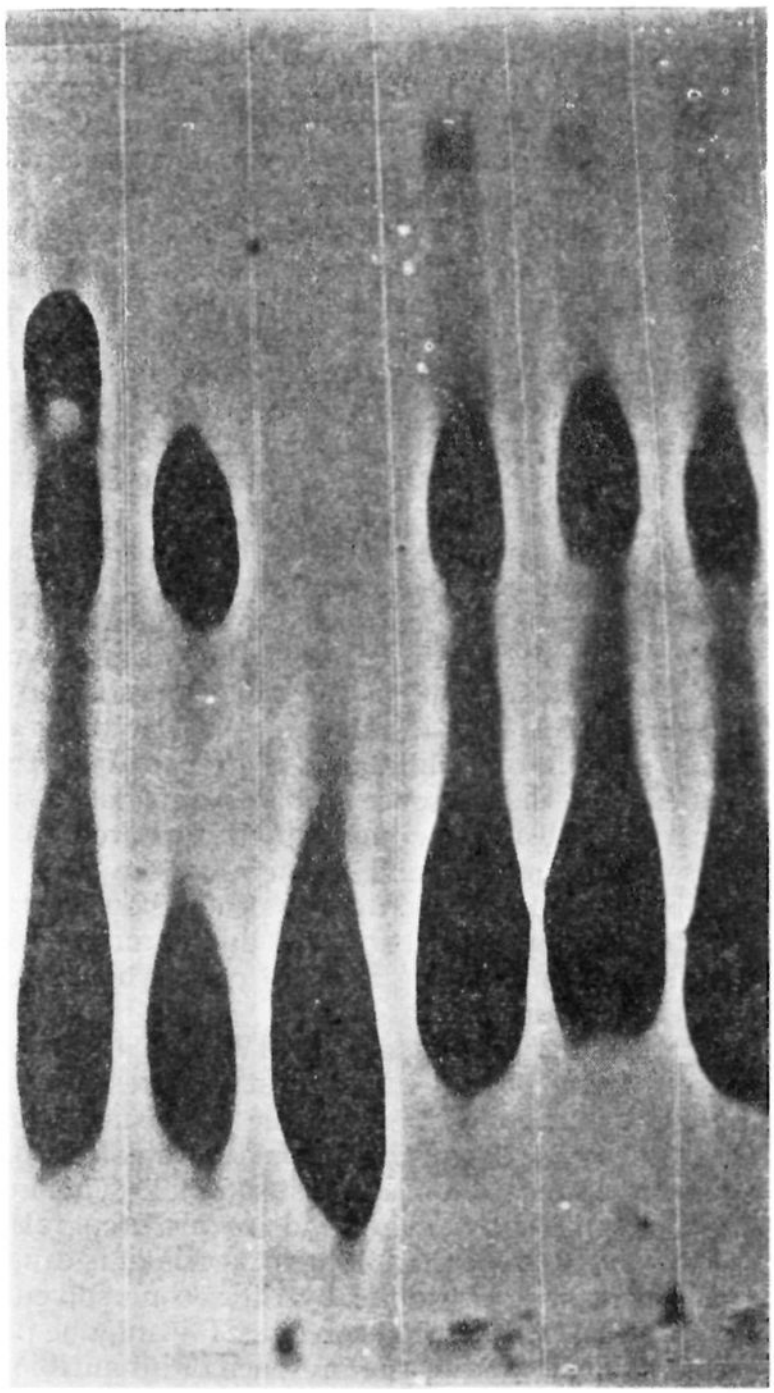

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Fig. 3.—Chromatography using butanol–2% piperidine–2% *p*-toluenesulfonic acid monohydrate as the developing liquid at room temperature; period of development forty-four hours: strip 1, preparation IV (3.0% solution); strip 2, preparation III (1.2% solution); strip 3, preparation V (0.8% solution); strip 4, preparation VI (3.0% solution); strip 5, preparation VII (3.0% solution); strip 6, preparation VIII (3.0% solution).

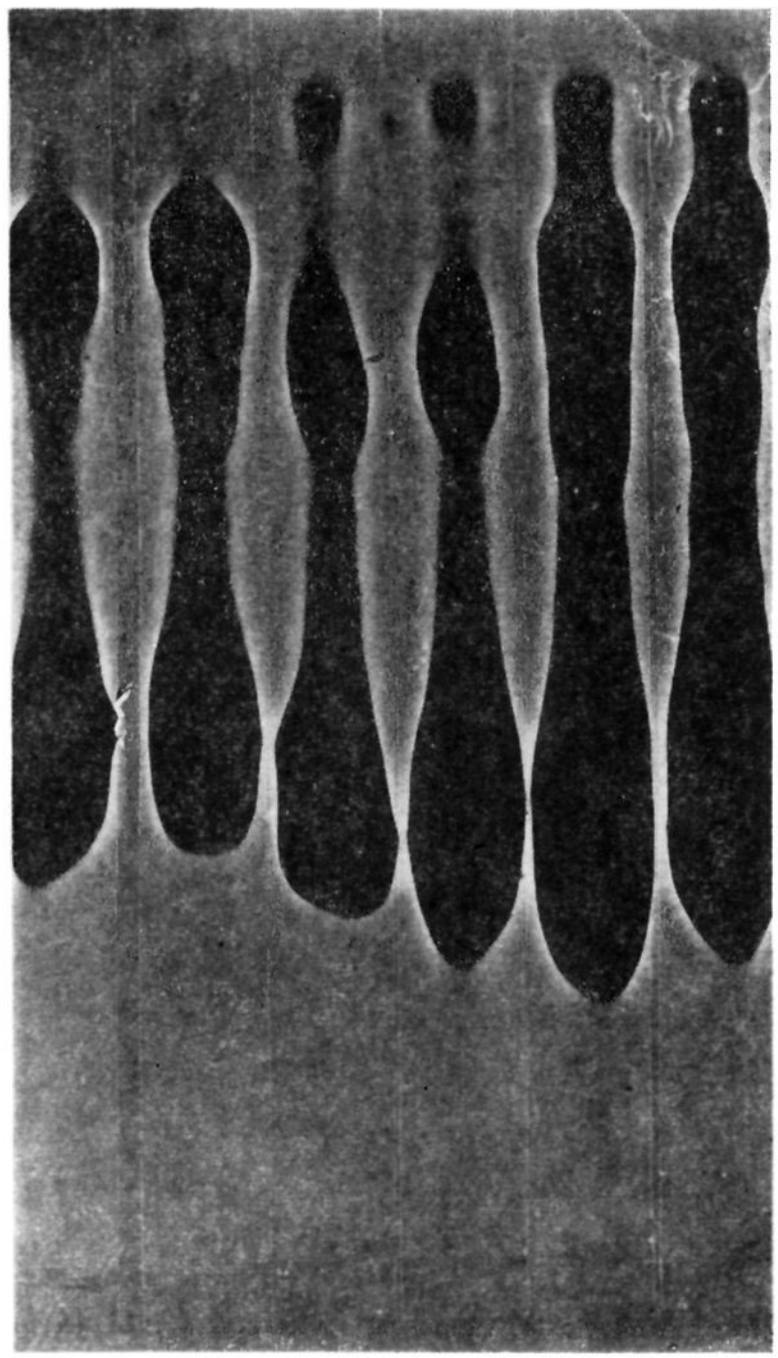

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Fig. 4.—Chromatography using butanol–2% piperidine–2% *p*-toluenesulfonic acid monohydrate as the developing liquid at room temperature; period of development forty-four hours: strips 1 and 2, preparation IV (3.0% solution); strips 3 and 4, preparation VI (3.0% solution); strips 5 and 6, combination of preparations IV and VI (one drop of a 3.0% solution of IV was applied to the strip; this was air-dried and one drop of 3.0% solution of preparation VI was applied directly over that of preparation IV.

ing band of inhibition. The presence of this band of inhibition suggests that still another new antibiotic may be present, although since it has not proved possible to separate such a substance more definitely from streptomycin B (lying above it) and streptomycin (lying below it) its existence may be illusory.

It was of interest to combine preparations IV and VI and chromatograph a mixture of the two. The results of such an experiment are given in Fig. 4.

Strips 1 and 2 for preparation IV indicate that the solvent had not moved the various antibiotics present as far down the column as in the case of strip 1, Fig. 3. (This may have resulted from the fact that a new batch of Whatman No. 4 paper was used.)

Strips 3 and 4 for preparation VI show very clearly the zone of inhibition due to the slowest antibiotic which barely moves from the site of application of the test sample. The zone is more intense than in strip 4 of Fig. 3 for the same preparation.

Strips 5 and 6 representing the combination of preparations IV and VI in such a way as not to di-

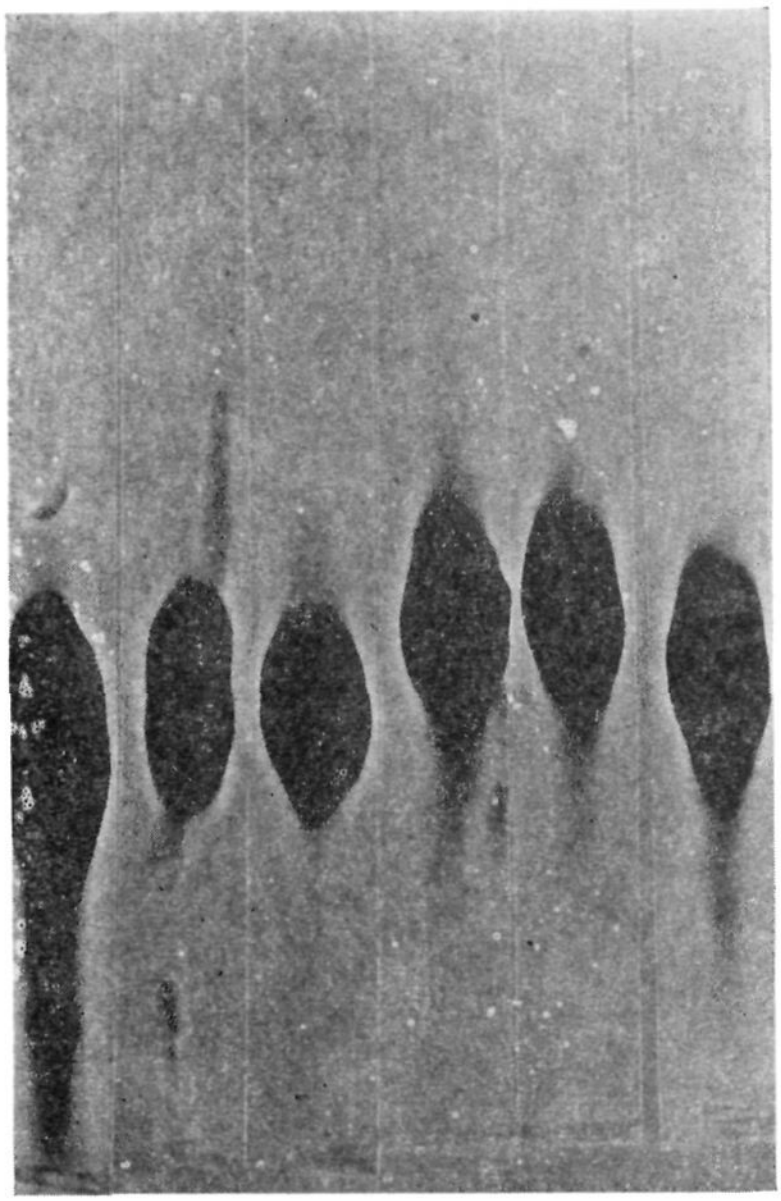

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Fig. 5.—Chromatography using lutidine–2% piperidine as the developing liquid at room temperature; period of development sixty-eight hours: strip 1, preparation IV (3.0% solution); strip 2, preparation III (1.2% solution); strip 3, preparation V (0.8% solution); strip 4, preparation VI (3.0% solution); strip 5, preparation VII (3.0% solution); strip 6, preparation VIII (3.0% solution).

lute them relative to the samples used on strips 1–4, show a connected zone of inhibition from which one can recognize, besides streptomycin and streptomycin B, a third antibiotic at the head of the strip and a fourth which immediately trails and abuts on streptomycin B.

From these data it is suggested that there exist at least two other antibiotics which are members of the so-called streptomycin complex of antibiotics.

It is of interest to compare the results obtained for the solvent butanol–2% piperidine–2% *p*-toluenesulfonic acid (to be referred to as solvent BPP) with those obtained using collidine–2% piperidine (to be referred to as solvent CP). For the sake of brevity only certain of the data will be compared.

Using solvent CP preparation III (Fig. 1, strips 3 and 4) shows in addition to the zones of inhibition caused by streptomycin and streptomycin B two more slowly moving trace zones of inhibition. Solvent BPP indicates but two zones, one for streptomycin B and one for streptomycin (Fig. 3, strip 2). The two trace zones in question are therefore not caused by the two new antibiotics found by use of solvent BPP as being present, the one in preparation IV (Fig. 3, strip 1), the other in preparation VI (Fig. 3, strip 4). If they were the same, then preparation III (Fig. 3, strip 2) should have been found to contain them on using solvent BPP. The two trace zones obtained on both strips 3 and 4, Fig. 1, are therefore either artefacts or represent antibiotics which solvent BPP cannot separate from streptomycin B or even from streptomycin itself.

The same considerations apply to preparation II, the highly purified streptomycin. The "tail" of inhibition on the main zone (see Fig. 1, strip 2) would suggest the presence of some streptomycin B and in addition one of the trace zones found in preparation III with solvent CP. However on using solvent BPP in analyzing preparation II no evidence of the presence of other antibiotics was observed and no streptomycin B appeared to be present in preparation II. Therefore the "tail" of inhibition is due to antibiotics other than the two new ones found with BPP or is due to an artefact. At the present writing it is not clear which is the correct explanation.

The results for preparation IV (Fig. 1, strips 5 and 6) using solvent CP suggest the presence of a slow-moving zone due to some substance which hardly moves down the chromatogram from the point of application of the test sample. This may be the same substance found in this preparation with solvent BPP (Fig. 1, strip 1) which is caused to move somewhat faster by the second solvent.

In other words, using solvent CP it may be possible to demonstrate the presence of all antibiotic demonstrable with solvent BPP and in addition the presence of those antibiotics which solvent BPP cannot demonstrate.

Since, moreover, using solvent CP a supposedly pure streptomycin appears to contain some streptomycin B from Fig. 1, strip 2, but does not contain it in a separate experiment using solvent BPP, the zone of inhibition represented by the "tail" is not due to streptomycin B but may either be an artefact or may be due to an antibiotic which solvent CP cannot separate from streptomycin B.

It has been pointed out earlier that the zone of inhibition formed in front of the streptomycin zone on chromatographing samples of preparation IV (Fig. 1, strips 5 and 6) using solvent CP is possibly due to the antibiotic suggested as a third form of streptomycin by Titus and Fried.[5] Solvent BPP gives no indication of such a substance possibly because this solvent lacks the specificity to separate it from streptomycin. It was decided therefore to try to find a comparatively rapid solvent which could separate this third form of

streptomycin suggested by the above mentioned authors and by our own results.

Lutidine–Piperidine as the Developing Solvent.—Using water-saturated lutidine to which was added 2% by volume of piperidine as the developing solvent, results such as those indicated in Fig. 5 were obtained.

The solvent lutidine–2% piperidine evidently has no ability to separate streptomycin from streptomycin B (see especially Fig. 5, strip 2). It does however indicate the presence of a rapidly moving antibiotic (Fig. 5, strip 1) in preparation IV and possibly also a smaller amount in preparation VIII. This finding of an antibiotic in preparation IV which moves more rapidly than streptomycin but which is absent from preparation III parallels a similar result for these preparations using collidine-piperidine (Fig. 1, strips 5 and 3).

It would appear from the findings presented and discussed above that various preparations of the streptomycin complex may contain, besides streptomycin and mannosidostreptomycin, a third antibiotic which may be that indicated by the work of Titus and Fried.[5,12] In addition, using a suitable solvent it is possible to demonstrate the existence of two other members of the streptomycin complex. Thus there would appear to be at least five antibiotic entities in the streptomycin complex. The existence of two other antibiotics is problematical.

It has been found expedient in examining a given streptomycin preparation to use two different solvents as the mobile phases. One of these consists of wet butanol–2% piperidine–2% *p*-toluenesulfonic acid. The other consists of wet lutidine–2% piperidine. Using both solvents it has proved possible to establish the complexity of various streptomycin preparations.

Studies are being conducted to determine the feasibility of applying paper chromatography directly to the determination of the types of streptomycin occurring in crude *S. griseus* fermentation broths. Preliminary experiments indicate the presence in such broths of substances which interfere with the normal development of a chromatogram.

It should be emphasized that no evidence has been presented above to the effect that the two new antibiotics found in certain complex streptomycin preparations are derivatives of streptomycin in the sense that streptomycin B is a derivative. In describing the two antibiotics as members of the streptomycin complex, a suggestion made by Waksman[4] is followed.

(12) Since submitting this paper for publication, an article by Titus and Fried (*J. Biol. Chem.*, **174**, 57 (1948)) has appeared in which these authors discuss the nature of the more rapidly moving third form of streptomycin, previously reported by them (reference 5 of this paper). These authors offer evidence indicating that the third most rapidly moving form of streptomycin is probably a tautomer of streptomycin itself. In the present paper, the most rapidly moving antibiotic was suggested as being identical with the third form of Titus and Fried. If it is true that the third form of Titus and Fried is a tautomer of streptomycin, then the most rapidly moving antibiotic reported as being present in preparation IV is probably not identical with the tautomer, but may represent instead an additional antibiotic. The reason for believing this rests on the fact that highly purified streptomycin preparations of different histories have never appeared to contain tautomers which could be revealed by use of the solvent systems described in this paper.

This finding was true independent of the equilibrium *p*H on the strip chromatograms, which was varied from *p*H 3.0 to *p*H 11.0. Similar results were found for preparation III which contains only streptomycin and mannosidostreptomycin; only two zones of inhibition were obtained independent of the equilibrium *p*H on the chromatograms.

It may be that the solvents employed by Titus and Fried are particularly suitable for demonstrating the existence of streptomycin tautomers, while the solvents employed in the present study (on paper chromatograms) may favor the formation of one of the tautomers of streptomycin to the exclusiion of the other.

Summary

A paper partition chromatographic method has been developed which is applicable to the identification of the antibiotic entities comprising the streptomycin complex.

Using the techniques described, it has been possible to recognize the existence of at least two other antibiotics as members of the streptomycin complex, in addition to the three already known.

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