XXIV. THE DIAZO REACTION IN URAEMIC SERA.

By LESLIE FRANK HEWITT.

*From the Biochemical Laboratories, Hospital for Sick Children,
Great Ormond Street, London, W.C. 1.*

(Received January 12th, 1925.)

THE general concensus of opinion is that uraemia is due to the inability of the kidney to deal with certain normal or abnormal products of metabolism. Foster [1923] emphasises the selectivity of this retention by the kidney as contrasted with general retention of metabolic products (urinaemia). Thus, whilst in normal blood the residual nitrogen comprises some 10–20 % of the non-protein nitrogen present, in uraemic blood this value reaches 30–50 %. Attempts to identify the substance or substances responsible for the symptoms of uraemia have, so far, proved unsuccessful. Foster [1915] claims to have obtained from uraemic blood a material which caused the death of guinea-pigs; full experimental details have, however, not been published, and the amount of the material obtained and its properties are not given.

The diazo reaction may prove a valuable means of identifying at least one of the contributory factors in uraemia. Ehrlich [1882] and Pauly [1904, 1905] have described the red colour produced when a diazotised solution of sulphanilic acid is added to certain urines and the mixture rendered alkaline. Van den Bergh [1918] applied the diazo reaction to the detection and estimation of bilirubin in serum. When a dilute hydrochloric acid solution of diazotised sulphanilic acid is added to serum containing bilirubin a pink colour, turning green on addition of alkali, is obtained (*direct* reaction). In certain cases the reaction is obtained only when an alcoholic extract of serum is used (*indirect* reaction). This indirect reaction is conveniently conducted [McNee, 1923] as follows: 2 cc. of serum are poured into 4 cc. of 96 % alcohol, the coagulated proteins are filtered off and to 2 cc. of the clear filtrate are added 1 cc. of 96 % alcohol and $\frac{1}{2}$ cc. of dilute freshly-prepared *p*-sulphophenyldiazonium chloride.

Andrewes [1924], on applying this test to uraemic sera, observed the formation of a buff colour in place of the usual pink. This buff colour deepened gradually when allowed to stand for twenty-four hours, and when made alkaline with caustic soda this deepened buff colour changed to a bright rose red, which faded rapidly. Andrewes obtained this reaction with the sera from eight patients suffering from severe uraemia, but failed to obtain it with the

serum of patients suffering from other diseases. This has been confirmed by the present author; the reaction has been obtained in the case of twelve uraemic patients¹ with blood urea concentrations of from 240 to 692 mg. per 100 cc. (mean value 366 mg.). As found by other workers [Van den Bergh, 1918; Andrewes, 1924; Feigl and Querner, 1919] these nephritic patients showed very marked hypo-bilirubinaemia². In view of the possible connection between the substance causing the diazo reaction and that causing the symptoms of uraemia it was decided to investigate the matter further.

The conditions affecting the reaction itself were first studied. The conclusions reached were as follows:

(i) The characteristic of the reaction is the evanescent pink colour obtained on adding caustic soda, since the buff colour is more difficult to detect in pigmented sera and may be obtained with blood other than uraemic.

(ii) In place of the tedious waiting for twenty-four hours for the development of the buff colour, the test may be made without delay by boiling the reaction mixture for 30 seconds before the addition of caustic soda.

(iii) Strong bases (*e.g.* potassium hydroxide and baryta) produce the pink colour when added to the buff solution, whereas weaker bases (*e.g.* sodium carbonate and ammonia) do not.

(iv) The buff solution has been kept for as long as two months without apparent decomposition, whereas the pink coloration may fade in a few seconds, or at most lasts an hour, according to the intensity of the colour. When the pink colour has once faded the buff colour cannot be recovered by addition of acid, whereas the pink solution becomes buff on acidification and pink again on being rendered alkaline. Thus the fading of the pink colour indicates a reaction not reversed by acids.

(v) Sera do not themselves give the reaction, an alcoholic extract must be used.

(vi) Oxalated plasma may be used in place of serum and is often more convenient. The cerebro-spinal fluid, however, appears not to give the test. In one case of severe uraemia the urea in the c.s.f. reached the concentration of 692 mg. per 100 cc., but the test was negative.

(vii) The intensity of the reaction does not follow the variations in the concentration of urea in the blood, although in general the intensity of the reaction roughly follows the severity of the uraemia.

(viii) The presence of acetic acid interferes with the reaction and may even inhibit the appearance of the pink colour entirely.

¹ The author is indebted to the following gentlemen for samples of uraemic blood: Dr G. A. Harrison, Dr R. D. Lawrence, Prof. Fraser, Dr Marrack and Dr Linder.

² Two infants of 4 and 6 months respectively with uraemia gave a negative test. This may possibly be due to a lower resistance than that of older patients, death therefore occurring at an earlier stage of the disease, before sufficient accumulation in the blood of the substance causing the reaction. It is noteworthy that these did *not* show hypo-bilirubinaemia.

EXPERIMENTS ON THE SUBSTANCE CAUSING THE REACTION.

(i) 24 cc. of an alcoholic extract of uraemic plasma (1 part of plasma to 2 of alcohol) were evaporated to dryness in an open dish; the residue was extracted with 2 cc. of absolute alcohol. Two drops of this extract were diluted to 1 cc. with a mixture of alcohol (2 parts) and water (1 part). This solution gave a strongly positive reaction with a diazotised solution of sulphanilic acid.

(ii) The substance is not removed from plasma or serum, when neutral, acidified or made alkaline, by ether.

(iii) 10 cc. of the alcoholic extract (66 % alcohol) were evaporated to one-third to remove the alcohol, made alkaline by the addition of a few drops of 40 % NaOH and extracted with 2 cc. of pure amyl alcohol. The aqueous layer showed a much diminished colour reaction, whilst the amyl alcohol gave a marked reaction. About six extractions of a deproteinised serum or plasma, made alkaline with either sodium hydroxide or sodium carbonate, with one-third the volume of amyl alcohol were sufficient to remove completely the substance causing the reaction. The amyl alcohol from such an extraction was removed by steam distillation, the material remaining in the aqueous residue.

(iv) The substance is entirely removed from an alcoholic extract by warming with a small quantity of absorbent vegetable charcoal and cannot be removed from the charcoal by warming with dilute acids or alkalis nor by extracting for ten hours with alcohol in a Soxhlet apparatus.

POSSIBLE IDENTITY OF THE SUBSTANCE CAUSING THE REACTION.

The substance possesses basic properties since it is extracted by amyl alcohol from alkaline solutions only. It is soluble in water, dilute acids and alkalis, in ethyl and amyl alcohols in the dilutions employed, but it is not soluble in ether. It is not volatile in steam and is stable to moderate heating. It is irreversibly adsorbed by charcoal. It cannot be detected in the blood of normal persons nor in that of patients suffering from diseases other than uraemia.

The simpler aliphatic amines are excluded on account of their volatility in steam and solubility in ether. Solutions of urea, creatinine and trimethylamine fail to give the reaction. It is probable that cyclic amines alone give this diazo reaction. In support of the hypothesis that the substance in uraemic blood causing the reaction is a toxic base containing the benzene nucleus or the iminazole grouping, *e.g.* tyramine or histamine, the following evidence can be adduced:

(1) Amines containing the phenyl or iminazole group give an evanescent pink coloration with an alkaline solution of diazotised sulphanilic acid [Koessler and Hanke, 1919, 1922].

(2) They are not volatile in steam [Barger, 1914].

(3) They are stable to moderate heating.

(4) They are not soluble in ether, but are extracted by amyl alcohol from alkaline solution [Koessler and Hanke, 1924, 1].

(5) Tyramine increases the blood pressure [Dale and Dixon, 1909], a

common symptom of uraemia; and tyramine produces renal disease and experimental nephritis in rabbits [Harvey, 1911].

(6) Koessler and Hanke [1924, 2] showed that over 60 % of the faeces they examined, both normal and pathological, contained bacteria which decarboxylated tyrosine and histidine to yield the corresponding toxic amines. Since these two amino acids are common products of digestion the presence in the body of the amines derived from them is readily explicable.

(7) Bain [1909, 1910] claims to have isolated tyramine from normal and pathological urines. Alcoholic extracts of urines frequently give (with the diazo reagent) a very similar reaction to that of uraemic blood.

(8) It has been shown [Koessler and Hanke, 1924, 3] that the liver plays a very subsidiary part in the detoxication of these amines, thus leaving the kidney to deal with them; so that, in case of kidney damage, such amines will not be readily eliminated from the body.

It would thus seem that the substance causing the diazo reaction in uraemic sera and plasma possesses many of the properties of the cyclic amines; further these amines are normally present in the intestine, and, as their elimination from the body depends on the kidney, nephritis would be expected to interfere seriously with their elimination. The accumulation of these amines in the blood in uraemia would thus be anticipated and may well account for the diazo reaction given by uraemic blood and for certain of the symptoms of uraemia.

SUMMARY.

(1) Andrewes' findings with regard to the diazo reaction in uraemic blood have been confirmed. It is possible to complete the reaction in one minute instead of twenty-four hours, by means of a slight modification.

(2) It is tentatively suggested that a cyclic amine, such as histamine or tyramine, may occur in uraemic blood and may cause the reaction.

The author wishes to express his deep indebtedness to Dr G. A. Harrison of the Hospital for Sick Children, Great Ormond Street, for providing the facilities whereby this work was carried out, and for his help and advice.

REFERENCES.

- Andrewes (1924). *Lancet*, i, 590.
- Bain (1909). *Lancet*, ii, 365.
- (1910). *Lancet*, i, 1190.
- Barger (1914). *Simpler Natural Bases* (London).
- Van den Bergh (1918). *Gallenfarbstoff im Blute* (Leiden).
- Dale and Dixon (1909). *J. Physiol.* **39**, 25.
- Ehrlich (1882). *Z. klin. Med.* **5**, 235.
- Foster (1915). *Trans. Assoc. Amer. Phys.* **30**, 305.
- (1923). *Arch. Neur. Psych.* **9**, 627.
- Feigl and Querner (1919). *Z. ges. exp. Med.* **9**, 153.
- Harvey (1911). *J. Path.* **16**, 95.
- Koessler and Hanke (1919). *J. Biol. Chem.* **39**, 507.
- (1922). *J. Biol. Chem.* **50**, 237.
- (1924, 1). *J. Biol. Chem.* **59**, 837, 839.
- (1924, 2). *J. Biol. Chem.* **59**, 835.
- (1924, 3). *J. Biol. Chem.* **59**, 901.
- McNee (1923). *Quart. J. Med.* **16**, 390.
- Pauly (1904). *Z. physiol. Chem.* **42**, 508.
- (1905). *Z. physiol. Chem.* **44**, 159.