XCI. THE NATURE OF THE "ETHER REACTION" OF URINE.

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(Received April 22nd, 1931.)

OVER forty years ago Plosz [1890] showed that when urine is strongly acidified with acetic acid and shaken with ether, chloroform or amyl alcohol, the mixture separates into two layers and on the boundary between the two a precipitate of protein is formed. He remarks: "The reaction is given by all urines, since all urines contain protein. The delicacy of the reaction is thereby shown¹. The protein so obtained is not a homogeneous substance since only a portion of it is soluble in acetic acid. Another portion is soluble in water, alkalis, and strongly concentrated solutions of common salt. The fraction insoluble in acetic acid dissolves, after being washed with acetic acid, in dilute alkali, and is again precipitated on addition of acetic acid. It behaves like mucin. Normal urine always contains protein and a body behaving in its reactions like mucin."

Two years later Smith [1892], who evidently had not seen Plosz's paper, described a reaction of urine with ether in which the acidified urine was shaken with ether, and a thick scum was formed as the layers separated. He stated that the scum was most abundant in the urine of those who had a good appetite and good digestion and was usually absent after long fasting or when the diet was greatly restricted.

Oriel and Barber [1930] have found that when acidified urine is shaken with one-fifth of its volume of ether the mixture separates into two layers with a scum between, which varies in extent and consistency according to the health of the person from whom the urine is derived. When the aqueous layer is discarded and alcohol is added to the ether and scum a precipitate is obtained which gives the biuret, Millon, Adamkiewicz and Molisch reactions. They state that the nitrogen content of the precipitate is also variable and in allergic conditions may rise from a few mg. to as much as 300 mg. daily with the onset of an attack, subsiding to its original value as the attack passes. The chief interest of Oriel and Barber's observations, however, lies in their discovery that the precipitate obtained from urine passed during an attack, contains some of the antigen responsible for the attack as shown by skin reactions and by reproduction of the attack when a solution of the precipitate is injected intradermally into the patient from whom it has been derived.

¹ That is, because the boiling test is negative in normal urines.

It appeared to be desirable to investigate the nature of this ether-alcohol precipitate more closely.

Isolation of the crude material.

The material was obtained by treating 500 cc. of urine with 5 cc. of 25 % sulphuric acid, adding 100 cc. of ether, shaking the mixture vigorously in a stoppered separating funnel for a minute and allowing the ether to separate for 15 minutes. The aqueous layer was then discarded and 100 cc. of 93 % alcohol were added to the ether and the scum. The precipitate was allowed to settle and was separated by centrifuging, washed with alcohol and with ether and dried in a desiccator or at 100° as required.

The material could also be obtained by adding to the urine 3 vols. of 93 % alcohol, and collecting the precipitate. The proportion of salts present was, however, much higher in this case.

The quantity of material obtained by means of the ether reaction as described above appeared to be higher in the case of urines from allergic patients than in the case of urines from apparently normal subjects. Thus an asthmatic in an attack yielded 1.067 g. per litre containing 0.047 g. N, whereas men employed on indoor work and apparently normal yielded 0.165 g. per litre, containing 0.004 g. N. Only a few allergic urines, however, have been examined quantitatively in this way, because Oriel and Barber have provided abundant data and also because the values so obtained are liable to certain errors. As will be shown the material consists largely of salts precipitated by the alcohol from the urine entangled in the ether, and some nitrogenous substances are similarly precipitated or adsorbed, which have no direct relation to the ether test. Further, only after being shaken several times with fresh quantities of ether does the urine cease to give the typical honeycomb layer. The vigour of the shaking also materially affects the degree of emulsification, and so the quantity of material obtained by a single treatment.

Ash content of the crude material.

The ash content of the precipitate (determined by ignition at a moderate temperature, moistening the ash with dilute sulphuric acid and then strong ignition) varied from 70 to 87 % of the material which had been dried at 100°. It consisted, as might be expected, of sodium sulphate with about 6 % of calcium sulphate and sometimes a little phosphate.

Fraction of the crude material soluble in cold water.

The greater part of the crude material was soluble in water and the solution obtained, using only a small excess of cold water above that required to dissolve the salts, gave only a moderate ether reaction. The p_H of this solution was about 3. The solution did not give the protein reactions distinctly but gave Molisch's reaction which was probably due to a trace of glucose in this

case. Evidently the material responsible for the ether reaction is sparingly soluble in concentrated salt solution at p_H 3.

Of the crude dry material from urine passed during a severe attack of asthma 89 % was soluble under these conditions. The crude material had 4.5 % N, of which 3.2 % was soluble, including 0.4 % of ammonia-N (these figures being all percentages of the crude material). The soluble portion contained practically all the inorganic matter and some organic matter. No appreciable precipitate was obtained on saturating the solution with ammonium sulphate and boiling. As the substance responsible for the ether reaction was almost entirely in the insoluble fraction the soluble material was not examined further.

The protein fraction.

The residue obtained on washing the crude material free from salts with cold water will be referred to as the "protein fraction" because it consisted almost entirely of protein or related substance. It was soluble to some extent in cold water, even after acidification, and could not therefore readily be obtained free from salts by shaking the ether with acidified water after removal of the urine. In its moist finely divided state it dissolved instantly in very dilute alkali to give an almost clear, slightly brown solution. When dried at 100° for some time it became insoluble in dilute ammonia. Addition of acetic acid to the filtered alkaline solution produced slight opalescence but no precipitate in the cold. On boiling the acidified solution a little coagulated protein was precipitated. When this had been removed the filtrate, although giving all the colour reactions of proteins, did not give a precipitate with potassium ferrocyanide. This applies both to material from asthmatic and that from non-asthmatic urine.

Half saturation of the acidified solution (freed from coagulable protein) with ammonium sulphate gave only a trace of precipitate in the cold even after 12 hours, but, on boiling, a flocculent precipitate was obtained and the filtrate from this, both in the case of asthmatic and normal urine was found to be free from protein. The precipitate was soluble in hot water and did not come down again on cooling.

When the acetic acid solution (freed from coagulable protein) was saturated completely with ammonium sulphate a precipitate was obtained in the cold. This was removed by centrifuging, dissolved in water, dialysed until free from sulphate and reprecipitated by addition of 3 vols. of alcohol. The precipitate was separated, washed with alcohol and ether and dried at 100°. It formed slightly pigmented transparent scales. It was acid to litmus and was readily soluble in water at p_H 8. It was precipitated by Esbach's reagent, but not by ferrocyanide. It contained no phosphorus, and had 12.5 % N (micro-Kjeldahl) on an ash-free basis. By an adaptation of Weiss's modification of Millon's reaction the tyrosine content of material from asthmatic urine was estimated to be about 2 %, and the tryptophan by the May and Rose colorimetric method as modified by Boyd [1929] was found to be 0.5 %. The

glyoxylic reaction was definite but faint. Cystine was present as shown by the lead acetate test. Molisch's test was positive. The low nitrogen content, together with the low tryptophan content, points to the presence of a large prosthetic group. This is confirmed by the behaviour of the material on heating with Fehling's solution, slow but distinct reduction taking place. A control test, using purified coagulated fish-muscle protein, showed no reduction under the same circumstances. It appears probable therefore that carbohydrate is present in combination with a protein.

The only difference observed hitherto between the material isolated from normal and that from asthmatic urines is one of quantity.

Determination of the protein nitrogen of urine.

If a sediment is present in the urine the sample is first made alkaline to dissolve uric acid and protein. 25 cc. of the filtered urine are acidified with acetic acid and 75 cc. of 93 % alcohol are added. After 12 hours the precipitate is separated on the centrifuge, dissolved in water, and the solution is dialysed in a thin collodion membrane for a week in presence of toluene, the water being frequently changed. The contents of the membrane are then transferred to an evaporating basin, evaporated to 5 cc. and transferred to a hard glass boiling-tube, and the nitrogen content is determined. Almost identical results may be obtained by direct dialysis of the urine without alcohol precipitation, but by the above method the urea, hippuric acid and other substances are eliminated first.

Protein nitrogen of normal and pathological urines.

A few normal and pathological urines have been examined by the above method, and the results are given in Table I. Further work is being carried out in this direction, but from the present results it is evident that there may be a normal urinary protein content even in the cachexia of cancer. The constancy of an increased protein excretion in allergic conditions in particular is being investigated.

Table I. *Protein (i.e. non-dialysable) nitrogen of some normal and pathological urines.*

Urine	Non-dialysable nitrogen (g. per litre)
1. Normal (apparently)	0.014
2. Normal (apparently)	0.024
3. Cancer (extreme cachexia)	0.011
4. Cancer	0.016
5. Cerebral tumour	0.009
6. Besnier Prurigo	0.053
7. Asthma (in attack)	0.030
8. Cow (healthy)	0.020

Discussion of the nature of the protein.

Considerable confusion exists as regards protein substances in urine. The term "proteose" has often been employed loosely. From the description of the product isolated from normal and asthmatic urines it appears to be a mixture of heat-coagulable protein and a mucoid. The practical differentiation between mucoids and glucoproteoses is not clear. Phosphorus was not found in the purified material, *i.e.* after precipitation and dialysis, but the crude protein fraction from one asthmatic urine contained 0.3 % of phosphorus. It appears that little or no nucleoprotein is present.

Mörner [1895] made a detailed study of protein substances in normal urine. He found a substance which he called "urinary mucoid" identical with the well-known "nubecula" of cooled urine and obtained by him by allowing the filtered fresh urine to stand in presence of chloroform. Its composition and reactions corresponded closely with those described above for the substance isolated by means of the ether reaction, after removal of irreversibly heat-coagulable protein. He also found that normal urine contains a little serum-albumin, which, on addition of acetic acid to urine and shaking with chloroform, is precipitated chiefly in combination with chondroitinsulphuric acid, but also in combination with glycuronic acid and nucleic acid. The properties of the mucoid and of the loose compound of serum-albumin with chondroitinsulphuric acid, as found by Mörner, were very similar, but the mucoid was not precipitated on shaking its solution with organic solvents in presence of chondroitinsulphuric acid (which is said to be present in all urines), although it was so precipitated in the absence of that substance. On this his separation of two different mucin-like substances depended. A consideration of the precipitation reactions of these bodies and of the phenomenon of the precipitation of proteins on the interfaces between solvents and water throws doubt on Mörner's conclusion that the mucoid was not precipitated from urine by shaking with solvents.

In order to settle this point 100 cc. of fresh normal urine were acidified with sulphuric acid and shaken repeatedly with fresh quantities of ether until a honeycomb layer was no longer obtained. The aqueous layer was then treated with 3 vols. of 93 % alcohol and after some hours the precipitate was removed on the centrifuge, dissolved in water, and the solution dialysed for a week in presence of toluene, the water being frequently changed. The non-dialysable nitrogen was then determined. The protein nitrogen of the urine was similarly determined omitting the treatment with ether. It was found that whereas the urine contained 0.014 g. of protein nitrogen per litre, the protein nitrogen left after repeated treatment with ether was 0.0026 g. per litre. Mörner repeatedly found mucoid corresponding to about 0.003 g. of nitrogen per litre, but it is probable that the precipitation was by no means complete. Further, it is not to be expected that shaking with ether can effect complete separation of the protein. It seems likely that the 0.0026 g. per litre of residual

non-dialysable nitrogen was due partly at least to a residue of the same kind of protein as that precipitated. These considerations support the view that Mörner was mistaken in supposing that he had effected a separation of two different kinds of mucin-like substances. Both, if present, are precipitated on shaking the acidified urine with solvents.

The irreversibly-coagulable protein (from asthmatic urine) was boiled repeatedly with water faintly acidified with acetic acid, allowed to stand in water at p_H 8 for 15 hours, washed repeatedly with distilled water by decantation, and then with alcohol and ether and dried at 100° . Its nitrogen content, allowing for 1.3 % of ash present, was 14.7 %. The alkaline treatment was intended to remove loosely combined precipitants such as chondroitinsulphuric acid, mentioned by Mörner. As the nitrogen content of serum-albumin is 15.9 % it is possible that the coagulated material was serum-albumin contaminated with mucoid (which, according to Mörner, is itself a protein precipitant) or with chondroitinsulphuric acid.

It is hoped by means of biological tests to establish the identity or non-identity of the proteins of normal and allergic urines with components of blood-serum and to confirm Oriel and Barber's observations on the specific antigenic properties of the urinary protein of allergic conditions, using purified material.

CONCLUSION.

The substances in normal and asthmatic urines responsible for the ether reaction and isolated thereby are an irreversibly coagulable protein, possibly identical with serum-albumin, and a mucoid containing 12.5 % of nitrogen, possibly derived from the urinary passages.

The author acknowledges the receipt of a personal grant from the Asthma Research Council. He is indebted to Dr W. Robson for advice and to Drs G. H. Oriel and G. W. Bray for pathological material.

REFERENCES.

- Boyd (1929). *Biochem. J.* **23**, 78.
- Mörner (1895). *Skand. Arch. Physiol.* **6**, 332.
- Oriel and Barber (1930). *Lancet*, 231.
- Plosz (1890). *Jahresber. Thier. Chem.* **20**, 215.
- Smith (1892). *Lancet*, 688.