THE NORMAL PIGMENT OF THE URINE.

IV. PRELIMINARY STUDY OF THE PROPERTIES OF THE PIGMENT OBTAINED BY THE NEW METHOD OF BUTYL ALCOHOL EXTRACTION.*

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In the preceding paper (1) a new method of preparing the normal pigment from urine has been described. It consisted essentially of extracting the pigment from acidified urine (pH about 4.0) with n-butyl alcohol, washing freely at different stages in the procedure with water, chloroform, benzo1, amyl acetate, and amyl alcohol and finally obtaining the pigment product in a dry form by washing with anhydrous absolute alcohol and anhydrous ether.

The purpose of this communication is to describe briefly a number of preliminary studies which indicate that the normal urinary pigment has not been altered significantly during the course of preparation and that it has been prepared in probably a higher state of purity than hitherto. Some further evidence also has been adduced to show that the pigment is a chemical entity and not a mixture of substances.

EXPERIMENTAL.

With the new method, the pigment was obtained in the form of a dry, brown powder, which no longer had the odor of urine. Microscopically, the freshly prepared precipitate of the urinary pigment in a drop of the mother liquor was usually refractile in character, and in a number of preparations, the pigment particles looked like very small needles and could be called semicrystalline.

* A preliminary report of part of this work has appeared in the Proceedings of the American Society of Biological Chemists (Drabkin, D. L., J. Biol. Chem., 74, p. xv (1927)).
Extraneous pigments, such as carotin, bile pigments, urobilin, and hematoporphyrin, could not be demonstrated spectroscopically in solutions of the purified pigment. The absence of uric acid was indicated by negative murexide tests upon the dried preparations. The method of preparation—extraction with butyl alcohol at room temperature and copious washing with water—precluded the presence of any except possibly minute traces of urea. The ammonia production, if any, from the action of urease upon 0.2 gm. of purified pigment, dissolved in water and digested at pH 6.8 (adjusted by buffer), could not be detected.

The physical properties of the pigment were: (a) The solubilities of the freshly prepared pigment were found to be practically identical with those of the pigment prepared by Garrod (2). It was very soluble in cold and hot water, reproducing the original color of the urine in these solutions. (The tint of aqueous solutions varied from golden yellow to reddish brown, depending on the concentration.) The pigment preparation was also very soluble in dilute ethyl alcohol, though much less readily soluble in commercial "absolute" alcohol. It was relatively insoluble in anhydrous alcohol and insoluble in anhydrous ether, chloroform, acetone, and benzene. The pigment was somewhat soluble in alcohol-ether and alcohol-chloroform mixtures. On standing 1 year over sulfuric acid in a vacuum desiccator, the pigment became relatively insoluble in cold water, although it was still fairly soluble in hot. This change in solubility was not accompanied by any change in physical appearance.

(b) Aqueous and alcoholic (ethyl and butyl alcohol) solutions of the pigment exhibited the absorption spectrum characteristics of a neutral filter, showing a uniform absorption in the violet and ultra-violet regions. In solutions in which the intensity of color was about two times that of normal urine, absorption began at 4560Å. and was strong at 4250Å. (violet region of spectrum). In more concentrated solutions there was slight absorption of light in the blue-green (beginning at 5430Å.), with definite absorption at 5000Å. Except for a displacement of the absorption about 50Å. towards the violet in the case of butyl alcohol solutions there was no difference between the spectral characteristics of the pigment in the above solvent and (in approximately similar concentration) in ethyl alcohol. During various stages of purification no change
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in spectrum was observed, definite absorption in dilute solutions still beginning at about 4250Å. Had urobilin been present in these solutions it would have been recognized by its absorption band (approximately 5000 to 4900Å). Findings in close agreement with the above were also obtained photographically, with a quartz spectrograph.

Moderately concentrated solutions of the pigment exhibited the property of fluorescence between 3680 and 3122Å. This phenomenon was brought out by a Wratten 18 filter and a mercury arc lamp. Although the characteristic 2-banded spectrum of urobilin did not appear, an aqueous solution of urochrome, illuminated by a Welsbach lamp, fluoresced strongly after treatment with NH₂OH and ZnCl₂ (followed by filtration). A strongly fluorescent solution (absorbing light definitely from 4120Å on) was also obtained after heating an aqueous solution with glacial acetic and iodic acids. The urinary pigment, however, was probably destroyed by this treatment.

(c) The pigment in aqueous solution did not dialyze through parchment, either into water or, when acidified, into butyl alcohol.

(d) A thoroughly washed, concentrated butyl alcohol solution of the pigment was subjected to "chromatographic analysis" by Tswett's method (3). In this method, when a solution of several pigments is slowly washed through a column of adsorbent such as anhydrous calcium carbonate, the pigments, due to their relative affinities for the adsorbent, are approximately separated in layers. This type of analysis indicated that the pigment preparation is not a mixture of substances. In control tests, bilirubin and carotin,¹ when mixed with the urinary pigment, were readily separated by this technique.

The chemical properties of the pigment were:

(a) The biuret reaction upon nearly colorless solutions of the purified pigment was negative. A very faint, unsatisfactory Adamkiewicz reaction was obtained. The Millon test, on the other hand, was consistently fairly strongly positive.

(b) The pigment from a washed butyl alcohol extract was transferred to water at approximately pH 9. From this solution the following metals were found to precipitate the pigment: Ag(NO₃⁻⁻

¹ Very kindly supplied by Professor Frank P. Underhill of Yale.
and \( \text{SO}_4^{=2} \), \( \text{Ba}(\text{Cl}^-) \), \( \text{Cu}(\text{SO}_4^{=}) \), \( \text{Fe}(\text{SO}_4^{=} \text{ and Cl}^-) \), \( \text{Hg}(\text{NO}_3^- \text{ and Cl}^-) \), \( \text{Pb}(\text{CH}_3\text{COO}^-) \), and \( \text{Zn}(\text{Cl}^-) \). The Cu, Fe, Hg, and Zn "salts" of urochrome were soluble in HCl, while the Ag precipitate was not. As originally found by Dombrowski (4) in the case of his copper-urochrome preparation, the Ag and Cu "salts" were soluble in \( \text{NH}_4\text{OH} \), the former producing a Burgundy red solution, the latter a blue-green. In two instances, with the compounds of Cu and Fe, refractile crystalline-like products were seen under the microscope, after careful evaporation of their ammoniacal and acid solutions.

(c) The statement in the preliminary report that the urinary pigment, prepared by the new method, was not precipitated by alkaloidal reagents must be modified to include a more extended experience. The above statement was based upon observations made upon an aqueous pigment solution, freshly transferred from butyl alcohol at pH 6.8. Under these circumstances, the urinary pigment was not precipitated by the addition of phosphotungstic, trichloroacetic, chromic, or picric acids.

The writer has recently found that the reaction of the solution must be very carefully adjusted, because urochrome is precipitated by phosphotungstic and silicotungstic acids only in a narrow range of pH. No precipitation took place when the solution was too alkaline or too acid. The optimum pH was found to be 3.8 and was insured by the use of a buffer solution. To a buffer-adjusted solution containing a weighed quantity of purified pigment 10 per cent silicotungstic acid was gradually added until the appearance of turbidity. Upon standing overnight in the refrigerator, the solution was appreciably decolorized with the formation of a dark brown precipitate, which was beautifully crystalline under the microscope. The crystals were quadrangular, flat plates, or prisms, their color presumably varying with thickness from a pale yellow-pink to a red-brown. Thus far, however, all preparations have contained some crystals which appeared to be practically colorless.

(d) Dombrowski (4) was the first to show that urochrome possesses reducing properties. That the pigment prepared by the new method is a mild reducing agent was suggested by the appearance of a blue color upon the addition of a solution of the pigment acidified with glacial acetic acid to a dilute solution of ferric chlo-
ride plus a dilute solution of potassium ferricyanide. Iodic acid, on the other hand, was not reduced, nor were other tests for reduction positive.

(e) An interesting, new observation was the complete decolorization of the pigment solution by heating with zinc dust and HCl, although whole urine could not be rendered completely colorless by this means. The aqueous pigment solution was also appreciably but not totally decolorized by treatment with sodium hydrosulfite. Bubbling a stream of hydrogen through the pigment solution produced no changes, nor was hydrogen rendered effective by the presence of platinized asbestos. Thus, decolorization was produced only with very powerful reductants.

Upon standing exposed to the air, the decolorized solution very gradually took on color. In the presence of hydrogen peroxide the restoration of color was very rapid. It should be noted, however, that, while the intensity of the restored color was equal to the original, the tint was appreciably changed—the new color being pinkish. The decolorization and restoration of pigment could be repeated a number of times upon the same solution, although there was evidence of loss of pigment during the process.

(f) Contrary to Dombrowski's findings (4), the non-specific sodium nitroprusside reaction (for the SH group) was consistently negative, both before and after the reduction of the pigment with zinc and hydrochloric acid. Although a faint, pinkish color was sometimes produced after the addition of the nitroferricyanide reagent, fading of color was not observed after the addition of glacial acetic acid. In only one instance out of a great many was a good test for soluble sulfides obtained (by precipitation as PbS), after the incineration of the pigment with metallic sodium.

Due to the unreliable nature of the above tests, the S was determined quantitatively by Osborne's (5) peroxide fusion method. In a single determination, 0.5 gm. of urinary pigment was found to contain 0.0032 gm. of sulfur (or 0.64 per cent). An equal quantity of high quality casein, run as a control, contained 0.0043 gm. of sulfur (or 0.86 per cent). No correction was made for the sulfur impurities in the reagents, the correction being negligible since only comparative values were sought.

The pigments of dog and rat urine could be extracted by n-butyl alcohol and maximum extraction took place at practically
the same pH (3.9) as in the case of human urine (1). With change in hydrogen ion concentration, these pigments also could be transferred from butyl alcohol to water and back.

It was of interest to evaluate provisionally the empirical standard which the writer had used in his earlier studies (6) in terms of mg. of purified pigment. A dilute alkaline solution of a weighed quantity of pigment was compared colorimetrically against the alizarin-aniline orange standard. 1 unit of pigment was found to be equivalent to 3.82 mg. of pigment. An average pigment output for an adult male was calculated to be 73 mg. (from 19.0 units) per 24 hours. On the same basis the output per square meter of surface area was calculated to be 42 mg. (from 11.0 units). Dombrowski's (4) figures for the daily excretion of urochrome are 6 to 10 times the above.

SUMMARY.

A preliminary study of the physical and chemical properties of the normal urinary pigment, prepared by butyl alcohol extraction, has indicated that the coloring matter of urine has been obtained relatively unchanged and free from most adventitious contaminants.

BIBLIOGRAPHY.

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