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Colorimetric Estimation of Penicillin in Aqueous Solutions.*†

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It was recently shown that acid hydrolyzates of penicillin gave a blue-violet coloration with the ninhydrin reagent.[1] Moreover, the same workers reported that the color intensity of this reaction ran parallel to the antibacterial activities of their preparations.

Colorimetric analysis of amino acids by the ninhydrin reagents has been reported by other investigators.[2,3]

The writer has modified the ninhydrin reagents so that small amounts of crystalline penicillin can be estimated with fairly consistent results, provided that temperature, duration of heating, and pH are rigorously controlled. These experiments have been performed on aqueous solutions of crystalline penicillin (Merck), with potency of 1650 units/mg.

The procedure is as follows:

To graduated 10 cc tubes are added 3 to 9 μg of freshly diluted solution of penicillin, 0.5 cc of 0.2 N H_2SO_4, 4 mg of ninhydrin (Eastman) treated with norite (Coleman and Bell). The tubes are then covered with glass vials and heated in boiling water bath for 20 minutes in subdued light. The tubes are then removed, cooled and treated with 0.2 cc of freshly prepared 10% pyridine (Mallinckrodt), 20 mg of norited $(NH_4)_2SO_4$ (Baker)† the volumes equalized with distilled water and then reheated for 15 minutes. The tubes are removed, cooled and made up to 6 cc with water.

The pH of these solutions should be from 4.7 to 5.0. They are then read in the Coleman spectrophotometer against reagent blanks at 550 μ. Blanks and penicillin solutions are prepared in triplicate.

The actual values obtained are not absolute but vary somewhat with the reagents and experimental conditions. However, straight line calibrations (semi-log)) may be obtained for any one set of reagents and conditions.

An illustrative set of values is as follows:

Concentration penicillin, gamma	Light transmission, %
3	88.5
6	79
9	71.5

Summary. A colorimetric technic for analysis of 9 μg or less of crystalline penicillin is described.

* The writer is indebted to Merck & Co., Rahway, N.J., for their generous gift of penicillin.

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1 Abraham, E. P., Chain, E., Baker, W., and Robinson, R., *Nature,* 1943, **151**, 107.

2 Harding, V. J., and Maclean, R. M., *J. Biol. Chem.*, 1915, **20**, 217.

3 Virtanen, A. I., and Laine, T., *Skand. Arch. f. Physiol.*, 1938, **80**, 392.

† 25 cc of 20% $(NH_4)_2SO_4$ is shaken with 250 mg norite and filtered.

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Parasympathomimetic Effect of Aqueous Humor in Human Eyes with and Without Chronic Simple Glaucoma.

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Velhagen[1] demonstrated that the aqueous humor of certain animals produced a parasympathomimetic effect on the isolated frog heart. Engelhart[2] one year later instilled eserine in the eyes of rabbits to prevent the destruction

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1 Velhagen, K., *Arch. f. Augenheil.*, 1930, **103**, 424.

2 Engelhart, E., *Arch. f. d. ges. Physiol.*, 1931, **227**, 220.

by cholinesterase of any acetylcholine present, and then exposed the eyes to light for parasympathetic stimulation. Under these conditions he proved the presence of acetylcholine in the aqueous humor. Pletneva, Raeva, and Voronina[3] studied the biological effect of the aqueous humor of human eyes and found that a sympathomimetic substance was present in some and a parasympathomimetic substance in others. They could demonstrate no consistent effect on the isolated frog heart by the aqueous in normal eyes or in any of the pathological ocular conditions they investigated. Apparently they did not use eserine or the standardized exposure to light according to the Engelhart technic.

The purpose of this study was to determine quantitatively the acetylcholine content of the aqueous humor of human eyes prepared according to the method applied by Engelhart to rabbits. Furthermore, since there is clinical evidence to suggest a deficiency of acetylcholine in glaucoma, determinations were made on a series of eyes with chronic simple glaucoma, and on a control group without history or signs of increased ocular tension.

Each eye to be studied was prepared by the instillation of 1 drop of 1% eserine salicylate into the conjunctival sac 3 times at 10-minute intervals beginning 1½ hours before operation. No other eye drops were used for 24 hours preceding the experiment, nor was systemic medication permitted. For 20 minutes to one-half hour before the procedure the bright Hammer lamp of the operating room was focused on the subject eye, with winking permitted. In this way, reflex stimulation of the parasympathetic nerves to the eye was produced. Anesthesia was obtained by retrobulbar injection of 2 cc of 4% novocaine. Preliminary studies on rabbits had shown that such procedure did not produce a demonstrable change in the biological effect of the aqueous humor. The conjunctival instillation of local anesthetic solutions was avoided since their passage into the aqueous might influence the results of the bioassay. Approximately 0.1 cc of primary aqueous humor was obtained from each eye, by paracentesis and aspiration with a hypodermic needle attached to a tuberculin syringe.

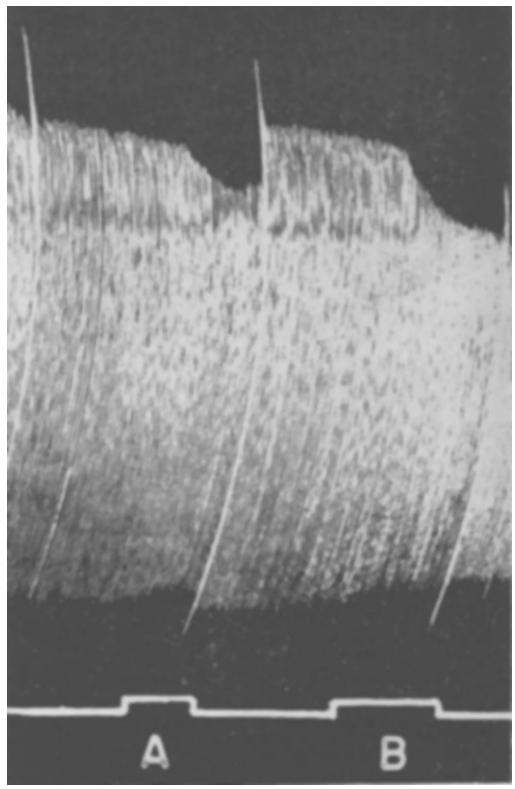

FIG. 1.
Kymographic record of response of frog heart preparation to aqueous humor from non-glaucomatous eye with diabetic cataract. A: Effect of 0.1 cc of undiluted aqueous humor. B: Effect of 0.1 cc of acetylcholine 1:100,000,000 on same preparation.

The effect of each sample was tested within 30 minutes on the freshly prepared isolated heart of the spring or summer frog (*Rana pipiens*) according to the perfusion method of Straub.[4] These preparations were usually found to be sensitive to acetylcholine in dilutions up to 1:1 billion. The aqueous was diluted 1:6 by the perfusion fluid to which it was added. A continuous kymographic record of the heart beat was made during each experiment. For an approximately quantitative estimation, comparisons were made between the effects of the aqueous humor samples

[3] Pletneva, N., Raeva, N., and Voronina, E., *Vestnik Oftal.*, 1938, **13**, 462.

[4] Sollman, T., *Laboratory Guide in Pharmacology*, p. 190, W. B. Saunders Co., Phila., 1917.

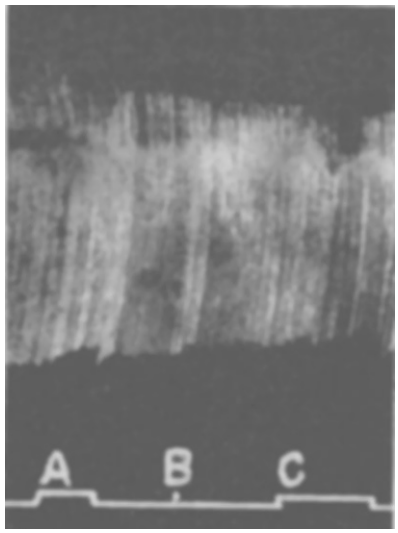

FIG. 2.
Inhibition of parasympathomimetic effect of aqueous humor by atropine sulphate. A: Effect of 0.08 cc of aqueous humor from normal eye. B: Addition of 0.1 cc of 1:10,000 atropine sulphate to perfusion fluid. C: Subsequent effect of 0.08 cc of same sample of aqueous humor as A.

and those of known dilutions of acetylcholine chloride on the same hearts.

Ten eyes with various non-inflammatory pathological conditions, but without glaucoma, and 7 eyes with chronic non-congestive glaucoma, not controlled by miotics, were studied. Two eyes in the latter group had previously undergone iridectomy without apparent effect on the disease. The aqueous humor of the 10 non-glaucomatous eyes produced an inotropic depression of the heart beat in each instance. In 8 of these experiments the intensity of this parasympathomimetic effect was approximately equal to that produced on the same heart by a similar amount of acetylcholine in 1 to 100 million dilution. The record of one experiment of this type is reproduced in Fig. 1. In the remaining 2 assays in this group the depression produced was sufficient to cause diastolic standstill for several seconds followed by resumption of the normal beat. This effect could be reproduced with known concentrations of acetylcholine of over 1 to 1,000,000.

Enough aqueous humor was obtained from 2 of these non-glaucomatous eyes to permit the testing of 2 samples from each. After one-half of the aqueous had been demonstrated to cause a depression in amplitude of the heart beat, 0.1 cc of 1 to 10,000 atropine sulfate solution was added to the cannula and the second half of the aqueous tested. In both cases complete inhibition of the parasympathomimetic effect previously noted occurred. The record of one of these experiments is shown in Fig. 2. Since it is characteristic of the pharmacological effect of acetylcholine that it is inhibited by atropine, this demonstration strongly suggests that the parasympathomimetic agent in the aqueous is similar to or identical with acetylcholine.[5]

The aqueous humor of each of the 7 eyes with chronic simple glaucoma was similarly tested. In no instance did a sample from these eyes produce a detectable parasympathomimetic depression of the cardiac contractions. The effect of the aqueous of an eye with chronic simple glaucoma is reproduced in

FIG. 3.
Kymographic record of response of frog heart preparation to aqueous humor from eye with chronic simple glaucoma. A: Effect of 0.25 cc of acetylcholine 1:100,000,000 to demonstrate sensitivity of preparation. B: Effect of 0.1 cc of aqueous humor from glaucomatous eye. C: Effect of 0.1 cc of acetylcholine 1:100,000,000.

[5] Chang, H. C., and Gaddum, J. H., *J. Physiol.*, 1933, **79**, 255.

Fig. 3. In the bioassay of the aqueous humor from 2 of these eyes, a slight augmentative effect was noted. It thus appears that in contrast to the aqueous of non-glaucomatous eyes, no acetylcholine-like substance is demonstrable in the aqueous humor of eyes with chronic simple glaucoma when tested by this method.

Conclusions. The results are presented of the bioassay of aqueous humor from human eyes prepared according to the method applied by Engelhart to animals. In a series of 10 human eyes without glaucoma, every sample of aqueous humor contained a parasympathomimetic substance which, in the 2 cases so tested, was found to be similar to acetylcholine. In the aqueous humor of 7 human eyes with chronic simple glaucoma, no such parasympathomimetic substance could be demonstrated.

The author wishes to express his appreciation to Dr. Robert K. Lambert for his cooperation, and to Miss Mary E. Carsten for technical assistance in this work.

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Renal Clearance of β-Hydroxybutyric Acid in a Dog.

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Urinary excretion of β-hydroxybutyrate (βB) commonly occurs in ketosis of starvation and diabetes and may be induced experimentally by injections of anterior pituitary extracts or of certain of the ketone bodies and fatty acids. Evidence has been obtained from studies on rats[1] and humans[2] which indicates that excretion of total ketone bodies increases markedly when their concentration in the blood rises above 15 to 20 mg % in humans and above 25 to 30 mg % in rats. However, variable amounts of acetone and acetoacetate are found associated with βB and analyses of total ketone bodies cannot yield conclusive information regarding excretion of βB. Intravenous injection of 0.5 g of βB per kilo per hour into a dog was found necessary to cause an appreciable excretion of βB. At a rate of 0.4 g per kilo per hour only slight amounts of βB were found to have been excreted. None was found in the urine when the rate of infusion was 0.3 g per kilo per hour.[3]

Procedure. The renal clearance of *dl*-βB was investigated in 2 experiments on a trained female dog weighing 13.6 kg. In the first experiment an intravenous infusion of 1.9 cc per minute of a 7.5% solution of sodium *dl*-βB and 1% creatinine was maintained for 45 minutes, following an initial priming injection of 3 g of sodium *dl*-βB and 1.5 g creatinine. Creatinine was infused to permit the measurement of the glomerular filtration rate. About 500 ml of water were given by stomach tube 45 minutes before the infusion was begun. In a second experiment 13 g of sodium *dl*-βB in 25% solution were given in a single initial intravenous dose. Creatinine, 100 mg per kg, was given subcutaneously 30 minutes before collection of urine samples. Diuresis was maintained with water given at 45-minute intervals. Because of the difficulty of obtaining the compound, the time allowed in each experiment for mixing was shortened to 12 minutes. The levels of creatinine and βB were followed in each experiment for about 1.5 hours with urine collections approximately every 10 minutes and blood samples every 15 minutes.

Analyses. The plasma proteins were precipitated by $CdSO_4$.[4] The carbohydrates in

[1] Shipley, R. A., and Long, C. N. H., *Biochem. J.*, 1938, **32**, 2242.

[2] Martin, H. E., and Wick, A. N., *J. Clin. Invest.*, 1943. **22**, 235.

[3] Wilder, R. M., *J. Biol. Chem.*, 1917, **31**, 59.

[4] Fujita, A., and Iwatake, D., *Biochem. Z.*, 1931, **242**, 43.