85. THE EXCRETION OF AN ANTI-PERNICIOUS ANAEMIA PRINCIPLE IN THE URINE

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RESEARCH on pernicious and other megalocytic anaemias has indicated that in normal gastro-haemopoiesis [Wilkinson, 1936] at least 3 unidentified chemical factors are involved. These are an "intrinsic" factor [Castle, 1929, 1] or haemopoietin [Wilkinson & Klein, 1932; Klein & Wilkinson, 1933; 1934] which has enzyme-like properties, and is present in the secretion and mucous membrane of the stomach and in the pylorus, and apparently reacts with an "extrinsic factor" present in foodstuffs such as beef protein [Castle, 1929, 2; Castle & Townsend, 1929; Castle *et al.* 1930; Jones *et al.* 1938], producing a third factor identical with or similar to the anti-pernicious anaemia principle present in liver [Klein & Wilkinson, 1934]. The latter principle is absorbed from the intestine and stored chiefly in the liver, but may be present in other tissues such as the kidney [McCann, 1928], lungs [Hitzenberger, 1934], brain [Ungley, 1931] and salivary gland and pancreas [Dakin *et al.* 1936].

The liver, however, appears to be the main depot and the active principle is transported in the blood to the bone marrow when required for normal erythropoiesis [Wilkinson & Klein, 1934; Wilkinson *et al.* 1937].

Under certain circumstances it would appear that this liver factor might be excreted in the urine. This has not been clearly established, although it is a matter not only of theoretical but also practical importance, for example in furnishing a possible means for the early diagnosis of pernicious anaemia. Some attempts have already been made to detect the liver factor in urine but they have only provided inconclusive evidence owing to the conflicting results obtained and the unsatisfactory nature of the tests employed. This may be due partly to the difficulty of separating without loss of potency small quantities of the anti-pernicious anaemia principle from large quantities of urine in a state suitable for parenteral injection, free from toxic substances, and partly to the fact that many of the examinations for anti-pernicious anaemia potency have been carried out on rats, guinea-pigs or pigeons which have been shown repeatedly by many reliable observers to be quite unsuitable for such tests, since a non-specific reticulocytosis (a) may arise spontaneously, by change of diet, or under the influence of substances present in liver other than the active anti-pernicious anaemia principle, or (b) may even fail to occur following the administration of a known active liver extract. In such a manner did Liener [1935], Walters [1936], Jéquier & Apsley [1938] test their extracts prepared from the urines of patients with pernicious anaemia collected before and during liver treatment and their results therefore are open to criticism.

The only reliable method of assay of the anti-pernicious anaemia factors so far is the clinical test applied under carefully controlled conditions [cf. Wilkinson, 1932]. Unfortunately, the very few such tests that have been carried out on urine extracts have given contradictory results.

Wakerlin [1935] and Wakerlin *et al.* [1935], after preliminary experiments on pigeons, obtained a positive clinical response in the treatment of one case of pernicious anaemia with a concentrated extract of normal urine, while Decastello [1935] obtained similar positive results. In later experiments, however, Wakerlin & Brunner [1936], and Wakerlin [1937; 1940] were unable to confirm their previous work, while Thornley [1938] obtained a negative result with an extract of normal urine tested on one case of pernicious anaemia. Wilkinson [1938] reported some preliminary results obtained in his clinic showing that haemopoietically active extracts could be obtained from normal urine and from urine collected from treated and remitting cases of pernicious anaemia with subacute combined degeneration of the spinal cord. In this paper we present further evidence regarding the presence of an haemopoietically active anti-anaemic factor in normal and certain other urines.

EXPERIMENTAL

Extracts were prepared from fresh urines by two methods, either by direct concentration *in vacuo* and subsequent extraction or, alternatively, by an adsorption technique. Three of these were made from normal urines, two from the urines of untreated patients suffering from pernicious anaemia in relapse, and four from the urines of treated patients with remitted pernicious anaemia, with or without symptoms and signs of subacute combined degeneration of the spinal cord. 20–40 l. of urine were used in each case and details of the extractions are shown in Table 1.

Table 1. *Extracts of human urines*

Extract no.	Urine	Method of extraction	Volume of urine extracted l.	Wt. of dry extract g.	Haemopoietic and clinical activity
U 3 }	Normal male	Direct concentration	40.0	32.9	Discarded
U 4 }		Norite adsorption	40.0	11.7	Active
U 9 }		Direct concentration	20.0	18.0	Active
U 1 }	Treated remitting pernicious anaemia	Direct concentration	46.5	41.0	Active
U 2 }		Direct concentration	49.0	100.0	Active
U 6 }		Norite adsorption	23.0	8.5	Discarded
U 8 }	Untreated relapsing pernicious anaemia	Direct concentration	20.0	54.0	Active
U 5 }		Norite adsorption	35.7	10.9	Inactive
U 7 }		Direct extraction	20.0	50.0	Inactive

(i) *Direct concentration method*

The samples of fresh urine were concentrated daily as soon as obtained to about one-tenth of their volumes at a temperature below 30° *in vacuo* using a Reich distillation apparatus. An equal volume of 90% alcohol was then added; the corresponding daily samples of the concentrates were mixed and later filtered. The filtrate was concentrated to one-tenth of its volume and two volumes of 90% alcohol were added. The resulting precipitate was filtered off and the filtrate after concentration to a syrup was poured into a large excess of absolute alcohol. The precipitate obtained was extracted with warm absolute alcohol to remove urea and other impurities and was then washed with ether, dried in a vacuum desiccator and weighed.

(ii) *Norite adsorption method*

The urine was treated in batches of 2 l. with 20 g. of norite at pH 5 and after standing for 24 hr. the norite was removed by filtration and put into 400 ml. of 60 % alcohol. The daily samples, left at this stage until all had been collected, were then mixed and boiled for 30 min. After filtration the norite was extracted twice more with 1 l. of phenol, containing about 5 % water and the extract was treated with ether and water. The aqueous layer was mixed with the 60 % alcohol filtrate and concentrated *in vacuo* to a syrup, which was poured into an excess of absolute alcohol. The resulting precipitate was washed with absolute alcohol, then ether and dried in a desiccator.

The clinical assay of the urinary extracts

The products were prepared for intramuscular administration by solution in the minimal amounts of distilled water, centrifuging and sterilization by passage through a Seitz filter.

The potency of the extracts was determined by the haematological and clinical effects following their intramuscular injection, after preliminary control periods, into well authenticated and specially controlled patients with relapsing pernicious anaemia. Haemopoietically active extracts produced firstly a sharp rise or peak in the number of reticulocytes in the circulating blood, and this was followed by an increase in the red blood cells and in the percentage of haemoglobin, with a simultaneous improvement in the clinical condition. The patients used for this purpose were all carefully chosen according to the rigid criteria laid down by one of us [Wilkinson, 1932]; estimations of the reticulocytes were made daily, and of the red cells, white cells and haemoglobin one or twice weekly as indicated by the state of the patient.

RESULTS

There were three groups of experiments.

(i) *Extracts from normal urines*

Extracts U 3, U 4 and U 9 were prepared from the urines collected from normal individuals in the Department, none of whom was taking liver in any form.

Extract U 3 gave painful reactions and was discarded, but the other two, U 4 and U 9, that were prepared differently, gave good positive clinical responses when given intramuscularly to test patients in doses equivalent to 8.5–20 l. of urine.

Case PA/833, with an initial count of, red cells 1,570,000 per μ l., haemoglobin 42 %, reticulocytes 0.1 %, received on 17 December 1937 2.5 g. of extract U 4 in one dose intramuscularly (equivalent to 8.5 l. of urine) and showed 14.8 % reticulocytes on the 9th day, and on the 17th day, red cells 2,340,000 per μ l., haemoglobin 55 %, with a corresponding good clinical improvement.

Case PA/851, with an initial count of, red cells 1,420,000 per μ l., haemoglobin 34 % and reticulocytes 0.2 %, received on 20 January 1938 2.4 g. of extract U 4 in 4 equally divided doses (equivalent to 8.2 l. of urine) followed by 0.6 g. on each of the 9th, 10th, 13th and 14th days later (total 4.8 g. extract equivalent to 16.4 l. of urine); unfortunately the maximal reticulocyte peak was accidentally missed but the reticulocytes were 11.6 % on the 12th day and on the 19th day the count was, red cells 2,040,000 per μ l., haemoglobin 52 %

and reticulocytes 1.4 %, with marked clinical improvement. Similarly, case PA/ADD/146, with an initial count on 6 October 1939 of, red cells 1,400,000 per μ l. and haemoglobin 28 %, received 18 g. of extract U 9 (equivalent to 18 l. of urine) and showed a reticulocyte response of 12.5 % on the 11th day and subsequent improvement in the blood and clinical condition.

(ii) *Urine extracts from treated patients with pernicious anaemia in remission*

These patients were in-patients under our control and had been given the usual anti-anaemic treatment; two of them had marked symptoms of subacute combined degeneration of the spinal cord. All had responded to anti-anaemic treatment and had red cell counts greater than 4,000,000 per μ l. at the times when the urines were collected.

Extracts U 1, U 2 and U 8 were prepared by the direct concentration method and all gave positive clinical responses when given intramuscularly to test patients in total dosages equivalent to 10–22.6 l. of urine. Extract U 6 gave toxic reactions and was discarded.

Case PA/627, with an initial count on 12 February 1936 of, red cells 1,280,000, haemoglobin 40 %, reticulocytes 1.0 %, received 20 g. extract U 1 in 8 days (equivalent to 22.6 l. urine); the reticulocytes rose to 12.2 % and on the 24th day the count was, red cells 2,100,000 per μ l., haemoglobin 63 % and reticulocytes 5.8 %, with very marked clinical improvement.

Case PA/633, with an initial count on 25 February 1936 of, red cells 1,420,000 per μ l., haemoglobin 38 % and reticulocytes 0.9 %, received 34.5 g. extract U 2 over 11 days (equivalent to 15 l. of urine); the reticulocytes rose to 29.6 % on the 15th day, and on the 21st day the red cells were 2,510,000 per μ l., haemoglobin 62 % and reticulocytes 6.9 % (on the 28th day, red cells 2,760,000 per μ l., haemoglobin 74 %), with considerable clinical improvement.

Case PA/975, with an initial count on 27 June 1939 of, red cells 1,730,000 per μ l., haemoglobin 43 % and reticulocytes 2.4 %, received 29 g. extract U 8 over 11 days (equivalent to 10.7 l. of urine); the reticulocytes rose to 15.2 %, and on the 21st day the red cells were 2,200,000 per μ l., haemoglobin 54 %; on the 25th day, the red cells were 3,200,000 per μ l. and haemoglobin 74 %, with a progressive clinical improvement.

(iii) *Urinary extracts from untreated patients with relapsing pernicious anaemia*

These in-patients were suffering from severe relapsing pernicious anaemia and did not receive any anti-anaemic treatment until about 40 l. of urine had been collected from each of them. Extracts U 5 and U 7 were prepared by absorption on norite and direct concentration respectively, both failing to produce any haemopoietic responses when injected intramuscularly into three test patients. Thus, case PA/853 (initial count on 15 March 1938 of, red cells 1,150,000 per μ l., haemoglobin 31 %, reticulocytes 0.2 %) received 2.5 g. extract U 5 on each of 3 successive days (a total of 7.5 g. equivalent to 24.7 l. of urine) without any response, the red cells falling to 800,000 per μ l., and the haemoglobin to 21 %; the subsequent response to an active liver extract was perfectly satisfactory.

Similarly, case PA/969, with an initial count on 24 June 1939 of, red cells 1,770,000 per μ l., haemoglobin 48 % and reticulocytes 0.8 %, and case PA/971, with an initial count on 4 July 1939 of, red cells 1,660,000 per μ l., haemoglobin 42 % and reticulocytes 0.8 %, failed to respond to intramuscular injections of extract U 7, but showed normal and prompt remissions when subsequently given ordinary active anti-anaemic preparation.

DISCUSSION

Our results show that from large quantities of normal urine it is possible to prepare extracts which, when administered to untreated patients suffering from relapsing pernicious anaemia, will initiate normal remissions in the same way that similarly prepared extracts of beef liver will do. While the urines from relapsing cases of pernicious anaemia failed to furnish such active extracts, when these patients had been adequately treated and their bloods and general conditions returned to normal they then passed urine from which active extracts could again be prepared.

There is, as yet, no evidence to show whether the anti-anaemic activity of these urinary extracts is due to a precursor of or to the actual so-called anti-pernicious anaemia liver principle, or to some other allied substance with similar haemopoietic properties; on theoretical grounds we would expect that the blood of normal individuals would contain the liver anti-anaemic principle in process of transport from the liver to the bone marrow. It is, therefore, reasonable to suppose that the principle present in the urine (admittedly in small quantities) is identical with this liver principle and represents that which has been removed via the kidneys from the blood. In relapsing pernicious anaemia, in which the liver stores are empty, there is none in the blood to be lost; on the other hand, in individuals receiving active anti-anaemia therapy the liver stores and presumably the blood level are restored [Wilkinson & Klein, 1934; Wilkinson *et al.* 1937] and the principle therefore appears once more in the urine.

In view of the difficulties, on the one hand, associated with attempts to express the activity of anti-anaemia extracts in a quantitative form on the basis of results of clinical tests on relatively few cases, and, on the other hand, encountered in preparing extracts readily from large amounts of urine, it is obvious that the examination of the urine for anti-pernicious anaemia potency does not offer a suitable or convenient diagnostic test for pernicious anaemia. If, however, an accurate chemical or biological test becomes available by which relatively small amounts of urine can be accurately assayed then such a test may be of great value in the early diagnosis of the disease, since absence of the anti-anaemia factor from the urine should be one of the first signs long before the blood picture becomes characteristic.

These considerations raise the possibility of a renal threshold for the anti-pernicious anaemia factor; the occurrence of a low renal threshold for the liver principle could under certain circumstances result in a continuous excretion of the liver factor and ultimately a deficiency sufficient to cause a megalocytic anaemia—this may indeed be the explanation of the occurrence of such types of anaemia in association with certain renal conditions.

SUMMARY

1. Extracts have been prepared from normal male urines and from the urines of treated patients with remitting pernicious anaemia which will initiate normal remissions of the disease when given intramuscularly to relapsing patients with pernicious anaemia.

2. Similar extracts prepared from the urines of untreated relapsing cases of pernicious anaemia did not possess such anti-pernicious anaemia activity when tested clinically.

3. This urinary anti-pernicious anaemic principle although showing a similar clinical action may or may not be identical with the liver principle.

4. This active material is present in the urine in relatively small amounts.

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