aminopropanesulfonic acid could be reversed by such natural amino acids as glycine, alanine, valine, or the leucines. None of these amino acids, however, produced an increase in growth at the concentrations used. In addition, simple mixtures of natural amino acids were ineffective in reversing the inhibition. The addition of from one to three milliliters of a solution of sixteen amino acids† to the basal medium in the presence of the aminosulfonic acid produced about a 2% increase in growth. However, addition of the same amino acid mixture to the basal medium in the absence of the aminosulfonic acid resulted in a similar increase in growth. This would indicate that the increased growth was due to a general improvement in the nutritive value of the medium rather than a reversal of inhibition by a specific amino acid.

Further investigations will be necessary to determine whether or not inhibition produced by 1,1-aminopropanesulfonic acid is reversed by substrates other than amino acids.

*Summary.* 1,1-aminopropanesulfonic acid was synthesized by the addition of ammonium bisulfite to propanal. The aminosulfonic acid inhibited the growth of *Lactobacillus casei* at levels from $10^{-4}$ to $10^{-2}$ molar. Single amino acids as well as combinations of amino acids were ineffective in relieving the inhibition.

† The amino acid mixture contained in each ml, 0.8 mg each of DL-leucine, DL-serine, DL-phenylalanine, DL-glutamic acid, DL-valine, DL-aspartic acid, L-cystine, DL-arginine hydrochloride, L-tryptophane, L-tyrosine, DL-threonine, DL-methionine, DL-alanine, DL-isoleucine, DL-lysine and L-histidine hydrochloride.

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## Growth of *Mycobacterium tuberculosis* in Egg Yolk Mediums. (17738)

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Egg yolk is widely used as the chief ingredient in a variety of solid mediums for the cultivation of *Mycobacterium tuberculosis;* in fact, no other nutrients need to be added(1). That it can be equally useful in liquid and semi-liquid mediums is not so well known. Besredka(2) described a liquid medium made by autoclaving egg yolk diluted with alkaline water; this preparation, however, does not support the growth of small inocula. Tubercle bacilli will grow well within the yolks of market eggs(3) even from inocula as small as 10 tubercle bacilli(4), as well as in the yolks of embryonated eggs(5). The widespread use of the Dubos Tween-albumen mediums has demonstrated the value of liquid mediums in metabolic and chemotherapeutic studies of the tubercle bacillus. The measure of the adequacy of such mediums is their ability to promote rapid growth from small inocula (10 organisms or less). Several easily prepared mediums that will do this can be made from egg yolk.

*Methods.* Strictly fresh chicken eggs were used. The yolks were removed aseptically and diluted with sterile water, salt solutions, or a basal medium made up according to a formula given by Dubos *et al.*(5):

| | g |
|---|---|
| Casein digest (N-Z Amine)* or asparagine | 2.0 |
| $Na_2HPO_4 \cdot 12H_2O$ | 6.3 |
| $KH_2PO_4$ | 1.0 |
| Na citrate $\cdot 2H_2O$ | 1.5 |
| Dissolve, then add | |
| Ferric ammonium citrate | 0.1 |
| $MgSO_4 \cdot 7H_2O$ | 0.6 |
| Distilled water to 1000 ml. Adjust to pH 6.8. | |

*N-Z Amine (Type B), Sheffield Farms Co., Inc.

1. Corper, H. J., and Cohn, M. L., *Am. Rev. Tuberc.*, 1942, v46, 560.

2. Besredka, A., *Ann. de l'Inst. Pasteur,* 1921, v35, 291.

3. Gelarie, A. J., *J. Lab. Clin. Med.*, 1944, v29, 532.

4. Eggerth, A. H., Dresher, E., and McOsker, V. C., *Am. Rev. Tuberc.*, 1948, v57, 632.

TABLE I.
Growth of *Mycobacterium tuberculosis* in Egg Yolk Mediums.

| Type of medium | Dilution of yolk | Growth in days | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 12 | 14 | 16 | 18 | 20 | 22 | 25 | 28 | 31 | 35 |
| Autoclaved in distilled water | 1-10 | 1+ | | 4+ | | | | | | | |
| | 1-50 | | | 1+ | | | 4+ | | | | |
| | 1-100 | | | | 1+ | | | 4+ | | | |
| | 1-300 | | | | | no growth | | | | | |
| Autoclaved in basal medium | 1-10 | 1+ | | 4+ | | | | | | | |
| | 1-50 | | | | 1+ | | | | 4+ | | |
| | 1-250 | | | | | | 1+ | | | 4+ | |
| | 1-1000 | | | | | | | 1+ | | | 4+ |
| | 1-2500 | | | | | | | | 1+ | | 2+ |
| | 1-5000 | | | | | no growth | | | | | |
| Unheated yolk in basal medium | 1-10 | 1+ | | 4+ | | | | | | | |
| | 1-100 | 1+ | | 4+ | | | | | | | |
| | 1-1000 | | 1+ | | 4+ | | | | | | |
| | 1-4000 | | | | | | | 1+ | | | 4+ |
| | 1-10000 | | | | | | | | 1+ | | 2+ |
| | 1-20000 | | | | | no growth | | | | | |
| Filtrates of yolk infusions (Coors) | 1-10 | 1+ | | 4+ | | | | | | | |
| | 1-30 | | 1+ | | | 4+ | | | | | |
| | 1-100 | | | | | no growth | | | | | |

The inoculum in each case was $10^{-8}$ mg of moist growth (about 10 tubercle bacilli). The data given are the averages of from 6 to 20 experiments.

The N-Z amine gave better growth with some of the yolk mediums than did asparagine, hence was preferred. The pH for optimal growth is not critical; reactions between pH 6.0 and 7.5 seem equally satisfactory. The yolk has a pH of about 6.0 and is not heavily buffered, so that adjusting the diluting fluid to the desired pH is usually all that is necessary.

*Preparation of the inocula.* Four different strains of human type tubercle bacilli were used: 3 of these were freshly isolated; the fourth has been cultivated for 6 years. Every significant experiment was repeated with all 4 strains; all 4 behaved essentially the same.

Ten to 20 day old cultures on solid medium were ground up in a tissue grinder with a few ml of the Dubos Tween-albumen medium. Clumps were removed by centrifugation at 1000 r.p.m. for 6 minutes; the supernatant suspension was diluted to a density equal to that of tube No. 3 of McFarland's standards (6). Such a suspension contains $10^0$ mg of moist tubercle bacilli per ml, or 1 billion single viable organisms. Microscopic examination showed that the bacilli were mostly single with a few small clumps.

Small tubes, each containing 1.5 ml of the medium, were inoculated in duplicate with doses of $10^{-4}$, $10^{-6}$, and $10^{-8}$ mg of moist culture contained in a volume of 0.1 ml. Colony counts were always made; a dose of $10^{-8}$ mg usually grew from 7 to 20 colonies. Cultures were incubated at 37 to 38°C. Growth in the cultures was determined by making smears at appropriate intervals. As growth is entirely subsurface, the smears were taken from the sediment. Scanty growth was recorded as 1+; numerous cords or clumps in every low power field as 4+. Incubation was continued for 35 days.

Egg yolk mediums. *1. Egg yolk diluted with distilled water and autoclaved.* When egg yolk is diluted and autoclaved at the usual pH, a coagulum is formed. If the dilution is 10-fold or greater, the most of the medium is liquid. Table I shows that when yolk is diluted 1 to 10 with distilled water and autoclaved, this medium supports growth of the tubercle bacillus very well, even when the inoculum is as small as $10^{-8}$ mg (about 10

5. Dubos, R. J., Davis, B. D., Middlebrook, G., and Pierce, C., *Am. Rev. Tuberc.*, 1946, v54, 204.

6. McFarland, J., *J. A. M. A.*, 1907, v49, 1176.

organisms). Growth is fully as rapid and profuse as with the Dubos liquid mediums. Dilutions of yolk up to 1 to 100 still support good growth, but higher dilutions are unsatisfactory. With larger inocula, such as $10^{-4}$ mg, smears from yolk dilutions up to 1-100 become positive in 5 days and heavily positive within 9 days; with this inoculum, yolk dilutions up to 1-600 will give growth.

*2. Egg yolk diluted with basal medium and autoclaved.* When yolk is diluted with the basal medium instead of with water, far less yolk is needed to obtain growth of the tubercle bacillus. As Table I indicates, as little as 1 part of autoclaved yolk in 2500 parts of basal medium has a demonstrable growth stimulating effect with inocula of $10^{-8}$ mg, while 1 part in 5000 is effective with inocula of $10^{-6}$ mg or more. When the inoculum is $10^{-4}$ mg, smears are positive in 4 to 6 days in yolk dilutions up to 1-250. The basal medium alone, without yolk, supports growth only when heavily seeded; with an inoculum of $10^{-4}$ mg sub-surface growth occurs in about 20 to 24 days and becomes moderately heavy in 35 days. Smaller inocula never grew out in the basal medium. In these autoclaved mediums, the growth stimulating substances are associated with the coagulum. If the fluid portion is completely freed from particulate matter (as by filtration through a Coors filter, or even through a retentive filter paper), it will not support growth. After centrifugation at 5000 r.p.m., supernatant fluids may sometimes support growth because all particulate matter is not removed. If the supernatant fluid is removed and replaced by adding sterile water or basal medium to the coagulated material, growth will occur. If a small amount of yolk (best diluted with an equal volume of water) is coagulated as a slant and covered with sterile water or basal medium, growth occurs in the liquid phase. In the autoclaved mediums, the organisms occur chiefly in loose clumps which often become completely dispersed and appear as clouds of single bacilli. Bacilli in "cords" as described by Middlebrook, Dubos, and Pierce (7) may also appear.

7. Middlebrook, G., Dubos, R. J., and Pierce, C., *J. Exp. Med.*, 1947, v86, 175.

*3. Unheated sterile egg yolk diluted with basal medium.* Unheated egg yolk is least soluble in distilled water and most soluble in 10% sodium chloride solution in the pH range used for these mediums. When diluted with the basal medium, much of it remains insoluble and settles out. The further addition of diluent does not bring more yolk into solution; instead, solid matter is precipitated out of the liquid phase. This indicates the presence of some powerful dispersing agent, the effectiveness of which is diminished by dilution. When the insoluble material is removed by centrifugation, the supernatant fluid contains active substances, though growth is usually better when the insoluble material is left in. In the experiments shown in Table I, the sediments were not removed. With mediums prepared in this way, the activity of unheated yolk can be demonstrated in dilutions as high as 1 part in 20,000 with inocula of $10^{-6}$ or greater, or 1 part in 10,000 with inocula of $10^{-8}$ mg. When the yolk content is 1 or more parts per 1000, growth is rapid and profuse, even with the smallest inocula (Table I). When raw egg yolk is diluted with distilled water, growth is both slower and less luxurious than when the same medium is autoclaved. Yolk diluted with the salts only of the basal medium (without the asparagine or N-Z Amine) supports growth well. The grouping of the tubercle bacilli in smears from these unheated yolk mediums is quite different from the loose clumps found with the autoclaved yolk. Here the arrangement is like that seen with the Dubos Tween-albumen medium: cords of bacilli, often very long and thick; also matted filaments or "Medusa heads"(7).

*4. Filtrates of unheated yolk infusions.* When egg yolk is diluted and infused with the basal medium (over night in the refrigerator, then 3 hours at 45°C), then centrifugated and filtered through a Coors filter, a strongly opalescent filtrate is obtained. If the filtrate from a 1 in 10 or 1 in 20 infusion is diluted further, a precipitate forms; this precipitate is mostly lipid but contains protein also. It is least soluble in distilled water and is more soluble in salt solutions. The precipitate contains most of the growth stimulating factors.

The filtrates from the more concentrated infusions in basal medium are excellent growth mediums for the tubercle bacillus (Table I). If more dilute infusions (*e.g.*, 1 part or less of yolk in 100 parts of basal medium) are made, very little of the growth stimulating material goes into solution and the filtrates do not support growth well.

These filtrates, while free from gross particles, are opalescent emulsions. An effort was made to secure a yolk filtrate that was water clear and would yet support vigorous growth of small inocula. This was done by extracting egg yolk repeatedly with acetone at room temperature; drying and powdering the residue; then making an infusion of the powder (0.66 g in 25 ml of basal medium) and filtering through a Coors filter. The filtrate was water clear. With an inoculum of $10^{-4}$ mg, smears were positive in 5 days and heavily positive in 8 days. With an inoculum of $10^{-8}$ mg smears were positive in 18 days and growth was heavy in 25 days. When the acetone-extracted yolk was further extracted with hot alcohol (which removes the phospholipids), the aqueous extract of the residue did not support the growth of tubercle bacilli. The fraction removed by the hot alcohol, when added to basal medium and autoclaved, did support growth. That the phospholipid fraction of egg yolk is largely responsible for the extraordinary capacity of the yolk to stimulate the growth of tubercle bacilli is clear from the reported observations of Boissevain and Schultz(8), Dubos(9), Finlayson(10), and others. Sphingomyelin, which constitutes about 0.3% of the weight of the yolk, is known to enhance the growth of the tubercle bacillus(11). Experiments still in progress indicate that other constituents besides the phospholipids may be equally important.

*Summary.* 1. Egg yolk can be used as the basis for several easily prepared liquid and semi-liquid mediums.

2. On such mediums, growth of the tubercle bacillus is rapid and heavy, even from small inocula.

3. By first removing the acetone soluble fats, water clear extracts of egg yolk can be prepared; these will support growth from small inocula.

8. Boissevain, C. H., and Schultz, H. W., *Am. Rev. Tuberc.*, 1938, v38, 624.

9. Dubos, R. J., PROC. SOC. EXP. BIOL. AND MED., 1945, v58, 361.

10. Finlayson, M. K., *J. Path. Bact.*, 1946, v58, 88.

11. Dubos, R. J., *J. Exp. Med.*, 1948, v88, 73.

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## A Radioautographic Method for Detailed Localization of Radioactive Isotopes in Tissues without Isotope Loss. (17739)

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Animal tissues are prepared for radioautographs by freezing in isopentane at –170°C followed by vacuum dehydration, infiltration with paraffin under vacuum, cutting with the technic described previously(1), and direct mounting of the section on a microscope slide or a thin cover glass. The tissue holder (slide or cover glass) is then placed in a vertical position in an oven at approximately 45°C; the paraffin liquefies and all exceess paraffin is drained from the slide and tissue section. An alternative method is to remove the paraffin more completely by means of a paraffin solvent such as xylol, warm dioxane, or warm absolute alcohol. The tissue is returned to the oven for a suitable length of time to ensure complete evaporation of the

1. Holt, M. W., Cowing, R. F., and Warren, S., *Science*, 1949, v110, 328.