IN THE LABORATORY

Intravenous Methylene Blue for Studying Fiber Degeneration in the Central Nervous System

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A technique based upon intravenous perfusion of methylene blue into an anaesthetized animal (*1*, *2*) permits a much wider application of the unique property of this dye in selectively staining nervous tissue *intra vitam*, and thus makes possible studies of both the general pattern and the cytological details of neural structure throughout the nervous system. It has been noted (*2*) that the value of this method is enhanced by its demonstration of degenerating fibers, both myelinated and nonmyelinated, in the peripheral nervous system, a feature of intravital methylene blue previously described in detail by Weddell and Glees (*3*), who used the technique of local injection of the dye.

The intravenous method has now been found to give a remarkably clear demonstration of degenerating fibers in

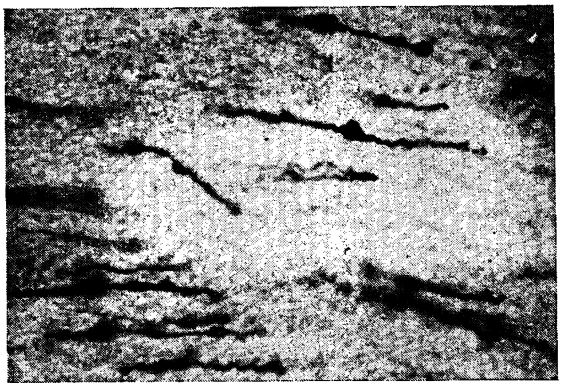

FIG. 1. Degenerating fibers in optic tract (×270).

the central nervous system. In rabbits perfused with methylene blue 5 days after section of one optic nerve, degenerating fibers in the optic pathways stand out distinctly as compared with normal fibers by reason of their more intense and metachromic staining. The contrast, even in macroscopic appearance, between the normal and degenerating optic nerves, is striking. In paraffin sections the degenerating fibers are rendered conspicuous both by their intense and characteristic reddish-purple shade of staining and by the morphological changes indicative of early degeneration, such as fibrillation and vesiculation of the axis cylinder, which they clearly exhibit (Fig. 1).

It is evident from these results that the method has a particular application to the problem of mapping out the fiber degeneration resulting from localized lesions in the central nervous system, and that it is therefore a technique likely to prove of great value in the field of experimental neurology.

The technical details of the method (*2*) have been modified to provide for more critical handling of experimental material from the central nervous system. The use of a more concentrated solution of dye has obviated the necessity of injecting a large volume of fluid which may result in cerebral edema. In addition, more satisfactory fixation has been obtained by taking the tissues through formol-saline after the ammonium molybdate stage. Neither of these modifications interferes with successful staining. Further studies are now being made on a series of rabbits in which section of an optic nerve has been selected as a control lesion, and details of these studies will be published elsewhere.

References

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3. WEDDELL, G., and GLEES, P. *J. Anat.*, 1941, **76**, 65.

A New Series of Reagents for the Colorimetric Determination of Steroids[1]

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In an investigation of the factors involved in the antimony trichloride reaction for certain ketosteroids described by Pincus (*1*), a new series of colorimetric reactions involving the use of antimony trichloride, bismuth trichloride, and aluminum chloride has been discovered. Using the Friedel-Crafts ketone synthesis as an analogy for combining catalysts and acyl chlorides, as well as other reactants, a remarkably specific and waried series of reagents has been prepared. Although only a few of the reagents have been developed far enough to give quantitative color responses following the Beer-Lambert law, it seems desirable to report the results obtained thus far.

[1] The steroids used in this study were generously provided by Ciba Pharmaceutical Products, Inc., through the courtesy of E. Oppenheimer.

Since we had observed qualitative differences in the colors developed by steroid extracts obtained from different individuals, we were especially eager to standardize the procedure in order to evaluate these individual differences. Furthermore, it was felt that a series of reagents which were more or less specific for certain steroids would give additional valuable information. These views led to the discovery of the reagents and conditions reported here. Conditions were maintained for study of only those reactions which were, theoretically at least, capable of practical application.

In general, the reagents are prepared by combining the anhydrous metallic chloride and the "solvent" in such a way as to give a maximum concentration of the metal chloride. The ratio between the metal and the solvent in a given reagent mixture is a critical factor in determining the course of the color reaction.

The conditions for the initial reaction of the reagent with the steroid vary from 20 min at 0° C for $BiCl_3$-acetyl chloride to 60 min at 100° C for $SbCl_3$-phenol. The speed of the reaction for a given reagent can generally be judged by the colors developed in the reaction mixture, although occasionally a given steroid may show little or no evidence of reaction until the mixture is diluted. About 0.2 ml of reagent is allowed to react with 100 μg (0.10 mg) of steroid. Following the initial reaction the tube is cooled to room temperature, if necessary, and diluted with 10 ml of an appropriate solvent. The colors developed depend upon the dilution solvent as well as the reagent used. It has been found that the most generally useful diluent is a mixture of 10% acetic anhydride (less in some cases) in anhydrous benzene; this produces colors which are remarkably stable, some lasting without appreciable change for several days.

The absorption spectra obtained for androsterone and dehydroisoandrosterone under the conditions described by Pincus[2] are shown in Fig. 1. The peak at 550 mμ, which is high immediately after dilution with 95% acetic acid, gradually drops as the color changes from reddish blue to blue over a period of 60–90 min. The colors shown in Fig. 1 were obtained 90 min after dilution. The peak at 550 mμ was not found by Pincus, probably because of the wide band width (30 mμ) of the spectrophotometer he used. The spectra shown here were obtained with the Beckman spectrophotometer, using a 1-cm Corex cell and a slit width of 0.04 mm. Factors which apparently influence the absorption at 550 mμ and 610 mμ are the amounts of water and acetic anhydride in the $SbCl_3$ solution. For example, if $SbCl_3$ dissolved in 95% acetic acid is used as the reagent, the absorption obtained with androsterone at 550 mμ drops rapidly, while the peak at 610 mμ reaches the same value as that shown in Fig. 1. If the $SbCl_3$ is dissolved in acetic anhydride, *no color is produced by androsterone.* This color-inhibiting effect of acetic anhydride solutions of $SbCl_3$ is further illustrated by the finding that androsterone gives only a light blue color when *anhydrous* (redistilled) $SbCl_3$ dissolved in the acetic anhydride-acetic acid solvent recommended by Pincus is used as the reagent. It would therefore appear that a more reproducible reagent can be prepared by

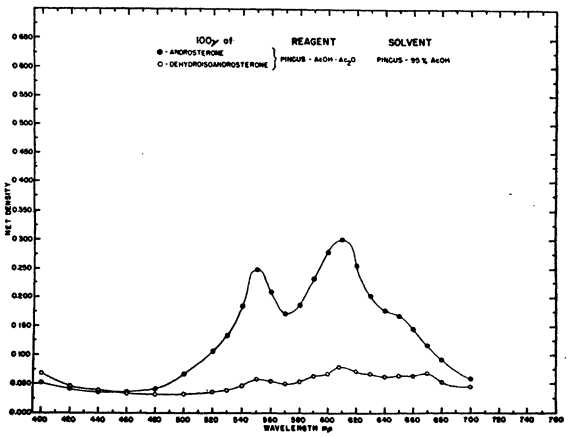

FIG. 1

using components of known water content rather than relying upon a certain amount of acetic anhydride to destroy variable amounts of water in the $SbCl_3$ being used.

These and other factors explain why some batches of $SbCl_3$ reagents fail to give colors following the Beer-Lambert law. To obtain further information concerning the relative importance of the different variables,

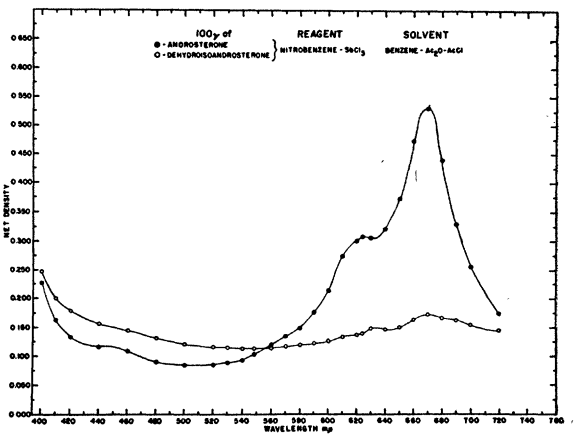

FIG. 2

many other conditions have been tested, and some of these results are summarized below.

Since nitrobenzene is an excellent solvent for $SbCl_3$ and, in fact, probably forms an oxonium complex with it, a reagent containing 26 gm of $SbCl_3$ in 5 ml of nitrobenzene was heated with steroid samples for 40 min at

[2] Thirty-eight gm of $SbCl_3$ was dissolved in 10 ml of a mixture of 9 parts of glacial acetic acid and 1 part of acetic anhydride. The antimony trichloride was used, as received from a freshly opened glass-stoppered jar (Coleman and Bell Company, Norwood, Ohio).

100° C. Upon dilution with benzene, containing 0.025% acetyl chloride and 0.025% acetic anhydride, an intense blue color is obtained with androsterone, while a light green color is given by dehydroisoandrosterone. The absorption spectra, shown in Fig. 2, illustrates the fact

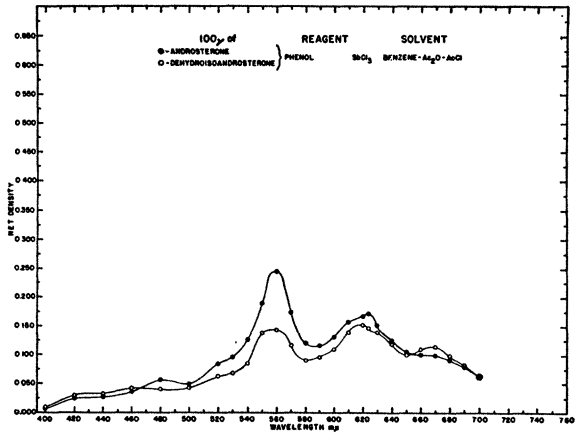

FIG. 3

that the color is extremely intense and that the peak has been shifted to 670 mμ. The colors obtained were stable for several days.

The use of a solution of $SbCl_3$ (52.1 gm) in phenol (10.2 gm) produces colors which have nearly equal intensity for androsterone and dehydroisoandrosterone (Fig. 3). The reddish blue colors produced upon dilution gradually become more blue and, again, are stable for

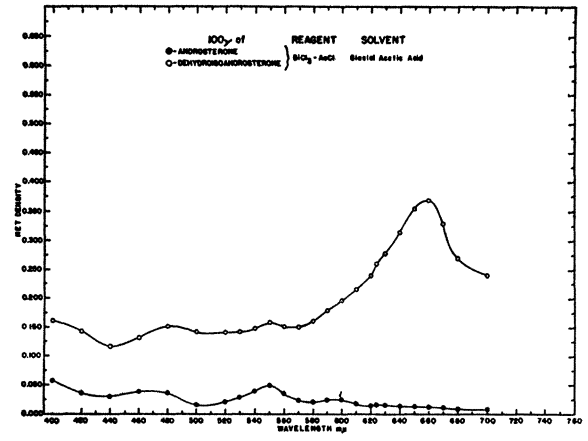

FIG. 4

long periods. If the acetyl chloride is not added to the benzene-acetic anhydride diluent, the colors are blue immediately upon dilution and dehydroisoandrosterone gives about 80% of the color of androsterone at the 624-mμ peak. Similar results are obtained using 95% acetic acid as diluent, but the colors fade more rapidly.

When $SbCl_3$ is dissolved in acetic anhydride, no color is produced with androsterone, as mentioned above, but an *intense blue color is obtained with dehydroisoandrosterone.* A similar effect is obtained using a solution of $BiCl_3$ in acetyl chloride, the spectra for which are shown in Fig. 4. This reaction shows a high degree of specificity and may prove valuable in measuring this steroid in certain pathological conditions.

A solution of $SbCl_3$ in succinic anhydride, using a 10% solution of acetic anhydride in benzene as the diluent, produces a deep blue bolor with androsterone, a very light blue color with dehydroisoandrosterone, a *blue color with a strong red fluorescence with testosterone,* a *pink color with a green fluorescence (like eosin) with* α*-estradiol,* and no color with Δ^4-androstenedione. Similar results are obtained using phthalic anhydride as the solvent for the $SbCl_3$.

A single reagent prepared by heating 10 gm of anhydrous aluminum trichloride with 10 ml of benzoyl chloride and dissolving the product in 40 ml of nitrobenzene illustrates a further extension of these reactions. In this case, the only steroid out of 15 tested which gave a color was Δ^4-androstenedione-3, 17, which produced an intense red solution (maximum at 570 mμ) when diluted with glacial acetic acid.

It seems plausible to expect that some of the other Friedel-Craft catalysts, such as $SbCl_5$, $SnCl_4$, $TiCl_4$, $TeCl_2$, $TeCl_4$, $ZnCl_2$, and $CbCl_5$, will give new color reactions having other degrees of specificity and intensity when combined with various solvents such as the acyl halides and anhydrides, organic acids, nitrobenzene, and phenols. As yet, the mechanism of the reaction is not understood. However, the fact that some of the colored products can be adsorbed on aluminum oxide, while others, particularly those obtained with $BiCl_3$ tend to precipitate on standing for 24 hrs, may facilitate isolation and identification of the colored end-products. The insolubility of the colored substance obtained with bismuth indicates that the colored product may be a metal complex with the steroid, since bismuth compounds are generally less soluble than antimony compounds.

Preliminary results indicate that at least some of the reagents mentioned can be applied to the measurement of steroid mixtures such as are obtained in the ketosteroid extracts of urine. It has been found that the colors can be destroyed by adding a small amount of moist U.S.P. ether to the colored solution, thus making possible the determination of the "blank" colors of the extract itself. If turbidity develops, the solution may be cleared by adding a drop of concentrated hydrochloric acid.

Further work, now in progress in this laboratory, includes testing the qualitative properties of various other reagents, refining those of promise, and applying the methods to the determination of steroids in urine extracts. The results will be published in detail at a later date.

Reference

1. PINCUS, G. *Endocrinology,* 1943, **32,** 176–184.