(c) In *n*-Butyl Alcohol.—The addition of mercuric acetate to allylurea in *n*-butyl alcohol was accomplished at a temperature below 70°. After the addition of sodium chloride to the reaction mixture, the solution was concentrated at room temperature. 3-Chloromercuri-2-*n*-butoxypropylurea was obtained in 16% yield. It was converted in 88% yield to the thioglycolic acid derivative, which resolidified after melting at 86–87° and subsequently decomposed at 140–145°.

Hydrolysis of N-(3-Chloromercuri-2-methoxypropylcarbamyl)-succinamic Acid (VIII, X = Cl).—A mixture of 3.0 g. of N-(3-chloromercuri-2-methoxypropylcarbamyl)-succinamic acid[13] and 10 ml. of 10% sodium hydroxide solution was warmed at 80° for two hours. The insoluble material was removed by filtration and the filtrate was acidified with 3 ml. of glacial acetic acid. The crude water-washed product weighed 1.7 g. (75%) and melted at 152–153°. Crystallization from ethyl alcohol did not alter the melting point.

Anal. Calcd. for $C_5H_{11}O_2N_2HgCl$: Hg, 54.63; N, 7.63. Found: Hg, 54.87; N, 7.70.

No depression of melting point was noted of a mixture of this solid with the 3-chloromercuri-2-methoxypropylurea obtained *via* the addition of mercuric acetate to allylurea. The products obtained by these two different routes were identical when compared in respect to crystalline appearance, diuretic response and toxicity.

Hydrolysis of N-(3-Bromomercuri-2-methoxypropylcarbamyl)-succinamic Acid (VIII, X = Br).—A mixture of 6.0 g. of N-(3-bromomercuri-2-methoxypropylcarbamyl)-succinamic acid[13] and 20 ml. of 10% sodium hydroxide solution was heated at 80° for one and one-half hours. After another one and one-half hours at room temperature the solid material was removed by filtration and the filtrate was acidified with acetic acid. The precipitate after crystallization from absolute ethyl alcohol melted at 162°; yield 2.9 g. (60%). The melting point was not depressed by mixture with the bromomercuri compound obtained *via* the addition of mercuric acetate to allylurea.

Anal. Calcd. for $C_5H_{11}O_2N_2HgBr$: Hg, 48.72; N, 6.81. Found: Hg, 48.66; N, 6.83.

(13) Prepared by the method of Pearson and Sigal which is similar to the method used above in the preparation of 3-chloromercuri-2-methoxypropylurea from the anhydride of 3-hydroxymercuri-2-methoxypropylurea.

Summary

A series of compounds of the structure $NH_2CONHCH_2CHORCH_2HgX$ has been prepared utilizing the addition of mercuric acetate and alcohols to allylurea. These compounds effect greater diuresis in dogs than do those mercurial diuretics in medical use.

MILWAUKEE 1, WIS. RECEIVED JANUARY 25, 1950

[CONTRIBUTION FROM THE RESEARCH DIVISION OF THE UPJOHN COMPANY]

A Paper Chromatographic Technique and its Application to the Study of New Antibiotics

BY D. H. PETERSON AND L. M. REINEKE

The necessity for early identification of antibiotics produced by a given culture has been well recognized by those working in the field. Past experience has shown that considerable time can be spent in purification studies only to find eventually by spectrum and toxicity tests that one has emerged with a known antibiotic such as, for example, streptothricin. It is increasingly apparent that the majority of cultures produce more than one antibiotic and hence antibacterial spectra and toxicity studies are generally of limited value in early isolation studies. This paper is therefore primarily concerned with a paper chromatographic method, its application to the early identification of antibiotics and factors affecting the procedure.

The classical rediscovery of paper partition chromatography by Consden, Gordon and Martin[1] has already contributed essential information to many biochemical and chemical problems. This unique and powerful tool has been widely adopted but its application to various problems has not yet been fully exploited.

The use of paper chromatography involves generally two basic principles. First it is necessary to have a chemical compound or its derivative of such nature as to distribute appropriately between one solvent as the stationary phase on the paper (water in this case) and another solvent as the mobile phase. Secondly, it is important to have some method of locating the position of the substance chromatographed by means of a chemical, physical or biological test. In addition adsorption on the paper plays a greater or lesser role depending upon the total system.

One of the most desirable features of the paper chromatographic technique is the resolution and identification using as little as 0.1–2.0 microgram of starting material.

Goodall and Levi[2] first applied a paper strip method successfully to the study of various penicillins in a mixture. Winsten and Eigen[3] modified the technique of the British workers[2] and applied it to studies on the streptomycin complex. Winsten and Spark[4] modified the original technique[2] and applied the method to the various penicillins contained in culture filtrates. Kleuner[5] has recently published a method and

(1) (a) In 1861 Schoenbein [*Verhandl. naturforsch Gesel Basel*, **III**, 249 (1861) and **IV**, 1 (1864)] in studying the formation of ozone with electrical discharges observed the different heights to which the components of a mixture rose when a strip of filter paper was dipped into the solution. He termed this use of the paper strip as "capillary analysis." (b) R. Consden, A. H. Gordon and A. J. P. Martin, *Biochem. J.*, **38**, 224 (1944).

(2) R. R. Goodall and A. A. Levi, *Nature*, **158**, 675 (1946).

(3) W. A. Winsten and E. Eigen, THIS JOURNAL, **70**, 3333 (1948).

(4) W. A. Winsten and A. H. Spark, *Science*, **106**, 192 (1947).

(5) R. G. Kleuner, *J. Bact.*, **57**, 101 (1949).

applied the procedure to penicillin culture filtrates as well as to preparations. Based on its movement with 3% aqueous ammonium chloride, Horne and Pollard[6] devised a method for identifying streptomycin; however, this method does not resolve the various types of streptomycin. Catch, *et al.*,[7] as well as Bell, *et al.*,[8] applied a paper strip method to a study of the polymyxin type of antibiotics. The former group used ninhydrin to locate the position of the zones, while the latter workers employed biological activity.

The use of *p*-toluenesulfonic acid in wet butanol was suggested by the work of Titus and Fried[9] who first used this solvent in the separation of mannosidostreptomycin from streptomycin. Winsten and Eigen[3] have dismissed this developing solvent on the basis of its inability to resolve a mixture of the streptomycins, and have recommended, from several which they studied, a developing solvent made up of piperidine and *p*-toluenesulfonic acid in wet butanol.

In the present work, the use of *p*-toluenesulfonic acid in wet *n*-butanol originally used by Titus and Fried[9] was studied further and found to resolve successfully many of the antibiotics derived from various *Streptomyces* cultures. When known antibiotics are used as controls, it has been possible to show the presence or absence of new antibiotics in crude preparations as well as in certain culture filtrates. The success of the method depends upon the control of several important conditions and for this reason a study of the various factors affecting the technique is also included. Descending chromatography was primarily used throughout this investigation since many of the antibiotics resolve rather slowly and their movement and resolution are not restricted by the solvent front as they are in the ascending technique.

Experimental

Although the general methods of Goodall and Levi[3] and Winsten and Eigen[3] were used, so many of the details are different that it appears worth while to describe them. A stainless steel stand, holding a flat glass dish or a Heresite[10] coated steel tray (5/8 in. in depth and 5 1/2 in. to 7 1/2 in. square) on top of the stand, is placed in a battery jar 12" × 24". Such an apparatus allows the use of paper as long as 20 in. from the point of application and allows the chromatography of fast as well as slow-moving antibiotics. The dish is placed in position and to it are added 150–175 ml. of the developing solvent, while 100 ml. of the same solution is added to the bottom of the jar. The developing solvent is prepared by adding 2% *p*-toluenesulfonic acid monohydrate to water saturated *n*-butanol. This solvent is approximately 96% saturated in respect to water at room temperature (25°).

p-Toluenesulfonic acid reduces the activity of streptomycin, but does not stimulate growth of the *B. subtilis* organism on a streptomycin assay plate. This suggests that the diffusion rate of the *p*-toluenesulfonate of streptomycin is slower than that of streptomycin sulfate.

Generally 0.002–0.004 ml. of the unknown sulfate containing 0.1 to 12.0 streptomycin units is applied at equal intervals (starting 1/2 in. from the edge) on Whatman No. 1 or Eaton–Dikeman No. 613 paper strips 5" × 22" at a position 15 in. from the end of the paper. Narrower widths obviously can be used if desired. A fold is made at 16.5 in. from the end and the remaining 5.5 in. of paper were carefully placed in the solvent so that a straight front was initiated. The paper is held in place of its own accord by adhesion to the bottom of the reservoir.

In the case of low potency culture filtrates, aliquots of the sample are applied several times with intermittent drying. As an alternative the culture filtrate can be dried from the frozen state and reconstituted in a smaller volume of water.

A piece of plate glass is placed over the jar to keep the system closed. It is important that preferably two such strips and no more than four be developed before the solvent system is renewed since water is lost primarily to the paper, the equilibrium is disturbed and poor resolution and slower mobility result. A period of approximately seventeen to twenty-four hours is required for the front to reach the end of the paper. After proper development, the strip is air-dried at room temperature and laid directly on agar plates (generally 12" × 18" Pyrex drying dishes were used although larger plates were used when required) seeded with *B. subtilis* according to the streptomycin assay technique of Loo, *et al.*[11] At the end of eight minutes the paper strip is removed and the plate allowed to incubate for sixteen to twenty-four hours at 37°. One of the most important parts of the procedure, as we have used it, is the proper preparation of the *B. subtilis* plates. Unless the plates are correctly made, considerable difficulty can be encountered in registering good zones and this can erroneously be blamed on the chromatographic technique. Our most successful plates have been prepared according to the following specifications. To sterile 12" × 18" Pyrex dishes, having loosely fitting copper lids, a 200-ml. layer of uninoculated stryptomycin agar (Difco) is poured. After solidification, 100 ml. of streptomycin agar inoculated with 1.0 ml. of a *B. subtilis* (Illinois) spore suspension containing 5 × 10^{10} spores per ml. is superimposed as a seed layer. The plates are stored at 4° until used. Condensed moisture on the inside of the copper lids is absorbed by means of a clean towel directly after removal from the refrigerator. This operation prevents condensate from dripping and disturbing the seed layer. Plates are incubated at 37° for fifteen hours to obtain a satisfactory growth. *E. coli* plates prepared according to the method of Murray, Tetrault, *et al.*,[12] were more appropriate for studying the mobilities of the rapidly moving antibiotics circulin,[12,13] aureomycin[14] and chloroamphenicol.[14] A development period of only sixteen hours differentiates these antibiotics. The streptomyces antibiotics streptomycin,[13] dihydrostreptomycin,[13] mannosidostreptomycin,[13] neomycin A[13] and streptolin-type antibiotics such as actinorubin[13] and antibiotic 136[13,15] required a development period ranging from twenty-four to one hundred and forty-four hours. The most reliable results could be ob-

(6) R. E. Horne and A. L. Pollard, *J. Bact.*, **55**, 231 (1948).

(7) J. R. Catch, T. S. G. Jones and S. Wilkinson, *Ann. N. Y. Acad. Sci.*, **51**, 917 (1949).

(8) P. H. Bell, J. F. Bone, J. P. English, C. E. Fellows, K. S. Howard, M. M. Rogers and R. Winterbottom, *ibid.*, **51**, 897 (1949).

(9) E. Titus and J. Fried, *J. Biol. Chem.*, **168**, 393 (1947).

(10) Heresite is a phenolic resin. Coating done by Heresite and Chemical Co., Manitowoc, Wisconsin.

(11) Y. H. Loo, P. S. Skell, H. H. Thornberry, J. Ehrlich, J. M. McGuire, G. M. Savage and J. C. Sylvester, *J. Bact.*, **50**, 701 (1945).

(12) F. J. Murray, P. A. Tetrault, O. W. Kaufmann, H. Koffler, D. H. Peterson and D. R. Colingsworth, *ibid.*, **57**, 305 (1949).

(13) Highly purified samples of these antibiotics as sulfates were prepared in this Laboratory. The actinorubin culture was kindly supplied to us by Harry E. Morton, The School of Medicine, University of Pennsylvania.

(14) Pure aureomycin obtained from H. Pierzma, Lederle Laboratories; chloroamphenicol from L. A. Sweet, Parke Davis and Company.

(15) N. Bohonos, R. L. Emerson, A. J. Whiffen, M. P. Nash and C. DeBoer, *Arch. Biochem.*, **15**, 215 (1947).

tained by interpreting several chromatograms of a given material.

The zones of inhibition were recorded by photographing the plates as described by Drake[16] of our laboratories after placing on the plate a 1-in. square of paper. This permitted measurement of the distances moved and a rough estimate of the amount of antibiotic present. Highly purified sulfates of known antibiotics were used as controls, streptomycin serving well for the faster moving antibiotics and streptothricin for the slower ones.

The effect of sodium chloride, sodium sulfate, ammonium sulfate, sodium acetate, ammonium citrate, sodium tartrate, potassium hydrogen phosphate, and potassium dihydrogen phosphate upon highly purified samples of streptomycin, dihydrostreptomycin, mannosidostreptomycin, streptothricin and neomycin sulfates was studied. The antibiotics were dissolved in 5% salt solution and the ratio of micrograms of salt to streptomycin units applied varied from 50 to 1 to 250 to 1.

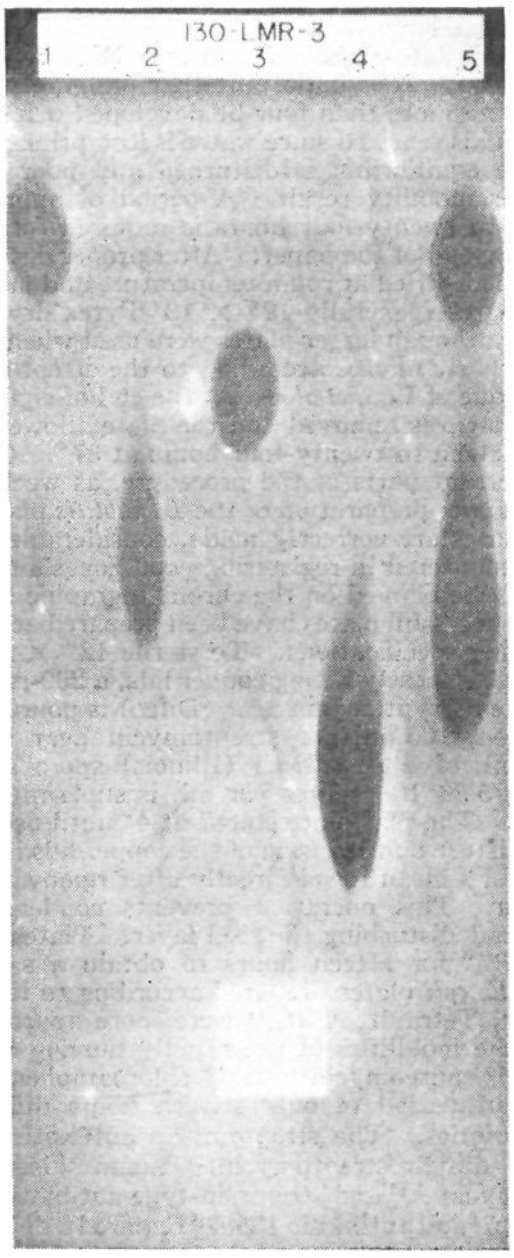

Fig. 1.—Paper chromatography of preparations using 2% *p*-toluenesulfonic acid monohydrate in water saturated *n*-butanol as the developing solvent at room temperature; development period 24 hours: zone 1, 0.4 unit mannosidostreptomycin; zone 2, 0.2 unit of streptomycin A; zone 3, 0.4 unit of dihydrostreptomycin A; zone 4, 0.4 unit of neomycin A, and zone 5, 0.4 unit each of streptomycin A and mannosidostreptomycin. All antibiotics are sulfates. A one inch square of paper is shown on each chromatogram.

(16) N. Drake, THIS JOURNAL, **72**, 3803 (1950).

Results and Discussion

Application of the Method.—Figure 1 shows a representative chromatogram indicating the characterization of streptomycin, mannosidostreptomycin, neomycin and dihydrostreptomycin. The method is applicable to crude preparations and directly to culture filtrates provided the salt concentration does not exceed approximately 50 micrograms per streptomycin unit applied. Differentiation of streptothricin and mannosidostreptomycin is more difficult. However, if the development period is extended to forty-eight to ninety-six hours streptothricin moves slightly but significantly ahead of mannosidostreptomycin.

It is of interest to note that studies on the streptomycin complex produced evidence of a third antibiotic of lower mobility than either of the well-known streptomycins. This unknown antibiotic was found in culture filtrates as well as in crude preparations of mannosidostreptomycin and streptomycin. Whether this third antibiotic is one of the two antibiotics described by Winsten and Eigen[3] is not known.

The Effect of Various Salts on Streptomycin, Mannosidostreptomycin, Dihydrostreptomycin, Streptothricin and Neomycin.—The object of this study was to establish conditions applicable directly to low potency beers (high in salt) and thus avoid spending valuable time making preparations low in salt. When 0.8 unit each of streptomycin hydrochloride and sulfate were placed together on the same spot and developed for thirty-four hours, there was only one zone present and the mobility was identical with that of streptomycin hydrochloride and streptomycin sulfate alone as controls. This experiment indicated that the *p*-toluenesulfonic acid, because of its excess concentration over sulfates and chloride ions, produced only one zone; *viz.*, that of the *p*-toluenesulfonate of streptomycin.

Shown in Table I are the effects of salts upon the resolution of highly purified sulfates of streptomycin, dihydrostreptomycin, mannosidostreptomycin, streptothricin and neomycin A. The mobilities for each antibiotic were related to the control and varied from run to run.

Streptomycin formed two zones with four out of the eight salts. Dihydrostreptomycin formed only single spots with all salts except sodium sulfate. Attempts to explain this latter observation on the basis of traces of streptomycin were unsuccessful since none was found in as much as 3.2 units of dihydrostreptomycin. With ammonium sulfate dihydrostreptomycin forms only one zone.

In the presence of sodium sulfate or ammonium sulfate, streptomycin exhibits a slower moving zone similar to that of salt-free mannosidostreptomycin. Mannosidostreptomycin gives two zones

TABLE I

THE EFFECT OF SALTS ON MOBILITY[a]

In each case the upper figure represents the number of zones formed and the lower figures the movement in centimeters.

Antibiotic (sulfates)	Control No salt	Ammonium citrate	Ammonium sulfate	Potassium hydrogen phosphate	K dihydrogen phosphate	Sodium acetate	Sodium chloride	Sodium sulfate	Sodium tartrate	Development period, hours
Streptomycin	1	2	2	1	1	1	2	2	1	24
0.8μ[c]	15–20	20–25	2.5–5	20–25	17–20	20–24	5–7.5	2.5–5	19–24	
		28–30	17–20				15–20	15–20		
Dihydrostrepto-	1	1	1	1	1	1	1	1	1	48
mycin	12.5–15	11–14	5–9	12–15	20–23	14–17.5	5–6	4–8	22–25	
0.8μ								19–23		
Mannosidostrep-	1	1	2	1	1	2	1	1	2	48
tomycin	5–6	9–11	2.5–5	6–9	6–9	1–1.5	7.5–10	5–7.5	4.5–5.5	
0.8μ			5–7.5			5–6			20–22	
Streptothricin	1	1	1	2	2	2	1	2	2	48
0.8μ	6–7.5	12–15	5–7.5	10–12.5	11–14	11–14	5–7.5	4–6	11–14	
				20–23	20–24	21–24		20–24	24–26	
Neomycin A	1	2	1[b]	1	1	1	1	1[b]	1	24
.005μ	20–24	17–20	1.5–2.5	20–22	20–22	22–25	6–7.5	1.5–2.5	0.5–1.0	

[a] A 5% salt solution of the antibiotic was used for each test. [b] Produces a characteristic horseshoe pattern. [c] Expressed as units of streptomycin present.

in the presence of ammonium sulfate, sodium sulfate, sodium acetate and sodium tartrate but not with the other salts. Streptothricin formed two zones with five out of the eight salts. Neomycin formed two zones with ammonium citrate but only one with the other salts studied. Titus and Fried[17] have given evidence for the existence of a tautomer of streptomycin by countercurrent distribution studies of fractions taken from a carbon column. Therefore, a possible reason for this phenomenon in streptomycin is the presence of a tautomer from the free aldehyde group which forms one zone and the original aldehyde which forms the second zone.

The *p*H of the sample has no effect because of the large amount of *p*-toluenesulfonic acid present. Ammonium sulfate decreased the activity of streptomycin 75% using the regular streptomycin assay of Loo, *et al.*,[11] while mannosidostreptomycin was not significantly affected. This fact may be associated with nitrogen utilization from the ammonium sulfate by the *B. subtilis* organism.

Optimum results were obtained with salt free preparations. However, by using a developing solution consisting of 2% *p*-toluenesulfonic acid in *n*-butanol previously saturated with 1–2.5% aqueous sodium chloride, it was found that most of the salt effects noted above could be compensated. While 1% sodium chloride worked well on many lower potency beers, 2.5% sodium chloride was found best for very low potency beers of 5–50 units per ml. Some irregularity was encountered with sodium sulfate and sodium tartrate but these salts are seldom present in culture filtrates in sufficient quantities to produce adverse effects.

In order to determine whether other substances in the fermentation liquor, besides salts, affected the mobility of streptomycin and mannosidostreptomycin, 200 units per ml. of mannosidostreptomycin were added to a beer containing 600 units of streptomycin per ml. The various salts were then added to this beer to make 5% solutions. Paper chromatograms of these samples showed results similar to the previous findings where no fermentation liquor was present and indicated that the main substances affecting paper chromatography of antibiotics were salts.

Application of this technique (using solvent to which sodium chloride was added) to several low potency beers showed that this modified developing solvent balanced the salt effect in the beer (0.5% sodium chloride added to the media) when the concentration of salts was no greater than 500 micrograms per streptomycin unit. In this case the antibiotics tested were similar to antibiotic 136.[15] See Figs. 2 and 3 for results showing the effect of salts, and the application of both developing solvents employed in this work. Other factors studied relating to the technique are listed below:

Effect of Temperature.—When papergram studies were done on streptomycin and mannosidostreptomycin at 25° and 37° very little difference was observed in the mobility during a twenty-four hour development period.

Degree of Saturation of the Chamber.—When the chamber was completely saturated by means of a solvent saturated cloth, resolution and movement of the antibiotics were very slow. For more rapid movement and best resolution the ratio of the amount of surface area to the volume of the chamber was important, and it was necessary to follow the instructions as previously given. Best results were obtained by replacing the solvent in the tray with new solvent after two 5″ × 22″ strips were chromatographed.

(17) Titus and Fried, *J. Biol. Chem.*, **174**, 57 (1948).

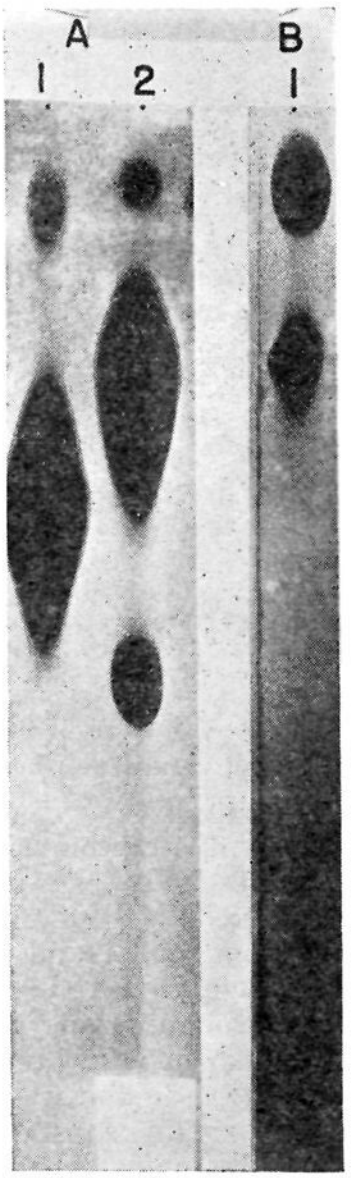

Fig. 2. A.—Streptomycin beer to which mannosidostreptomycin has been added: spot 1, 4.2 units of naturally occurring streptomycin (fast-moving zone) and 0.42 unit of mannosidostreptomycin; spot 2, same as spot 1 but also contains 270 mcg. of sodium chloride, note the typical salt effect on streptomycin, developing solvent is 2% *p*-toluenesulfonic acid monohydrate in water saturated *n*-butanol, developing period, 40 hours. B.—Same beer as A, spot 2 but containing 1.2 units of naturally occurring streptomycin, 1.2 units of added mannosidostreptomycin and 270 mcg. of sodium chloride; note neutralization of salt effect; developing solvent is 2% *p*-toluenesulfonic acid monohydrate in *n*-butanol previously saturated with 2.5% aqueous sodium chloride; developing period, 43 hours.

Fig. 3. A.—Spots 1 and 2 represent low potency unknown beers (20 and 50 streptomycin units per ml., respectively) containing slow-moving antibiotics of which one is streptothricin: spot 1, 1.0 unit; spot 2, 0.8 unit; spot 3, 0.5 unit of streptothricin sulfate; developing solvent is 2% *p*-toluenesulfonic acid in *n*-butanol previously saturated with aqueous 2.5% sodium chloride; developing period was 96 hours. B.—Spot 1, 1.2 units of mannosidostreptomycin; spot 2, 1.0 unit of antibiotic 136 from which streptothricin has been isolated (*Arch. Biochem.*, **15**, 215, 1947); spot 3, 0.8 unit of known streptothricin sulfate; developing solvent is 2% *p*-toluenesulfonic acid in water saturated *n*-butanol; developing period was 168 hours.

It has been found convenient and satisfactory to empty the used solvent from the tray into the bottom of the jar after each chromatogram until five pairs of strips have been chromatographed.

Concentration of the Antibiotics Applied.—If the concentration of an antibiotic mixture to be applied is high, the zones will be larger and naturally resolution will be slower. However, as Winsten and Eigen[3] pointed out, it is necessary to use high concentrations when a study of trace antibiotics is desired.

Acknowledgment.—We are indebted to Dr. G. F. Cartland for suggestions and criticism in this work.

Summary

This study has indicated the successful use of a paper chromatographic technique using 2% *p*-toluenesulfonic acid monohydrate in water saturated *n*-butyl alcohol for the resolution and identification of various antibiotics in salt-free preparations and in culture filtrates where the salt concentration is not in excess of 50 micrograms per streptomycin unit. A modified solvent containing sodium chloride is applicable

to low potency culture filtrates.

Employing 2% *p*-toluenesulfonic acid monohydrate in water saturated *n*-butanol the effect of various salts on the mobilities of streptomycin, dihydrosteptomycin, mannosidostreptomycin, streptothricin and neomycin A sulfates has been studied. Two distinct zones were formed by each of the antibiotics studied in the presence of one or more salts.

KALAMAZOO, MICHIGAN RECEIVED JANUARY 7, 1950

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, THE UNIVERSITY OF TEXAS]

Ortho Esters, Imidic Esters and Amidines. The Identity of N-Phenyl-N'-*p*-tolylformamidine

BY ROYSTON M. ROBERTS

Walther[1] first reported the preparation of N-phenyl-N'-*p*-tolylformamidine and claimed to have obtained four "isomeric" compounds (m. p.'s. 98°, 102°, 120°, 132°) by various methods. This work was criticized by Wheeler,[2] who claimed that all of Walther's "isomers" were actually mixtures and that the pure compound melted at 103.5–104.5°. However, a short time later, Wheeler and Johnson[3] described two new routes for the preparation of this compound (equations A and B).

$$C_6H_5N{=}CHOEt + H_2NC_7H_7 \rightleftharpoons C_6H_5N{=}CH{-}NHC_7H_7 + EtOH \quad (A)$$

$$C_7H_7N{=}CHOEt + H_2NC_6H_5 \rightleftharpoons C_7H_7N{=}CH{-}NHC_6H_5 + EtOH \quad (B)$$

The products of the two reactions were said to be identical, m. p. 86°. Although it conflicted with Wheeler's own previous results, this was the work which was accepted and recorded by "Beilstein."

When we set out to prepare this unsymmetrical diarylformamidine, we used the method of Wheeler and Johnson (equation A). To our surprise, we obtained a product which melted sharply at 105–106°. Further investigation of the literature led us to Walther's work and to the earlier paper of Wheeler. Since it was necessary to have a very pure material for our purposes, we studied the conditions of the reaction and the isolation of the product quite carefully. We found that the reaction was greatly affected by a small amount of acid; when a small crystal of aniline hydrochloride was added to a mixture of ethyl N-phenylformimidate and *p*-toluidine at room temperature, a vigorous evolution of heat occurred and the mixture set to a mass of crystals within a few minutes. Products of all such acid-catalyzed reactions were found to melt lower than those from mixtures which had been carefully protected from acid. When very small amounts of acid were used, products were obtained which melted sharply at around 85°, and the melting point was not raised by further recrystallization from petroleum ether or ethanol (the solvents employed by Wheeler and Johnson). We have now shown that the product of the reaction in the absence of acid, which melts at 105–106°, is N-phenyl-N'-*p*-tolylformamidine, and that the product formed in the presence of very small amounts of acid, which melts at about 85°, is a mixture of the unsymmetrical formamidine and the two corresponding symmetrical formamidines, N,N'-diphenylformamidine and N,N'-di-*p*-tolylformamidine. Thus, it seems likely that Wheeler actually had the pure compound in his first work, but in the later work used a reaction which is extremely sensitive to acid-catalyzed disproportionation.

We obtained the same pure "high-melting" product from ethyl N-*p*-tolylformimidate and aniline (equation B) when care was taken to avoid traces of acid. Again, when very small amounts of *p*-toluidine hydrochloride were added to the reaction mixtures, the "low-melting" product was obtained. This "low-melting" product is a very unusual mixture. It melts over a narrow range at around 85° (various preparations after several recrystallizations melted at 83–84°, 84–86°, 85–86°, etc.) and we were not able to raise the melting points of such products by repeated recrystallization from several different solvents. It thus appears to be a eutectic mixture of minimum solubility or, perhaps, a solid solution of the three compounds.

The evidence that the "low-melting" product is a mixture of N-phenyl-N'-*p*-tolylformamidine, N,N'-diphenylformamidine, and N,N'-di-*p*-tolylformamidine, and that the "high-melting" product is pure N-phenyl-N'-*p*-tolylformamidine is: (1) Reactions according to both equations A and B *in the absence of acid* led to the same product, m. p. 105–106°. (2) Reactions according to both equations A and B *in the presence of small amounts of acid* led to products with lower melting points. From both pairs of reactants products were obtained with rather sharp melting points around 85°. (3) Treatment of the "high-melting" product with small amounts of acid in alcohol solution produced the "low-melting" product. (4) The "high-melting" product gave a picrate which melted over a rather narrow range, 174–176°.

(1) Walther, *J. prakt. Chem.*, [2] **55**, 41 (1897); Zwingenberger and Walther, *ibid.*, [2] **57**, 209 (1898).

(2) Wheeler, *Am. Chem. J.*, **19**, 367 (1897).

(3) Wheeler and Johnson, *Ber.*, **32**, 35 (1899).