acetone 2,4-dinitrophenylhydrazone gave no depression.

Oppenauer Oxidation of Obliquol.—A solution of 0.4 Gm. of obliquol in 5 ml. of cyclohexanone and 10 ml. of toluene was treated with 0.8 Gm. of aluminum isopropoxide and refluxed for 2 hours. This solution was cooled and 5 ml. of 10% sulfuric acid added. The solution was then extracted with ether and the residue recrystallized repeatedly from methanol, m.p. 160–165°. Further attempts to purify this compound were unsuccessful. The above procedure was repeated using aluminum tertiary butoxide. It was refluxed for 9 hours. A substance was obtained that was identical to the one obtained by the previous method. It also resisted further purification. Both compounds gave identical infrared spectra. In both cases the compounds gave positive Zimmermann tests but no absorption in the ultraviolet. The infrared spectra showed bands at 3450 cm.$^{-1}$ (O—H), 1715 cm.$^{-1}$ (keto carbonyl), and 1040 cm.$^{-1}$ (C—O).

Chromic Acid Titration of Obliquol.—Obliquol (221 mg., 0.0005 mole) was titrated with a chromium trioxide solution in sulfuric acid that was prepared according to the procedure described by Curtis (7) and Bowers (8). The volume of solution consumed was equivalent to the conversion of 1.95 hydroxyl groups to carbonyl groups.

Chromic Acid–Acetic Acid Oxidation of Obliquol.—A solution of 100 mg. of obliquol in 10 ml. of benzene was treated with 130 mg. of chromium trioxide in 1.5 ml. of water and 3 ml. of glacial acetic acid and allowed to stand for 18 hours. The benzene solution was separated, concentrated, and placed on an alumina column. A small fraction that was eluted with benzene-methanol remained as a viscous oil. The infrared spectrum showed no hydroxyl group absorption but showed a strong carbonyl peak at 1715 cm.$^{-1}$. The substance gave a positive Zimmermann test.

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New Method for Location of Organic Acids on Paper Chromatograms

By GILBERT C. SCHMIDT, CARL FISCHER†, and JOHN M. McOWEN

A modification of Riegler's nitrite test, employing betanaphthol, sodium nitrite, and naphthylamine or sulfanilamide, has been adapted for location of organic acids on paper chromatograms. Most of the twelve acids tested are detectable at a level of 5 mcg. or less although 50–75 mcg. are required in isolated cases. The reagent may be used with all four of the common solvent systems employed, with no notable differences in sensitivity. The sensitivities of this reagent and four previously described detection reagents have been compared. The new reagent is more sensitive than the best of the previously described procedures.

The introduction of paper chromatography as a method for identifying organic acids (1) has been followed by the use of numerous detection reagents. The acid-base indicators have been most frequently used, but colors are transient, complete solvent removal is essential, and it is difficult to distinguish true spots from artefacts. Better results have been reported if the indicator is added to the solvent system (2, 3), but this modification has not been generally accepted. Potassium permanganate, usually used to locate unsaturated and hydroxy compounds (4), has been combined with indicators to produce a general location reagent. The reported sensitivity is poor, but final colors and development times vary for different acids. The combined reagent has therefore been recommended to distinguish acids having similar R_f values (5).

The acid catalyzed condensation of aniline and reducing sugars to form colored compounds has been used to locate both sugars (6) and acids (7). Sugars are detected with an aniline-phosphoric acid mixture, while acids are detected with an aniline-xylose mixture. The aniline-xylose reagent and acridine appear to be the most useful of the remaining detection reagents (7), but both are carcinogenic and little has been written about them.

Nessler's reagent and starch-iodine-iodate reagent have been used to locate the ammonium salts of acids, but neither is a satisfactory general location reagent (7, 8). Aqueous ferric chloride

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may be used to locate hydroxy acids (4), but it is highly insensitive and is not generally applicable. A variety of diazonium salts are useful in selected instances, particularly for the detection of phenolic acids (8–10), but many metabolically important acids do not give colors. The same difficulty arises with ammonium vanadate, dinitrophenylhydrazine, *p*-dimethylaminobenzaldehyde, phosphomolybdic acid, potassium ferrocyanide-ferric ammonium sulfate, and dichlorophenolindophenol.

Chloranilic acid, previously used to detect inorganic ions and certain nitrogenous compounds, has been introduced as a highly sensitive reagent for locating sodium salts of organic acids (11). Volatile acids are stabilized and tailing is reduced, but buffered papers or chelating agents cannot be used. A neutral or basic solvent is required and the paper must be thoroughly dried to prevent interference by "ghost" spots.

Riegler's nitrite test (12) has been modified by the authors and introduced as a location reagent for acids (13). In Riegler's test, nitrites give a color with alphanaphthol and sodium naphthionate in acid solution. Acids give a positive test in the presence of alphanaphthol, sodium naphthionate, and sodium nitrite. Both tests result in a deep red color and apparently depend on the formation of an azo dye. We reasoned that paper chromatograms sprayed with a mixture of alphanaphthylamine and betanaphthol, followed by a sodium nitrite spray or dip, should form nitrous acid only in those spots occupied by migrated free acids. Consequently, only the acid spots should undergo diazotization, coupling, and color development.

The original method is highly sensitive and has been successfully used for more than a year, but replacement of the highly carcinogenic alphanaphthylamine is desirable. This report describes our original procedure and a recent modification in which sulfanilamide replaces the carcinogen. Sensitivities of these two procedures and some other available methods are compared.

EXPERIMENTAL

Standard Solutions.—Choice of reference acids was based on our own interests and on general biochemical importance. Commercial acids of the best available grade were used without further purification. Stock aqueous 1% solutions were prepared as needed. The quantity of acid applied to each spot was controlled by dilution of the stock solutions and by varying the sizes of micropipets throughout the range 1–25 μl.

Chromatogram Development.—Acids were chromatographed on Whatman No. 1 paper. Areas of applied spots were controlled by rate of sample delivery and by drying with unheated air from a hair dryer during application of the samples. Heat was avoided because some of the acids are unstable. Air dried chromatograms were then developed for the prescribed period by the ascending method.

Solvent Systems.—Several common solvent systems were chosen to determine if solvent composition would severely limit use of the alpha naphthylamine or sulfanilamide location reagents. Butanol-acetic acid-water (7), propanol-eucalyptol-formic acid-water (14), ethanol-ammonia-water (15), and an 8:1:1 propanol formic acid-water system were used for this purpose. The propanol-formic acid-water system was used for comparing sensitivities of the proposed reagents and several previously described reagents. Solvents were freshly prepared each day and were equilibrated for 1 hour before transferring to the chromatographic tanks. An additional 1 hour equilibration preceded introduction of the chromatograms. Chromatograms were routinely dried at room temperature for at least 4 hours, but in most instances location reagents could be applied after drying for 1 hour.

Alphanaphthylamine Reagent.—Chromatograms were sprayed with a solution of alphanaphthylamine (1%) and betanaphthol (0.5%) in 95% ethanol. They were then air dried at room temperature until the ethanol had evaporated, but for no longer than 1 hour. Dried chromatograms were then sprayed with 1% aqueous sodium nitrite. Acids immediately appear as dark orange spots on a pale pink background. Intensity of the background color increases on standing but is less pronounced if the naphthylamine-naphthol solution is allowed to age for a day or two before use. This solution is stable for at least two weeks at room temperature, but the sodium nitrite solution is preferably prepared just before use since it is stable for only 12 hours at room temperature.

Sulfanilamide Reagent.—Chromatograms were treated as described for alphanaphthylamine reagent, except that the initial spray contained 2% sulfanilamide and 0.5% betanaphthol in 95% ethanol. Following the sodium nitrite spray, acids immediately appear as orange spots on an almost colorless background. Background color does not appreciably increase when chromatograms are stored for several weeks. The use of aged reagent has no advantage.

Other Location Reagents.—Four previously described location reagents were chosen for sensitivity comparisons. Aniline-xylose (7), acridine (7), and combined indicator-permanganate reagent (5) were prepared and used as previously described. Bromcresol green reagent (16) was purchased in spray cans.[1]

RESULTS AND DISCUSSION

Preliminary Experiments.—Filter paper circles were spotted with 10 μl. quantities of tropic acid solutions, representing different amounts of acid. These test plates were then developed under a variety of experimental conditions to establish the feasibility, potential sensitivity, and certain requirements for color development.

[1] "Spraytec" reagents, available from Aloe Scientific Co., St. Louis, Mo.

In support of the assumption that diazotization and coupling are responsible for color development, a variety of diazotizable amines and a variety of coupling phenols or amines produced colors. Of the combinations tested, the naphthylamine-naphthol and sulfanilamide-naphthol reagents gave the best results. Only these combinations were further studied.

These experiments also disclosed that the diazotizable amine and the coupling agent must be applied as a single solution, that the sodium nitrite must be freshly prepared to prevent intense coloration of the entire chromatogram, that the use of ice cold reagents decreases sensitivity, and that application of reagents by spraying is preferable to dipping.

Sensitivity of the Proposed Method.—Preliminary estimates of sensitivity were obtained from the previously described spot plates, but these tests were confined to tropic and mandelic acids and did not involve migration. Accurate sensitivity comparisons were therefore conducted under more probable experimental conditions.

Twelve commonly encountered aliphatic acids, and the propanol-formic acid-water system were arbitrarily chosen. Graded amounts of each acid were applied to a series of chromatograms. The chromatograms were then developed and were sprayed with naphthylamine or sulfanilamide reagent as previously described. Sensitivities shown in Table I represent the lowest levels at which color could be visually detected when chromatograms were held against a window and viewed by daylight. At levels somewhat higher than those recorded, spots were immediately visible after spraying with sodium nitrite.

TABLE I.—SENSITIVITIES OF NAPHTHYLAMINE AND SULFANILAMIDE REAGENTS

Acids Tested[a]	Mcg. of Acid Detected per 10 μl. Spot: Sulfanilamide	Mcg. of Acid Detected per 10 μl. Spot: Naphthylamine
Citric	2	7
Lactic	2	4
Succinic	2	3
Malic	2	5
Malonic	2	6
Tropic	2	3
Mandelic	3	2.5
cis-Aconitic	5	70
Oxalacetic	40	60
Glyoxylic	2	1
Tartaric	5	9
α-Ketoglutaric	2	4

[a] All chromatograms were developed in propanol-formic-water.

The reported sensitivities were further checked by chromatographing mixtures of acids in propanol-water-formic acid. Mixtures were prepared as stock solutions containing 50 mcg. of each component in 10 μl. of solution. Each mixture, and dilutions of it, were spotted on a single chromatogram which was then developed and sprayed as previously described. Figure 1 shows a representative chromatogram. It is apparent that the individual components of a mixture are visible in a photograph when the concentrations are approximately twice the minimal detectable levels shown in Table I. The same situation occurs with chromatograms of single acids, consequently sensitivities are not appreciably altered when a mixture is separated.

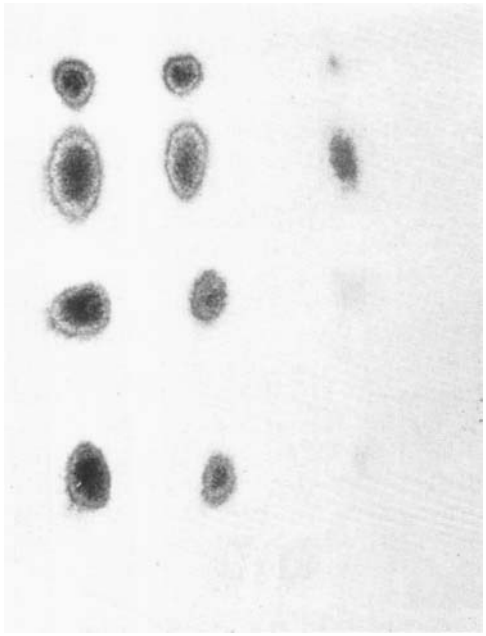

Fig. 1.—Representative separation of acid mixture at different concentration levels: solvent, propanol-formic acid-water (8:1:1); acids in order of increasing R_f value, tartaric, malic, succinic, and mandelic; acid concentrations, from left to right, 50, 25, and 10 mcg. of each component.

Although this report is not primarily concerned with separation techniques, it may be noted that the propanol-formic acid-water system produces excellent separation of many acids. Spots are compact and there is no tailing.

Comparative Sensitivities.—Sensitivities of the naphthylamine and sulfanilamide reagents were compared with four previously described general acid location reagents. For the sake of simplicity, all comparisons were made after migration in the propanol-formic acid-water system. Sensitivities of the previously described reagents for the twelve acids used in this study are shown in Table II. The data in Table II provide a controlled comparison of these four methods and, in conjunction with data in Table I, illustrate the excellent sensitivity of the new reagents.

In obtaining these data, it was noted that the degree of increased sensitivity obtained by reading acridine stained chromatograms by ultraviolet light is extremely time dependent. The more quickly chromatograms are read, the greater is the increase in sensitivity. Tabulated values were obtained by reading immediately after dipping. The sensitivity of the combined indicator-permanganate reagent of Paskova and Munk was confirmed, but we were unable to obtain the color variations reported by these authors.

Effect of Solvent Composition on Sensitivity.—Since the requisite use of a single solvent system would severely limit usefulness of the proposed reagents, the effects of four common solvent systems were investigated. The sensitivity of both alpha-

TABLE II.—SENSITIVITIES OF SELECTED DETECTION REAGENTS

Acids Tested[a]	Mcg. Acid Detected per 10 μl. Spot: Paskova Munk	Aniline Xylose	Bromcresol Green	Acridine	Acridine (u.v.)
Citric	10–50	5	5	50	20
Lactic	60	15	8	150	20
Succinic	50	8	15	20	15
Malic	125	8	10	50	15
Malonic	30	5	8	20	10
Tropic	30	5	5	No spot	No spot
Mandelic	80	10	15	50	20
cis-Aconitic	125	10	3	50	10
Oxalacetic	>150	>50	20	100	15
Glyoxylic	50	5	25	100	20
Tartaric	80	5	15	20	15
α-Ketoglutaric	50	4	8	20	10

[a] All chromatograms developed in propanol-formic-water.

TABLE III.—SENSITIVITY OF NAPHTHYLAMINE REAGENT USED AFTER DIFFERENT DEVELOPING SOLVENTS

Acids Tested	Mcg. Acid Detected per 10 μl. Spot: Propanol Formic Water	Butanol Acetic Water	Ethanol Ammonia	Propanol Formic Eucalyptol
Citric	7	7	5	10
Lactic	4	7	8	8
Succinic	3	3	3	5
Malic	5	5	6	9
Malonic	6	7	7	10
Tropic	3	3	3	5
Mandelic	2.5	3	3.5	5
cis-Aconitic	70	80	55	70
Oxalacetic	60	55	No spot	70
Glyoxylic	1	2	3	10
Tartaric	9	9	12	10
α-Ketoglutaric	4	4	4	7

TABLE IV.—SENSITIVITY OF SULFANILAMIDE REAGENT USED AFTER DIFFERENT DEVELOPING SOLVENTS

Acids Tested	Mcg. Acid Detected per 10 μl. Spot: Propanol Formic Water	Butanol Acetic Water	Ethanol Ammonia	Propanol Formic Eucalyptol
Citric	2	6	9	2
Lactic	2	8	10	4
Succinic	2	1	4	1
Malic	2	4	8	3
Malonic	2	8	6	2
Tropic	2	1	9	4
Mandelic	3	6	10	5
cis-Aconitic	5	No spot	9	10
Oxalacetic	4.0	30	60	20
Glyoxylic	2	1	1	1
Tartaric	5	8	10	9
α-Ketoglutaric	2	9	12	2

naphthylamine reagent and sulfanilamide reagent was determined for each of the twelve reference acids in each solvent system, in the manner described above. The results, shown in Table III and Table IV, demonstrate that none of the four solvents would prohibit use of the proposed reagents. Both reagents could be used with any of these systems, and both may be directly applied to chromatograms that have been developed with ammoniacal solvents. The effect of buffered papers is unknown, since no attempt was made to evaluate this variable.

The failure to obtain a spot with sulfanilamide reagent in the presence of 100 mcg. of *cis*-aconitic acid, using butanol-acetic acid-water as the developing solvent, is unexplained. The same is true for naphthylamine reagent and oxalacetic acid in the ethanol-ammonia system. It should also be noted that some spots may develop slowly with the sulfanilamide reagent. Chromatograms sprayed with this reagent should therefore be re-examined for latent spots after standing overnight.

SUMMARY AND CONCLUSIONS

Two new reagents have been developed for the location of organic acids on paper chromatograms. Color development apparently depends on an acid catalyzed diazotization and coupling to form a highly colored compound, since colors are produced in the presence of a wide variety of diazotizable amines and coupling amines or phenols. The proposed reagents differ in the nature of the diazotizable amine, one employing naphthylamine and the

other sulfanilamide. The sulfanilamide reagent is generally, but not universally, the more sensitive. It usually produces less background color than naphthylamine and the spots are more stable because the background darkens much less rapidly. The sulfanilamide reagent does not have the disagreeable odor of naphthylamine and does not have the carcinogenic action of the latter compound.

Both reagents have been tested against twelve reference acids in four commonly used solvent systems. Sensitivities vary somewhat from one solvent to the next, but the variations are minor and do not prevent use of any solvent tested. Both reagents are much more sensitive than the indicator-permanganate or acridine procedures. The sulfanilamide reagent is notably more sensitive than aniline-xylose or bromcresol green and does not possess the carcinogenicity of aniline-xylose. Both reagents give more reproducible sensitivities than any reagent tested, and neither produces "ghost" spots.

Intense background colors may develop with either sulfanilamide or alphanaphthylamine if chromatograms are exposed to high concentrations of laboratory fumes, but minimal care will prevent this occurrence.

An 8:1:1 propanol-formic acid-water system has been used to develop most of these chromatograms. It has not been extensively studied as a solvent for separating organic acids, but may certainly be used to advantage. It produces excellent separations of many compounds tested, causes little or no tailing, and gives compact spots.

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Protective Coatings XVI

Disintegration of Protective-Coated Tablets as Determined by Urinary Excretion in Humans

By TADAO IDA, SHOJI TAKAHASHI, KAZUO NODA, SHUZO KISHI, SETSUO NAKAGAMI, and ISAMU UTSUMI

A disintegration test of protective coated preparations in human bodies was carried out by determining the riboflavin amount excreted in urine after administration of the riboflavin tablets coated with the previously reported protective-coating agents. Though the rate of the riboflavin excretion was somewhat slow, there was no indication of unusual disintegration of the coated tablets in human bodies.

IN PRECEDING PAPERS, studies on the protective-coating agents were reported in which the amino derivatives and the amino acid derivatives of cellulose, saccharides and polyhydric alcohols, polyvinylamines, polyvinylaminoacetals, polyvinylpyridines, and others were synthesized and examined (1–12). Polyampholites of the vinylpyridine-methacrylic acid system were also studied for the protective-coating agents which solubilize in both gastric and intestinal juice (13). All preparations coated with these agents showed excellent results in the tests—water-resistance, *in vitro* disintegration, and others.

In this report, the *in vivo* test was examined in human bodies for the disintegration of the preparations. Since riboflavin absorbed in excess is rapidly excreted in urine, disintegration rate was determined by the riboflavin amount in urine excretion after the administration of the coated riboflavin tablets.

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