CCXXI. THE ERROR DUE TO BLOOD ARGINASE IN THE UREASE METHOD FOR THE DETERMINATION OF BLOOD UREA.

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THE possibility of an error in the determination of urea in blood by urease methods was brought to our notice by the fact that figures obtained by the direct estimation of urea in human blood corpuscles were obviously too high. This error appears to have been first reported by Behre [1923], who observed that when the soya bean urease method was applied to whole blood the urea obtained was partially determined by the concentration of urease extract employed, and that the substance responsible for this "extra urea" was chiefly contained in the corpuscles. She did not come to any conclusion as to the exact nature of this substance. Addis [1928], when estimating the urea in liver tissue with Jack bean urease, obtained a linear relation between the quantity of urea produced and the quantity of urease used. He considered that the extra urea was derived from the action of arginase in the liver on arginine in the Jack bean. He also found that the apparent concentration of urea in human blood might be varied by adding different amounts of urease. He does not state that the variation in the case of blood was due to arginase. Peters & Van Slyke [1932], in describing the urease method as applied directly to whole blood, state "...unnecessarily prolonged contact between blood and enzyme is to be avoided because of the possibility of slow formation of ammonia by the action of arginase in the blood cells on arginine in the enzyme preparation" and refer to the work of Addis and Behre which did not actually prove that the error was due to the action of blood arginase.

This source of error in a standard method appeared to be of sufficient importance to justify a further investigation.

EXPERIMENTAL.

Method. Urea was determined by the aeration-titration method as described by Kay and Sheehan [1934] except that the acid was titrated to the nearest 0.05 ml. in a standard 50 ml. burette instead of to 0.01 ml. as in the original method. All determinations were done in duplicate and the figures for blood are given to the nearest 0.5 mg. urea per 100 ml. blood.

Urease. 1. A watery extract of soya bean powder prepared by extracting 10 g. of powder with 100 ml. of water for 1 hour as described by Kay & Sheehan [1934]. 2. Jack bean urease powder, acetone precipitate [Peters & Van Slyke, 1932]. 3. Crystalline urease prepared by the method of Sumner [1926, 1, 2]. It was found that good crystals could be obtained by using Jack Bean Powder (B.D.H.), which had been thoroughly extracted with ether in a Soxhlet apparatus and then dried, in place of the "Arlco" Jack bean meal used by Sumner.

Blood. Blood was taken from a limited number of normal men by venipuncture and oxalated in the usual manner.

Preliminary experiments confirmed the findings of Behre and Addis that extra urea was formed when the concentration of enzyme was increased. The action of a strong solution of urease was then compared with that of a preparation of crystalline urease on blood and plasma simultaneously. It will be seen from Table I (Nos. 1-4) that the 100 mg. of powder gave 6.5 mg. of urea more than the crystalline enzyme with whole blood, but that the two preparations gave identical results with plasma.

Table I.

Subject	Substrate	Urease preparation	Urea mg. per 100 ml. blood or plasma
1. S. L. T.	Blood	100 mg. powder	25.5
2. "	Blood	Crystalline	19
3. "	Plasma	100 mg. powder	23
4. "	Plasma	Crystalline	23
5. A. D.	Blood inactivated	Soya bean extract (5 ml.)	25
6. "	Blood inactivated + 2 mg. arginine	"	25
7. "	Blood	"	30
8. "	Blood + 2 mg. arginine	"	39.5
9. A. B. A.	Blood inactivated	"	19.5
10. "	Blood inactivated + 2 mg. arginine	"	20.5
11. "	Blood	"	31
12. "	Blood + 2 mg. arginine	"	34
13. R.	Plasma inactivated	"	29.5
14. "	Plasma inactivated + 2 mg. arginine	"	28.5
15. "	Plasma	"	29.5
16. "	Plasma + 2 mg. arginine	"	29.5
17. T. P.	Blood	100 mg. powder	24.5
18. "	Blood + 2 mg. CoCl_2 , $6\text{H}_2\text{O}$	"	49.5
19. "	Blood inactivated	"	21.5
20. "	Blood inactivated + 2 mg. CoCl_2 , $6\text{H}_2\text{O}$	"	41.5
21. S. L. T.	Blood	100 mg. powder	32
22. "	Blood + 2 mg. CoCl_2 , $6\text{H}_2\text{O}$	"	60.5
23. "	Plasma	"	30.5
24. "	Plasma + 2 mg. CoCl_2 , $6\text{H}_2\text{O}$	"	32.5
25. A. B. A.	Blood	"	37.5
26. "	Blood + 2 mg. glutamic acid, 2 mg. histidine hydrochloride, 2 mg. glycine	"	38.5
27. "	Blood + 2 mg. arginine	"	49.5

It was then assumed that these results were due to the action of blood arginase. The addition of arginine in solution to the blood was found to increase the apparent urea. Now Hunter and Dauphinee [1933] have shown that arginase is rapidly inactivated at a pH less than 4.0. Accordingly blood arginase was inactivated in the following manner: to 2 ml. of blood washed into the reaction tube with water instead of buffer was added 1 ml. of $N \text{H}_2\text{SO}_4$ and the tube heated to 50° for 10 min., the tube was then cooled and the acid neutralized by 1 ml. of $N \text{NaOH}$ and then the buffer and urease added. An equivalent amount of a mixture of equal volumes of $N \text{H}_2\text{SO}_4$ and $N \text{NaOH}$ was added to the other tubes in the series. Not only was no extra urea produced by the addition of arginine to the "inactivated" blood but the apparent urea in the "inactivated" blood was less than in the untreated blood, while the addition of arginine to the untreated blood produced a considerable increase in the urea found. (Table I, Nos. 5-8.) Blood from a different individual showed the same effect except that there was a very slight increase of urea on adding arginine to

the "inactivated" blood. (Table I, Nos. 9-12.) On the other hand the values for plasma urea were unaffected by this "inactivation" nor did the plasma give any extra urea on the addition of arginine. (Table I, Nos. 13-16.)

In a recent paper Hellerman and Perkins [1935] demonstrated the activation of arginase by certain metal ions Co^{++} , Ni^{++} , Mn^{++} . The effect of the addition of a solution of cobalt chloride to the reaction tubes was therefore investigated. Cobalt chloride 2 mg. in 2 ml. of water produced an increase of approximately 100% in the apparent urea of the blood. (Table I, Nos. 17, 18.) This extra 25 mg. of urea per 100 ml. of blood would be equivalent to the hydrolysis of 1.45 mg. of arginine in the reaction tube. The "inactivated" blood was also reactivated by the cobaltous ions and gave an extra 20 mg. of urea. (Table I, Nos. 19, 20.) With blood and plasma the addition of cobalt again produced a large increase in the urea found in the blood and only a very small increase with plasma which was probably due to a small amount of corpuscle arginase contaminating the plasma as there was slight haemolysis. (Table I, Nos. 21-4.) As a control tubes were put up without blood. Both urease with cobalt and also urease with arginase and cobalt failed to form ammonia. The titrations were identical with that for the urease blank.

Other amino-acids were tried in place of arginine. In one experiment glycine gave no extra urea and in another a mixture containing glutamic acid 2 mg., histidine hydrochloride 2 mg. and glycine 2 mg. produced only the slight increase of 1 mg. per 100 ml. in the apparent urea, while the addition of 2 mg. of arginine gave an increase of 12 mg. (Table I, Nos. 25-7.)

Urease solutions were incubated with a preparation of liver arginase—made from calf liver by the method described by Hellerman & Perkins [1935]—instead of blood, and it was found that the arginase produced ammonia from the strong urease solutions. It will be seen from Table II that 10 mg. of urease powder

Table II. *The reaction between arginase and urease preparations.*

	ml. 0.01 N H_2SO_4 equivalent to NH_3 formed after incubation at 50° for 30 min. with urease powder	
	10 mg.	100 mg.
1. Arginase solution 2 ml.	0.0	0.35
2. Arginase solution 2 ml. + arginine 2 mg.	0.9	0.7
3. Arginase solution 2 ml. + CoCl_2 , $6\text{H}_2\text{O}$ 2 mg.	0.3	2.15

incubated with arginase gave no appreciable ammonia though the arginase solution was active and hydrolysed added arginine. When the arginase was activated by cobalt a small quantity was produced. By contrast 100 mg. of urease powder, though only giving a small quantity of ammonia with arginase alone, with cobalt-activated arginase gave a large amount of ammonia equivalent in this case to the hydrolysis of 1.85 mg. of arginine. The quantities of ammonia produced from this strong urease solution are of the same order as those equivalent to the "extra" urea obtained with blood under similar conditions.

DISCUSSION.

The experiments reported here show that the factor responsible for the production of the extra urea from the urease solutions is the arginase in the corpuscles, because it is activated and inactivated by the same methods as arginase and can be replaced by a preparation of arginase from liver. Addis, in

his experiment with liver and urease, considered that it was unlikely that the arginine in some of his urease preparations was free, and suggested that either proteolytic enzymes in the liver set arginine free, or that one of the proteins of the Jack bean has arginine so situated in its structure that urea might be formed from it by arginase. We have no direct analytical evidence of the amount of free arginine in our urease preparations but free arginine cannot be excluded on general grounds. Arginine is precipitated by acetone in the strength used for precipitating the soluble urease powder and according to Sasaki [1932] arginine nitrate 9.55 g. was isolated from the hot water extract from 5 kg. soya bean which had previously been extracted with ether.

According to Edlbacher *et al.* [1927] arginase is present in the blood of man, cattle, sheep and pig, and absent from the blood of the dog, cat, rabbit, porpoise, mouse, rat, hen, pigeon and goose [Edlbacher & Rothler, 1925]. On the other hand Weil & Russell [1934] report the presence of arginase in rat blood in both the cells and plasma and that it is higher in adult males than in young males, castrates and females, but there is no obvious relationship between the blood urea level and the arginase activity. There seems to be general agreement that arginase is present in human blood and that it is confined to the cells.

In the experiments reported above the reaction mixtures were all incubated for 30 min. The effect of the time of incubation has not been investigated in detail but bloods from some subjects were found to give appreciable amounts of extra urea after only 8 min. incubation. The original observations of Behre were made after incubation for 10 min. on bloods obtained from hospital cases which showed considerable variation in the magnitude of the increase in urea with concentrated extracts. It is of interest to note that even with the weak extracts used by Behre [1923] the figure for whole blood urea was in some cases a little higher than that for plasma indicating a small production of extra urea. The urease preparations used in the present work though described as strong solutions were actually of the strength advised for routine use.

It seems unlikely that this error is of significance in the routine estimation of blood urea for clinical purposes. When however an accurate estimation is necessary the variable error introduced by the action of blood arginase may be far greater than any experimental error and will vitiate the result unless precautions are taken to eliminate the arginase action. The question would not arise with an animal such as the rabbit whose blood is said to be free from arginase. In the case of human blood the most satisfactory solution appears to be the use of plasma for analysis. In the presence of cobaltous or other divalent ions which activate arginase such precautions would be essential.

SUMMARY.

1. The reported production of "extra" urea when human blood is incubated with concentrated solutions of urease is confirmed.
2. The substance responsible is considered to be the blood arginase. It is confined to the corpuscles and is inactivated by incubation at 37–50° for 10 min. after the addition of acid to the blood and is activated by the addition of cobaltous ions which may produce an increase of 100% in the apparent blood urea. A similar production of ammonia is observed when the concentrated urease solutions are incubated with a preparation of liver arginase.
3. The significance of this error in urea analysis is discussed and the use of plasma is advised for the accurate estimation of urea in human blood.

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