10755

Further Observations on the Red Pigments of Pellagra Urines.

C. J. WATSON.

From the Division of Internal Medicine, University of Minnesota Hospitals, Minneapolis, Minn.

In an earlier study[1] it was noted that the amount of urinary coproporphyrin in 4 cases of alcoholic pellagra was not correlated with the Beckh-Ellinger-Spies test.[2] The latter investigators had found this test positive in many cases of pellagra and they believed that it indicated the presence of porphyrinuria. The test was employed by them in a quantitative as well as qualitative manner, and, unfortunately, has been recommended by others[3] as a quantitative procedure for urinary porphyrin. In recent reports Spies[4, 5] has stated that the test revealed the presence of porphyrin or porphyrin-like substances. In the writer's previous communication red pigments were described, differing markedly from porphyrins, particularly in their ready solubility in toluene and the difficulty with which they were extracted from organic solvents by hydrochloric acid. The Beckh-Ellinger-Spies (B.E.S.) test, however, was not correlated with the presence of red pigment in the toluene preservative. Subsequent study of the urines of additional cases of pellagra has shown that one or both of 2 red pigments may be present in varying amount. The first of these occurs as a chromogen and changes into a pink or red pigment upon the addition of hydrochloric acid to the urine. Particular attention has been given recently to the study of this chromogen in the urines of 2 cases of endemic pellagra. Two 24-hour samples were available from each of these cases.* The urine samples were sent to the writer from the

1 Watson, C. J., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 514.

2 Beckh, W., Ellinger, P., and Spies, T. D., *Quart. J. Med.*, 1937, **30**, 305.

3 Bray, W. E., *Synopsis of Clinical Laboratory Methods*, C. V. Mosby Company, St. Louis, 1938, p. 47.

4 Spies, T. D., Aring, C. O., Gelperin, J., and Bean, W. B., *Am. J. Med. Sc.*, 1938, **196**, 461.

5 Vilter, R. W., Vilter, S. P., and Spies, T. D., *Am. J. Med. Sc.*, 1939, **197**, 322.

* A small portion was removed from each sample, for the purpose of porphyrin and flavin determinations, in Birmingham. According to the note received with the urine specimens these patients were complaining of sore tongue, cracked lips, and abdominal distress of a burning type. They were evidently classified as pellagra cases at the time of shipment of the urines. In a subsequent communication, however, Dr. Spies stated his belief that both patients were suffering from riboflavin deficiency. They had not received nicotinic acid since last year at which time they were treated for pellagra.

Hillman Hospital in Birmingham, Alabama, through the courtesy of Dr. Spies. It had been noted in Birmingham that the B.E.S. test was positive in each urine. This was likewise true in Minneapolis. The substance responsible for the red color in this test, however, was not porphyrin in any of the 4 urines. The amount of coproporphyrin in each 24-hour sample was estimated fluorimetrically with the stufenphotometer. The values were unusually low: Case 1, 1st 24 hours: trace, too small to estimate; second 24 hours, 17.8γ; Case 2, 1st 24 hours: trace, too small to estimate; 2nd 24 hours, 14.1γ. It should be emphasized again that all of the porphyrin (as determined by red fluorescence in ultraviolet light), was extracted by 5% HCl from the primary ether extract of the urine. The subsequent 25% HCl, containing the red pigment responsible for the positive B.E.S. tests, failed to show any trace of red fluorescence. Further study has revealed that this red pigment exhibits the various characteristics of urorosein, first described by Nencki and Sieber.[6] The chromogen of this substance was identified by Herter[7] as indolacetic acid. Nencki and Sieber had noted that this chromogen changes rapidly to urorosein if concentrated hydrochloric or sulphuric acid is added to the urine. The substance can then be concentrated readily in amyl alcohol by shaking with a small amount of this solvent. The alcohol assumes a rose red color and shows a fairly well defined absorption band at 552-559 mγ (max. 555-7). It should be noted that the spectroscopic absorption of urorosein in 25% HCl, as obtained in the B.E.S. test, is characterized by 2 weak bands: I 544, II 511.† The first of these is somewhat more intense, but both are relatively weak, broad and diffuse. These characteristics were noted in each of 4 urine samples received from the Birmingham cases. It was further noted that the chromogen, after extraction from the urine with ether and subsequent removal of the ether by evaporation on the water bath, gave the Salkowski reaction for indolacetic acid, as stressed by Herter.[7]

After preliminary extraction of the urine with ether, as in the B.E.S. test, the Nencki-Sieber test for urorosein on the extracted

[6] Nencki, M., and Sieber, M., *J. f. Prakt. Chem.* (N.F.), 1882, **26**, 333.

[7] Herter, C. A., *J. Biol. Chem.*, 1908, **4**, 253.

† Crystalline indolacetic acid has been found to exhibit the same color reactions with identical absorption spectra. This is true, however, only if a few drops of a dilute potassium nitrite solution have been added to the aqueous (or urine) solution prior to carrying out the B.E.S. or Nencki-Sieber tests. This supports Herter's opinion[7] that the presence of nitrite in the urine is important to the development of the urorosein reaction.

urine, was negative. If the Nencki-Sieber test was first carried out, and the rose colored amyl alcohol, showing absorption at 556 mγ, was then separated, mixed with several volumes of ether and shaken with a saturated aqueous solution of sodium acetate, the pink color disappeared entirely. It was quickly regenerated by shaking with 2-3 cc of 25% HCl, as in the B.E.S. test.

Although the Nencki-Sieber test was not carried out in urine samples obtained from earlier cases of pellagra,[1] the entirely similar behaviour of the red substance in the 25% HCl of the B.E.S. tests leaves little doubt that it was the same which has been described above.

In none of the urines from the Birmingham cases has the toluene preservative so far become red or pink (toluene was added to a portion of each sample after arrival in Minneapolis). This is further indication that the red pigment previously noted in toluene preservatives differs from the substance giving the B.E.S. test. Some reference was made to this difference in the writer's earlier communication.[1] The possibility was considered then that the pigment going into toluene was a derivative of the B.E.S. pigment, although no direct evidence was obtained in favor of this concept. The similarity of the toluene soluble pigment to indirubin, or indigo red, was mentioned in the earlier report.[1] Further study has strengthened the belief that this pigment is indirubin, or a very closely related substance. It has now been observed in the urines of 7 additional cases, 3 of whom were pellagrins. One case of mild pellagra was, in fact, first recognized after the red toluene preservative had been noted. This patient was on the urological service receiving treatment for prostatic hypertrophy with pyonephrosis. He was 79 years of age and was found to be suffering as well from chronic fibroid pulmonary tuberculosis. The urine in this case was one of a number collected from various patients in a search for red pigments extracted by the toluene preservative. Urine samples from 30 patients have been observed in this respect for a considerable period of time. Red pigment in the toluene has been noted after from 2-10 days in 5 of these; 2 of these had extensive pulmonary and intestinal tuberculosis, one had Pott's disease with a large paravertebral abscess, and one had carcinoma of the prostate, suprapubic cystotomy and infected urine. The diagnosis in the fifth case is still obscure; this individual, a man of 60, was malnourished and had been on a deficient diet for over a year; hypochromic anemia, a mild hemorrhagic tendency, mild diarrhea and vomiting were present.

No organic lesion has been discovered. The urine in this case has repeatedly exhibited the red pigment in toluene; most interesting was the observation that the fresh urine at times gave strong B.E.S. and Nencki-Sieber tests for urorosein and simultaneously with the disappearance of these reactions the toluene gradually became pinkish red. A similar sequence of events was reported previously for a pellagra urine obtained from Baltimore.[1] In the present study the red pigment was also observed in the toluene preservative of urines from 2 cases of endemic pellagra.‡ In one of these the amount was relatively large permitting isolation of the substance in crystalline form. The characteristics of the pigment extracted by toluene in all of these cases corresponded closely with those described by Rosin in an extensive study of indirubin or indigo red.[8] As noted previously the substance crystallizes in the form of long narrow prisms of dark crimson color. The crystals sublime above 300°C in accordance with Rosin's description of indirubin. The lower sublimation temperature noted previously[1] may have been related to impurity. In contradistinction to urorosein, this substance goes into ether with a red or pink color, and cannot be removed in appreciable amounts by 25% HCl or 10% sodium hydroxide. The spectroscopic absorption is much less characteristic than in the case of urorosein; broad, diffuse, but relatively weak absorption is noted in the yellow and green, maximum from 530 to 550 mγ. In the Nencki-Sieber test, indirubin goes into the amyl alcohol but colors it a darker red, and the characteristic urorosein absorption band at 556 is not seen. Reduction to "white" indirubin by means of glucose and gentle heating, in an alcoholic solution, is readily effected; if the test tube containing this solution is shaken vigorously the upper portion coming in contact with the air again assumes a red violet color which fades when the shaking is discontinued ("Küpenreaktion").

The question of relationship of the 2 red pigments to one another cannot be answered at the present time. Assuming that they are urorosein and indirubin, as indicated in the foregoing, it is probable that a close relationship exists. This is suggested by their common origin from tryptophane, their chemical structure,[9] and the repeated observation that as the urorosein reaction disappears the indirubin

‡ The writer is indebted to Drs. J. H. Musser and Alden Graves of the Charity Hospital, New Orleans, for their cooperation in arranging for the transmission of these urines to Minneapolis.

8 Rosin, H., *Virch. Arch.*, 1891, **123**, 519.

9 Dalmer, O., in Oppenheimer's Hand. d. Biochem. des Menschen u. d. Tiere. II. Aufl. Bd. I, pp. 257 and 261, 1924, G. Fischer, Jena.

color appears in the toluene preservative. Further investigation of this question and also of the question of relationship of vitamin deficiency to the appearance of these substances in the urine, is in progress. It seems noteworthy that both urorosein and indirubin have been encountered in a variety of diseases such as cancer, tuberculosis, and diabetes,[6, 7, 8] all of which, however, are frequently associated with various deficiency states. Nencki and Sieber[6] first encountered urorosein in the urine of a diabetic patient; it is therefore of interest that Spies and his associates[5] have recently suggested correlation between cozymase deficiency in diabetic patients, and the presence of a positive B.E.S. test.

Conclusions. 1. The color noted in positive Beckh-Ellinger-Spies tests is not due to porphyrin. While a marked increase in urinary porphyrin, such as occurs in porphyria, would be productive of color, the color reaction as observed in pellagra and other diseases, is due to urorosein, first described by Nencki and Sieber. There is no evidence that the color reaction is due to any bile pigment derivative. 2. The urines of pellagra patients may contain either the chromogen of urorosein, or a red pigment extracted by the toluene preservative. It appears highly probable that this pigment is indirubin, although exact identification has not yet been made. 3. Both of the red substances may be noted in the urines of patients not having clinical pellagra. Further investigation is necessary to decide whether their appearance is related to deficiency of nicotinic acid or other essential substances.

10756 P

Metabolism of Two Di-deuterobutyric Acids as Indicated by Deuterium Content of Excreted Beta-Hydroxybutyric Acid.

MARGARET MOREHOUSE.* (Introduced by H. J. Deuel, Jr.)

From the Department of Biochemistry, School of Medicine, University of Southern California, Los Angeles.

In order to obtain direct information as to what portions of the fatty acid molecule are convertible to the acetone bodies, a study has been made of the metabolism of the di-deuterobutyric acids. In the first series of tests alpha-beta and beta-gamma deuterobutyric

* Aided by a grant of the Rockefeller Foundation.