no KCN. Aliquots of the dialyzed enzyme containing 0.05 mg of protein were tested for nitrate reductase activity using DPNH in the assay (*1*) and purified buffer and KNO_3 (*7*). Other aliquots of the enzyme solution were preincubated at 2°C with 0.01 μM of various metals, and then were assayed for enzyme activity using purified reagents. The mean increases or decreases in activity in percentages from the addition of the various metal salts were: $FeSO_4$, +15; Na_2MoO_4, +47; $FeCl_3$, +15; $MnSO_4$, +4; $CuSO_4$, −43; and $ZnSO_4$, −2 for two experiments. Dialysis of the enzyme against buffers at *p*H value below 7.0 failed to remove Mo from the enzyme. These samples, however, consistently showed a small increase in activity from the addition of $FeCl_3$ or $FeSO_4$.

In view of the results reported here, it seems apparent that Mo is a constituent of the soybean leaf nitrate reductase. The results suggest that iron may partially replace Mo as a metal factor in the enzyme (*8*).

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20 June 1955

Comparative Tonographic Study of Normotensive Eyes of White and Negro Persons

Opinion is divided concerning a predilection of Negroes for glaucoma. The pertinent literature is reviewed in our extensive publication (*1*).

More pigment being present in the Negro eye, pigment granules may more readily be deposited in the trabecular meshwork, in the canal of Schlemm, and in its outlets, causing an obstacle to aqueous outflow.

The purpose of the work reported here (*2*) was to determine, with the aid of an electronic tonometer, whether there are any differences in intraocular pressure (IOP), facility of outflow (c), and production of aqueous humor (K), between

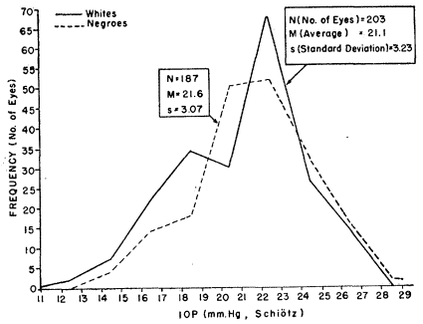

Fig. 1. Intraocular pressure distribution in 203 eyes of white persons and 187 eyes of Negro persons.

normotensive eyes of white and Negro persons. In other words, we have looked for a possible difference in the aqueous humor dynamics of Caucasians and Negroes.

One hundred seven white persons, representing a total of 203 eyes, and 97 Negro subjects (187 eyes) were studied. Of the white subjects, 52 were female (100 eyes) and 55 were male (103 eyes). Of the Negro subjects, 45 were female (86 eyes) and 52 were male (101 eyes). All persons were between 40 and 92 years of age. All eyes were free of manifest ocular disease.

It was, of course, impossible to determine the percentage of Negro blood in our subjects. We did take the precaution of selecting subjects with very dark skins, because their eyes are richer in pigment. We believe that our colored examinees are representative of the typical American Negro.

We performed tonography on each eye, using a Mueller electronic tonometer without recording attachment. The technique used was the standard one described by various authors; we followed all the precautions recently outlined by Stepanik (*3*). The values were recorded every 30 seconds; the total duration of each tonometry was 4 minutes. The facility of outflow was calculated from the formula

$$c = \frac{V_\Delta}{(\mathrm{Av}\, P_t - P_0)\, t},$$

and the rate of production of aqueous humor was calculated from the formula

$$K = P_0 \times c.$$

For interpretation of these formulas, the reader is referred to Grant's original papers (*4*).

All our data were evaluated statistically, using the F test of Snedecor and the t test of Student. First we compared the values of intraocular pressure, facility of outflow, and production of aqueous humor of the eyes of the white and Negro persons studied. Figures 1, 2, and 3 show the curves of distribution for intraocular pressure, facility of outflow, and production of aqueous humor, respectively, of these two groups with the relative average values (M) and the standard deviations (s). By comparing our data with the appropriate values from the F and t tables, we found that the difference between the samples was attributable to chance. In other words, no significant difference between intraocular pressure, facility of outflow, and production of aqueous humor of normotensive eyes of white and Negro persons exists.

The same statistical analysis was made on the values of intraocular pressure, facility of outflow, and production of aqueous humor for the eyes of white and Negro men and women. The calculations and the values for the F and t tests proved that all data were homogeneous for both female and male groups. Intraocular pressure, facility of outflow, and production of aqueous humor of the eyes of Negro women and men do not differ significantly from those of the eyes of white women and men.

Our investigations proved that (i) there is no significant difference between intraocular pressure of normotensive eyes of Negro and of white adults; (ii) the facility of outflow of aqueous humor is not significantly lower in the Negro eye than it is in the white eye; and (iii) the production of aqueous humor is the same in the eyes of members of both races.

Since the resistance to outflow is the reciprocal of the facility, we conclude that the eyes of Negroes do not present more resistance to outflow than do the eyes of Caucasians. The eyes of persons of the white and Negro races differ ana-

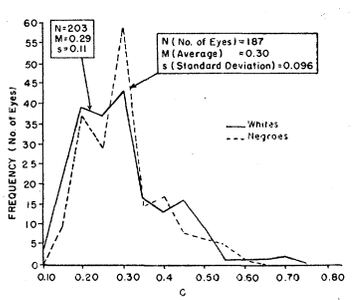

Fig. 2. Facility of outflow in the same eyes.

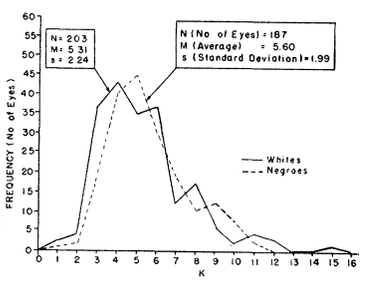

Fig. 3. Production of aqueous humor in the same eyes.

tomically, but the dynamics of the aqueous humor are equal in both. Therefore, it seems impossible to maintain the assumption that there is a predilection of Negro eyes for glaucoma.

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10 June 1955

Staining Methods in Chromatography of Acidic and Neutral Mucopolysaccharides

Staining of complex carbohydrates following separation by paper chromatography or electrophoresis requires reagents other than those commonly used for detection of simple sugars. Such reagents as potassium permanganate (*1*), ammoniacal silver nitrate (*2*), acidic naphthoresorcinol (*3*), sodium metaperiodate, and lead tetraacetate (*4*) have been used to detect nonreducing sugars. The Hotchkiss periodate method has also been employed to stain protein-bound carbohydrates on filter paper after electrophoresis (*5*), although difficulty has been encountered in the rather intense staining of the paper itself and in applying this method to the detection of acidic mucopolysaccharides after chromatography (*6*). Acidic mucopolysaccharides have also been detected after paper electrophoresis (*7*) or ascending chromatography (*8*) by virtue of a metachromatic reaction with toluidine blue.

Mucoproteins of biological origin may consist of mixtures of acidic and neutral mucopolysaccharides. Toluidine blue stains substances metachromatically with free anionic groups. Neutral mucopolysaccharides and acidic mucopolysaccharides in which the anionic groups are combined—as, for example, with proteins, or otherwise masked—stain orthochromatically (*9*) or may fail to stain at all on paper.

Mucicarmine is frequently used empirically in staining procedures for the demonstration of "mucin" from various epithelial secretions (*10*). This stain may be effective histologically in sites where toluidine blue fails to stain (*11*). The chemical basis for its staining action is uncertain (*12*).

Mucicarmine has been adopted by us as a stain for locating the position of acidic and neutral mucopolysaccharides following paper partition chromatography. Heparin, potassium chondroitin sulfate from cartilage (*13*), and potassium hyaluronate from umbilical cord (*13*) served as acidic mucopolysaccharides. For a neutral mucopolysaccharide, dialyzed and concentrated blood group substances A and B (Sharp and Dohme) from hog and horse stomach were used. The product was subjected to hydrolysis with $2N$ H_2SO_4, and chromatographic examination for simple sugars revealed fucose, galactose, and hexosamine. Hexuronic acid was not detected on the chromatograms. Only traces of hexuronic acid could be detected by use of the naphthoresorcinol method (*14*).

Duplicate ascending chromatograms of solutions of blood group substances and acidic mucopolysaccharides were run on Whatman No. 1 paper for 22 hours at 23°C. The solvents found most satisfactory consisted of 55 percent of $0.05M$ Na_2HPO_4 and 45 percent of redistilled isopropyl alcohol, and 58 percent of a phosphate-sodium chloride buffer ($0.0007M$ NaH_2PO_4, $0.09M$ Na_2HPO_4, and $0.07M$ NaCl) (*15*) and 42 percent of redistilled isopropyl alcohol.

After the chromatograms have dried, fixation of the paper is desirable before staining. This was suggested by Leitner and Kerby (*6*) who used 95-percent alcohol and ether for this purpose. We have obtained somewhat sharper delineation of spots and more brilliant colors after staining by using formalin and ethanol as a fixative. Each chromatogram was immersed in a mixture of 20 ml of neutral formalin and 80 ml of absolute ethanol for 15 minutes. After drying, one chromatogram was sprayed with toluidine blue, the other with mucicarmine. Both sides of the paper were sprayed and care was taken to avoid running of the dye on the paper.

Recent reports have described many fractions in commercial toluidine blue of varying staining ability (*16*). In addition to a metachromatic staining fraction, much of the dye lot may either stain chromotropes orthochromatically or lack staining power entirely. Hence, commercial toluidine blue was recrystallized several times from hot water and alcohol before use. Forty milligrams of the recrystallized dye was added to 80 ml of acetone and 20 ml of distilled water. The solution was stored in a dark bottle at 5°C. Acetone effectively prevented spreading of the spots and did not inhibit metachromasy as alcohol does at this concentration. Other cationic dyes (pinacyanol, methylene blue, and crystal violet) were tried, but toluidine blue was the most satisfactory in brilliance of metachromatic color and in showing the least background discoloration of the paper.

Mucicarmine stock solution was prepared according to Lillie's method (*17*). The carmine was not recrystallized. Different dye batches gave similar staining results. One gram of carmine, 0.5 g of anhydrous $AlCl_3$, and 2 ml of distilled water were heated together in a porcelain crucible for 2 to 3 minutes. It was found desirable to drop the crucible directly into 100 ml of 50-percent ethanol. After 24 hours the solution was filtered and stored at 5°C. The spraying reagent was made up prior to use. Five milliliters of the stock solution were added to 17 ml of absolute ethanol and 3 ml of distilled water.

After the chromatograms had been sprayed, they were repeatedly rinsed in distilled water acidified with a small amount of glacial acetic acid (*6*) until the background discoloration was largely removed; then they were washed in distilled water and hung up to dry. More vivid metachromatic color changes were noted on chromatograms that had been sprayed with toluidine blue when they were dried in the cold.

Toluidine blue revealed acidic mucopolysaccharides as bright pink (heparin) or purple (chondroitin sulfate and hyaluronate) spots on a light blue background. Blood group substances did not stain. Mucicarmine stained both acidic mucopolysaccharides and blood group substance bright pink on a very light pink or colorless background.

The use of these two sprays on duplicate chromatograms thus permits the separate identification of acidic and neutral mucopolysaccharides in materials of biological origin (*18*).

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