Development of a Paper Strip Test for Detection of Niacin Produced by Mycobacteria

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The development of a reagent-impregnated paper strip test for niacin is described. The test system is based on the formation of cyanogen chloride by the reaction of chloramine-T and potassium thiocyanate in the presence of citric acid. Rupture of the pyridine ring of niacin by cyanogen chloride yields γ-carboxy glutaconic aldehyde and coupling with a primary aromatic amine produces a yellow color. Sensitivity to niacin, both in known solutions and from extracts of 378 clinical mycobacteria isolates, equalled or exceeded that of other methods for detection of niacin. Correlation with other tests for mycobacterial niacin was excellent.

Since about 1960 the niacin test has been the most widely used laboratory procedure for differential identification of the mycobacteria. Pope and Smith (15), Bird (2), and Konno et al. (8) have demonstrated that human tubercle bacilli produce considerably more niacin than other mycobacteria. This characteristic is sufficiently regular for human strains to be accurately differentiated from other acid fast organisms (7, 9).

Several modifications of the niacin test have been made to increase sensitivity, ease performance, or both (1, 5, 10, 12, 14, 16, 18, 19); all are based on the reaction of niacin with a cyanogen halide, usually in the presence of a primary amine. In most tests, niacin is extracted from a mature culture on a solid medium with a small volume of water or saline.

In the procedure described by Runyon (16), equal volumes of an aqueous extract of a culture, 4% ethanolic aniline, and 10% cyanogen bromide are mixed in a test tube in a fume hood. Alternatively, chloramine-T and sodium or potassium cyanide may be used and the unstable ethanolic amine omitted, although this modification results in a somewhat less sensitive reaction (16). The Runyon procedure is the most widely used in this country but has several significant procedural disadvantages. Ethanolic aniline is particularly sensitive to moisture and light, and cyanogen bromide when vaporized is a potent irritant and is extremely toxic (17). Its hydrolysis product, hydrocyanic acid, is also highly toxic.

Kilburn and Kubica (6) have described a test for niacin production by mycobacteria in which reagents are dried on a strip of filter paper. This lessens or eliminates many of the hazards and inconveniences previously associated with the niacin test. The present report describes the development of a reagent-impregnated test strip with improved stability, sensitivity, and ease of use (PathoTec-Niacin; General Diagnostics Division, Warner-Lambert Co., Morris Plains, N.J.). Data are presented which demonstrate a high degree of correlation between the results of conventional and strip tests in the identification of mycobacteria.

MATERIALS AND METHODS

Preliminary evaluation. Sample test strips (6) and details of their preparation were generously made available to us by J. O. Kilburn (Center for Disease Control, Atlanta, Ga.). Similar strips were prepared to evaluate reactivity with known concentrations of niacin. To strips of heavy filter paper (No. 623, 70 lb., Eaton-Dikeman Co., Mt. Holly Springs, Pa.) measuring 6.3 by 60 mm, approximately 0.05 ml of the following solutions were applied to areas measuring 6.3 by 12 mm by means of a capillary pipette. Area 1 (bottom zone) received 10% ethanolic suspension of sodium p-amino salicylate (NaPAS; Henley & Co., Inc., New York, N.Y.). Area 2 was untreated. Area 3 was treated with 60% potassium thiocyanate (Allied Chemical Corp., Morristown, N.J.) in 8% aqueous citric acid (Mallinkrodt, St. Louis, Mo.). Area 4 was untreated. Area 5 received 50% aqueous chloramine-T (Eastman Organic Chemicals, Rochester, N.Y.) heated to 60°C to dissolve. The strips were allowed to dry, stored under dessication, and tested for sensitivity to niacin.

Serial dilutions (0.6 ml) of aqueous niacin (333 μg/ml to 2.5 μg/ml; Nicotinic acid, Matheson Coleman and Bell, East Rutherford, N.J.) were added to test tubes (13 by 100 mm). Test strips were added, partially immersing the aromatic amine reagent (area

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1) in the solution, and the tubes were sealed with a waxed cork. The system was allowed to react at ambient temperature for 15 to 20 min with occasional gentle agitation. The development of a yellow to orange color in the solution was interpreted as a positive reaction, and the absence of this color was considered a negative reaction. Approximately 0.5 ml of 10% NaOH was added to each tube before disposal to inactivate any residual cyanogen chloride.

Modified formulations. NaPAS (10%) in 95% ethanol is a thick suspension of flocculent crystals. Application of this mixture to paper is difficult and the volume applied is not easily reproducible. A mixture of absolute ethanol and dimethyl sulfoxide (DMSO; 84:16) was found to dissolve NaPAS to a final concentration of 15%. The NaPAS solution, thiocyanate-citric acid solution, and chloramine-T solution were applied in parallel bands 12 mm wide to a 1-meter strip of filter paper. An area of waterproof acrylic lacquer (Krylon Inc., Norristown, Pa.) was applied to one edge of the paper so that strips could be handled without touching the reagents. Since dried reagents on paper are essentially colorless, a 0.2% xylene solution of sudan IV (Allied Chemical Corp., Morristown, N.J.) or sudan red GGA, (GAF Corp., New York, N.Y.) was also applied to this edge for identification purposes. After drying, the paper was cut transversely to give strips of the configuration shown in Fig. 1. These strips were tested for sensitivity to niacin immediately after preparation and again after storage at 4, 25, and 35 C for 2 to 8 weeks.

When some difficulty was encountered with stability of the NaPAS area, 28 aromatic amines were evaluated for reactivity in the test system. Approximately 0.1 ml of a 10% solution or suspension of several crystals of each amine were added to 0.6-ml samples of niacin solutions and reacted with test strips prepared as in Fig. 1 except that area 1 was removed. Five compounds showing high sensitivity and low blank color were dissolved in water or ethanol to a final concentration of 10 to 15%; 0.02 ml was applied to area 1 of paper strips (Fig. 2) and retested.

In another experiment intended to improve reagent stability, 20% aqueous sodium p-amino benzoate (NaPAB) was (i) diluted to 10% with water; (ii) diluted to 10% with 95% ethanol; (iii) adjusted to pH 10.5 with 0.1 NaOH and diluted to 10% with water; and (iv) adjusted to pH 10.5 with 0.1 NaOH and diluted to 10% with 95% ethanol. These four solutions were allowed to stand at 20 to 25 C in closed containers for 10 days and were then scanned with a Beckman DB-G spectrophotometer from 600 to 340 nm against water or water-ethanol blanks. An increase in optical density at 450 nm was taken to indicate deterioration of the NaPAB.

Test strips were also prepared with 60% KSCN and 50% citric acid applied to separate areas of the paper; 10% NaPAB was applied to area 1. This configuration is shown in Fig. 2. These strips were tested for sensitivity to niacin and evaluated for appearance and sensitivity after storage at temperatures as high as 45 C.

To insure that the reagent application pattern shown in Fig. 2 was optimal, reagent areas 1, 3, 5, and 7 were arranged in each of the 24 theoretically possible linear sequences and tested with niacin solutions.

Quantitative aspects of test system. Test strips as shown in Fig. 2 were reacted with niacin solutions at various concentrations for 8 to 10 min. The solutions from 10 tests were pooled, and the absorbance at 470 nm against saline was determined spectrophotometrically. Reagent blank was determined and subtracted from each determination. For comparison, the same reaction was carried out in a liquid-reagent system. The following reagents were added in sequence to 2.4 ml of several niacin solutions in a 13- by 100-mm tube: 0.1 ml of 60% KSCN, 0.1 ml of 0.5% citric acid, 0.2 ml of 15% chloramine-T, and 0.2 ml of 10% NaPAB. Tubes were stoppered, mixed, and reacted for 7 to 8 min at ambient temperature; absorbance was determined against a reagent blank. In some tests, the acid was omitted or was replaced by 0.1 ml of 0.1 M citric acid-sodium citrate buffer.

The procedure of Malatesta (13) was modified to determine the amount of cyanogen chloride formed by the reagents on test strips shown in Fig. 3. In this assay, pyridine is converted to glutaconic aldehyde by CICN and reacted with diethylcarboxylate.
to give an orange to violet color. Methods and results will be reported in greater detail elsewhere.

Clinical evaluation. Three hundred seventy-eight cultures of mycobacteria were tested for niacin production with four-zone test strips (Fig. 2) and by the cyanogen bromide-aniline method of Runyon (16). Fresh isolates were grown for 3 to 6 weeks on Middlebrook 7H-10 agar or Lowenstein-Jensen egg medium and extracted with about 1.5 ml of sterile distilled water. Approximately 0.6 ml of extract was placed in a screw-cap tube (13 by 100 mm), a test strip was added in such fashion that the end opposite the orange marker was immersed in the extract, and the tube was closed securely with a cap having a rubber liner. The system was allowed to react at room temperature for 15 to 20 min with occasional gentle agitation and was observed for color development. Aqueous cyanogen bromide (10%) and 5% alcoholic aniline were added to a second sample of extract and similarly observed for color development. After use, the entire closed system was autoclaved and discarded.

 Cultures which were positive by the strip test but negative by the Runyon method were restested by substitution of 3% benzidine base in 95% ethanol for the aniline reagent (3) or by repetition of the Runyon procedure after sufficient reincubation to give a total incubation time of 4 weeks or more. Niacin-negative cultures were identified as strains other than Mycobacterium tuberculosis by means of additional differential tests (20–22) (Table 5).

RESULTS

Preliminary evaluation. Sample strips received from Kilburn and similar strips prepared in this laboratory demonstrated a sensitivity to niacin substantially in agreement with published results (6). Strips made with NaPAS in ethanol-DMSO

![Graph](image-url)

**Fig. 3.** Relationship between niacin concentration and development of color at 470 nm. The regression coefficients for the strip test eluate and the liquid reagent determination did not differ significantly ($b = 0.04633$ versus 0.04604, respectively), and the regression equation for the combined data is: $Y = 0.049 + 0.0462X$ where $Y$ is the predicted optical density at 470 nm and $X$ is niacin concentration in µg/ml.

| Compound                                      | Niacin (µg/ml) |
|-----------------------------------------------|----------------|
| 8-Amino-2-naphthalene sulfonic acid, sodium salt | >333           |
| 5-Amino-1-naphthalene sulfonic acid, sodium salt | >333           |
| 5-Amino-1-naphthalene sulfonic acid, sodium salt | >333           |
| 8-Amino-1-naphthalene sulfonic acid            | >333           |
| 4,5-Dihydroxy-2,7-naphthalene disulfonic acid, disodium salt | >333 |
| m-Phenylenediamine                             | >333           |
| Sulfadiazine                                   | >333           |
| Sulfamethizole                                 | >333           |
| 5-Amino-2-naphthalene sulfonic acid            | >333           |
| p-Amino dimethylaniline oxalate                | 167            |
| p-Phenylenediamine                             | >333           |
| m-Phenylenediamine dihydrochloride             | 83             |
| Sulfanilamide, sodium                          | 83             |
| Barbituric acid                                | 83             |
| o-Tolidine hydrochloride                       | >333           |
| 8-Amino-1-naphthalene sulfonic acid, sodium salt, 5% suspension | 42  |
| Sodium barbital                                | 42             |
| Sodium barbital                                | 42             |
| 4-Amino-1-naphthalene sulfonic acid, sodium salt | 21f            |
| 5-Amino-2-naphthalene sulfonic acid, sodium salt | 21f            |
| Sulfadiazine, sodium                           | 21f            |
| o-Phenylenediamine                             | 21f            |
| Sulfamethizole, sodium                         | 21f            |
| Sulfanilic acid, sodium                        | 21f            |
| Sulfathiazole, sodium                          | 21f            |
| Sulfanilamide                                  | 21f            |
| p-Amino acetophenone                           | 21f            |
| p-Amino benzoic acid, sodium, sodium            | 21f            |

* Neutralized with NaOH to phenolphthalein end point.
* Positive color is red-brown.
* Positive color is purple.
* Adjusted to pH 11.5 with NaOH.
* Positive color is pink.
* Some color developed in reagent blank.
* Adjusted to pH 10.0 with NaOH.
* Acid form adjusted to pH 7.3 to 7.5 with NaOH.

(Fig. 1) initially gave strong positive reactions when tested with 12.5 µg of niacin (0.6 ml of 21-µg/ml solution). After being stored for 2 weeks at 25°C, however, color formation was less intense and strips stored at this or higher temperatures developed a brown discoloration of the NaPAS area with a concomitant loss in sensitivity.

Modified formulations. Table 1 shows the sensitivity of 28 primary aromatic amines tested as solutions or suspensions. Twenty-three of these compounds were either nonreactive, lacked sensitivity, or reacted with other components of the
test system. Five compounds were selected on the basis of sensitivity, relatively low blank color, and solubility characteristics. They were applied to paper and retested (Table 2). NaPAB was the most sensitive, equal to NaPAS. NaPAB is readily soluble in water and yields a clear, faintly yellow solution which turns brown on storage. Although not soluble in absolute ethanol at 10% concentration, a 10% stock solution in 50% ethanol had less tendency to discolor on standing than a 10% aqueous solution. Spectrophotometric examination of stored stock solutions of NaPAB indicated that both pH adjustment and dilution with ethanol reduced the amount of discoloration of these solutions.

When three-reagent test strips (Fig. 1) were stored for 2 weeks at 25 and 35°C, the acidified KSCN reagent zone (Fig. 1, area 3) became yellow and the test strip showed reduced sensitivity.

### Table 2. Niacin-sensitivity of test strip made with several aromatic amines

| Compound                        | Niacin (µg/ml) | Control |
|---------------------------------|----------------|---------|
|                                 | 83 42 21       |         |
| Sulfanilic acid, sodium salt    | ±  -  -        |         |
| Sulfathiazole, sodium           | 2+ ±  -        |         |
| Sulfanilamide, sodium           | ±  -  -        |         |
| p-Aminoacetoephene              | ±  -  -        |         |
| p-Aminobenzoic acid, sodium salt| 3+ 2+ +       |         |
| Sodium PAS                      | 3+ 2+ +       |         |

to niacin. It was also found that a stock solution of acidified KSCN became yellow and developed an odor of H₂S when stored at ambient temperature for 2 weeks, whereas 60% KSCN alone remained clear and colorless when similarly stored. Strips made with KSCN and citric acid in separate bands and NaPAB at pH 10.5 in water-ethanol solution (Fig. 2) showed minimal discoloration of all reagent areas. The data indicated that strips prepared in this way were stable in appearance and reactivity for 1 to 2 months when stored at 45°C and in excess of 12 months at 35, 25, or 4°C (Table 3).

When reagents were applied to paper in sequences 1, 3, 5, 7, and 1, 5, 3, 7 (Fig. 2), test strips were acceptable in appearance and sensitivity. All other sequences were less satisfactory in one or more respects.

**Quantitative aspects.** The lowest concentration of niacin tested was 2.5 µg/ml. At this level, test strips (Fig. 2) developed a pale yellow color in the solution (absorbancy at 1-cm light path was 0.15, measured at 470 nm), which was visually classified as a ± reaction. Color development in the liquid test system was made optimal by using a buffer of pH 4.0 to 4.5. Within this range, sensitivity of the reaction was essentially the same as that of the test strip. Sensitivity was reduced at higher pH, and a precipitate formed in the test system at lower pH values. The optical density is linear with respect to niacin concentration within the sample range tested (2.5 to 20 µg/ml). Color development in strip and liquid-reagent systems is compared in Fig. 3.

Colorimetric assay by the modified Malatesta

### Table 3. Reaction of stored strips with 21 µg of niacin solution per ml

| Batch no. | Temp (°C) | 1 wk | 2 wk | 4 wk | 2 months | 3 months | 6 months | 12 months |
|-----------|-----------|------|------|------|----------|----------|----------|----------|
| 6         | 4         | ±    |      |      |          |          |          | ±        |
|           | 25        |      | ±    |      |          | ±        |          | ±        |
|           | 35        |      |      | ±    | ±        | ±        | ±        | ±        |
|           | 45        |      |      |      |          | ±        |          | ±        |
| 7         | 4         | ±    |      |      |          | ±        |          | ±        |
|           | 25        |      | ±    |      |          | ±        |          | ±        |
|           | 35        |      |      | ±    | ±        | ±        | ±        | ±        |
|           | 45        |      |      |      |          | ±        |          | ±        |
| 8         | 4         | ±    |      |      |          | ±        |          | ±        |
|           | 25        |      | ±    |      |          | ±        |          | ±        |
|           | 35        |      |      | ±    | ±        | ±        | ±        | ±        |
|           | 45        |      |      |      |          | ±        | ±        | ±        |

*All strips are as shown in Fig. 2. Batches 7 and 8 are duplicates; 6 is the same except that only half as much NaPAB was applied to area 1.*

*Symbols: · · ·, not tested; +, positive reaction; ±, weak positive reaction; and −, negative reaction.*
method showed that each strip generates about $0.8 \times 10^{-6}$ to $10^{-6}$ mole of cyanogen chloride. This amount is in excess of the molar concentration required to react with niacin produced by mycobacteria. The yield was increased to approximately $7 \times 10^{-5}$ mole when a large excess (1,000-fold) of chloramine-T was incorporated into the assay system.

Clinical evaluation. As shown in Table 4, 378 clinical isolates were tested; 296 were positive with both the strip and cyanogen bromide-aniline methods. Of these cultures, 264 were positive when first tested at an age of about 3 weeks. The remaining 32 were niacin-negative by both methods when tested at the same time and positive when older Lowenstein-Jensen egg medium cultures were tested. Eight cultures were positive by the strip test and negative by the Runyon method. One was a pigmented culture in which color from the culture was precipitated by the Runyon reagents but not by the test strip. The other seven were retested and found niacin-positive by the benzidine test or by the aniline test after additional incubation. No cultures were strip-negative and aniline-positive. Additional testing of the 74 negative cultures resulted in identification as shown in Table 5.

**DISCUSSION**

Several difficulties were encountered in attempting to develop a stable and sensitive system. NaPAS at 10% concentration in ethanol is considerably in excess of the limit of solubility, and application of this suspension to paper depends heavily on technique to give workable results. In addition, the resultant button of crystals is mechanically fragile and is easily detached or pulverized during storage and handling. For these reasons, application of NaPAS to paper as a solution is more desirable. Since NaPAS is unstable in aqueous solution (6), several nonaqueous solvent systems were examined; ethanol-DMSO was found to dissolve NaPAS at the desired concentration. Stability of strips made with this solution was poor, however, and other amines were examined in search of an alternative reagent. Of those tested, NaPAB was the most sensitive color developer. Stability of this reagent was measurably improved by the use of a pH-adjusted ethanol-water solvent. Since acidified KSCN is also unstable, KSCN and citric acid were isolated in two separate bands on the strip. This configuration substantially increased stability, eliminated discoloration of KSCN, and maintained chemical sensitivity of the strip even under severe and prolonged storage conditions.

When the 24 possible sequences of reagents on the strip were evaluated, only one modification yielded results equivalent to those obtained with strips as shown in Fig. 2. This modification was inversion of the citric acid and KSCN positions. All other trials yielded inferior results. It was found that NaPAB must be in position 1 to develop color in the solution in a positive test. Other sequences led to development of pink or orange colors on the strip, loss of sensitivity, and interfering reactions between the reagents on the strip.

In the development of a yellow color, the formation of a Schiff base as described by Feigl (4) appears to be the reaction involved in this test system; the strip test differs from other procedures in the use of cyanogen chloride instead of cyanogen bromide. The production of cyanogen chloride from acidified KSCN and chloramine-T have been described by Kraus and Krausova (11) and by Kilburn and Kubica (6). The postulated reactions are shown in Fig. 4. Color development is linear within the range tested, and response slopes of the two systems are not significantly different (Fig. 3).

The method for using the strip is similar to that described by Kilburn and Kubica (6), but with a slightly increased reaction time to insure full color development. In our experience, both methods are quick, easy, reliable, and safe. CICN is highly toxic, but the strip method keeps this reagent confined within a closed tube at all times, in contrast to other methods in which bromine and BrCN must be handled and can escape into

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**Table 4. Reactions of 378 mycobacteria with aniline-BrCN and test strip reagents**

| Aniline-BrCN reaction | Test strip reaction | No. of cultures |
|-----------------------|---------------------|-----------------|
| +                     | +                   | 296<sup>a</sup> |
| -                     | +                   | 8               |
| +                     | -                   | 0               |
| -                     | -                   | 74              |

<sup>a</sup> Procedure of Runyon (16).

<sup>b</sup> Includes 32 cultures initially negative by Runyon procedure and test strip, but positive by both methods when older cultures were tested.

**Table 5. Identification of niacin-negative cultures after additional incubation**

| No. of organisms | Identification |
|------------------|----------------|
| 20 378           | Mycobacterium kansasii |
| 20 378           | Group III Battey-avium-swine |
| 20 378           | Group III not Battey |
| 10 378           | Group IV M. fortuitum |
| 2 378            | Group II M. aquae |
| 1 378            | Group III M. terrae |
| 1 378            | Group III M. gastri |
the atmosphere. Reagents such as CICN should not, however, be used in greater quantities than are needed. The assay of CICN demonstrated that an average strip generates about $9 \times 10^{-7}$ mole. This represents about 5% of theoretical yield, but a comparison with the amount of niacin present in 0.6 ml of a 20 $\mu$g/ml solution (1.63 $\times$ 10$^{-7}$ mole) indicates a reagent to sample ratio of 5.5 to 1 or better at expected sample levels. Quantitative niacin tests demonstrate that this ratio is sufficient to give a linear response within the range tested. The NaPAB is present in larger excess; each strip contains approximately 2 $\times$ 10$^{-6}$ mole.

When compared to the aniline test in the identification of mycobacteria, the strip method gave equal or superior results. Of 378 cultures tested, 370 (98%) gave identical results by both methods. The remaining eight cultures were initially strip-positive and aniline-negative. Retesting after additional incubation showed that seven of the eight were in fact niacin-positive. The remaining organism was not readable by the strip method due to heavy pigmentation of the culture. This was not considered significant, since the utility of the niacin test is to differentiate among nonpigmented cultures.

Thirty-two cultures first grouped as niacin-negative by both methods were positive by both methods when retested after additional incubation. This experience serves to emphasize the importance of adequate incubation time for development of a mature culture. In the present study, initially niacin-negative cultures were retested after additional incubation if they were less than 4 weeks old, if less than 50 colonies were present, or both. Our data thus agree with that of Ellner (3) who has recommended the use of cultures at least 3 weeks old and noted that a test at 4 weeks will detect 95% of niacin-positive cultures. Konno (7), however, recommends incubation times of 1 to 3 months.

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Statistical analyses were performed by Douglas Dancz.

ADDITIONUM IN PROOF

Since preparation of this paper, the authors have received notice that the paper "Determination of Cyanogen Chloride in Activated Test Strips" by M. S. Lefar, A. Maslansky, W. D. Young, and D. F. Kronish has been accepted for publication in The Analyst, London.

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