CXXXV. NEW SOURCES OF UREASE FOR DETERMINATION OF UREA

By MANAYATH DAMODARAN AND
PALGHAT MANUVADHYAR SIVARAMAKRISHNAN

From the University Biochemical Laboratory, Chepauk, Madras

(Received 24 May 1937)

ALMOST every method in general use to-day for the determination of urea in biological material involves as its first step the hydrolysis of the urea into ammonia and carbon dioxide by the action of soya bean or jack bean urease. That this procedure under certain conditions leads to abnormally high values has been occasionally pointed out, though little account of the fact seems to be taken in routine analytical practice. Excessive values for urea were reported for whole blood using concentrated soya bean extract by Behre [1923], for whole blood and for liver treated with jack bean urease by Addis [1928], and for kidney tissue with jack bean urease by Rehberg & Blem [1932]. The source of this "extra urea" formed when urease preparations are brought into contact with blood or tissues still awaits systematic investigation. But it was inferred by Addis, from the fact that the urea value increased with increasing quantities of urease added, that the abnormal values were due to the action of arginase in liver on arginine in the enzyme preparation. Experiments in the same sense have been reported by Kirk [1933] for kidney and by Anderson and Tompsett [1936] for blood. In view of the fact that the amino-acid canavanine isolated from jack bean by Kitagawa & Yamada [1932] has been shown by them to be decomposed by minced liver yielding urea, it is more correct to say with Kirk that the "extra urea" arises from the action of an enzyme in the blood or tissue on a substrate in the urease preparation. The findings reported in the present paper make it quite clear that the urea does not arise in every case from the same substrate. Thus soya bean preparations give rise to a small "extra urea" formation with liver and a rather higher value with blood. Jack bean gives on the other hand with blood a value lower than that obtained using soya bean, while with liver it gives a urea content many times the amount originally present in the tissues.

According to Peters & Van Slyke [1932], these abnormally high values can be avoided, at least for human blood, by (i) using only minimum quantities of the urease preparation, (ii) carrying out the digestion at room temperature (20°), and (iii) reducing the reaction time to 5 min. In securing these conditions in practice difficulties were experienced in this laboratory chiefly because commercial jack bean or soya bean preparations were found to vary in potency and to deteriorate in the Indian climate so that for quantitative decomposition of urea in 5 or 15 min. at room temperature (30°) quite large quantities of the preparations had to be used, thus introducing into the reaction mixture considerable amounts of the substrates that give rise to "extra urea". The present investigation was undertaken to find out a suitable indigenous source of urease from which a fully active preparation could be made as and when required.

The urease content of Indian seeds was investigated by Annett [1914], who seems to have been the first to point out the very high potency of jack bean in this respect. The only one among the seeds which is shown in his studies to be rich in urease which is readily obtainable in South India, viz. horse-gram (*Dolichos biflorus*), was found by him to deteriorate rapidly in activity on keeping. As Annett's investigation was confined to the Papilionaceae, in the present study attention was mainly directed to the Cucurbitaceae in which family also the occurrence of urease has been often detected [cf. Klein, 1933]. Though the original object of our investigation was only to find a readily available substitute for jack or soya bean which could be used under the limitations stated by Van Slyke, in the sequel it was found that the seeds of the water melon (*Citrullus vulgaris*) was not only an excellent source of urease, being more active than soya bean (though less so than jack bean), but also possessed the advantage that it gave rise to insignificant amounts of urea on incubation with blood or liver.

Defining a unit of urease as that quantity of it which decomposes 1 mg. of urea per hour under experimental conditions described later, defatted seed meals of jack bean, soya bean and water melons contained in a typical experiment, 9139, 1374 and 2351 units respectively per g. of dry material. Urea values obtained when the three preparations were used under standard conditions were in excess of the correct figures (obtained by the xanthidrol method) by 0.58, 2.14 and - 0.02 mg. respectively with ox blood, and 42.1, 0.56 and - 0.20 mg. with ox liver. Blood and liver from other sources were not tried, but results of a research still in progress on the nitrogenous constituents of the seeds show quite definitely that the water melon seeds contain only traces of free arginine and no canavanine and that, in so far as these substrates are concerned, there is no possibility of appreciable "extra urea" formation when urease from this seed is brought into contact with blood or tissues.

There is no doubt therefore that the use of water melon seeds as a source of urease is free from the errors that pertain to the now widely used jack bean or soya bean. It is true that such errors can be avoided in the latter case also by using purified preparations prepared free from the proteinogenous bases which as far as we now know are the sources of the "extra urea". But the methods of preparation in common use, extraction with water and precipitation by acetone [Van Slyke & Cullen, 1914] or extraction with 30% aqueous alcohol [Folin & Wu, 1919] are hardly likely to attain this object. The processes involved are as likely to concentrate the free amino-acid precursors of urea as the urease protein itself. The only justifiable method of purification would be the preparation of crystalline urease according to Sumner. This would however deprive the urease method of the ease and simplicity which have brought it into such general use, and the substitution of urease from *Citrullus vulgaris* for the jack bean and soya bean preparations appears to be the most satisfactory way of avoiding the errors mentioned.

Two other seeds of the Cucurbitaceae, the common gourd (*Cucurbita maxima*) and the snake gourd (*Trichosanthes anguina*), also show similar properties, having high urease activity and producing little or no "extra urea" on incubation with biological materials. From our standpoint these seeds are however of less importance than water melon seeds as they are not so abundantly available. Among the few seeds of the Papilionaceae examined, *Canavalia obtusifolia* is nearly as rich in urease as the jack bean but like the latter gives rise to considerable amounts of "extra urea" in presence of liver.

EXPERIMENTAL

Methods. All urea determinations were carried out according to the standard methods described by Peters & Van Slyke [1932] except that (i) digestion with urease and aeration were carried out at 30° instead of at 20°, and (ii) in blood urea estimations the maximum period considered permissible by these authors, viz. 15 min., was allowed for urease action, instead of the 5 min. generally recommended by them.

Urease activity of different seeds

Preparation of seed meal. The seeds were crushed, freed from husk and finely ground. While seeds like *Canavalia ensiformis*, *C. obtusifolia*, *Soja hispida* and *Dolichos biflorus* yielded fine powders by this treatment, more fatty varieties (*Citrullus vulgaris*, *C. colocynthis*, *Trichosanthes anguina*, *Cucurbita maxima*) were obtained in a pasty condition. In all cases, weighed samples were taken out simultaneously for determinations of urease activity and of moisture. Defatted preparations were obtained by grinding up the powder or paste with light petroleum (B.P. 50–60°) washing at the pump 3 or 4 times with the latter and finally drying at room temperature in a current of air. In the case of soya bean a freshly obtained commercial preparation was also used for comparison.

For experiment the materials were made up to uniform suspensions containing 1 g. in 100 ml. of water, 1 ml. of the suspension being added to each 5 ml.

Table I. *Urease content of different seeds*

Reaction mixture: 5 ml. of urea solution, 1 ml. of enzyme suspension,
allowed to act 15 min. at 30°

No.	Seeds tested	mg. dry weight of seed meal	ml. N/10 NH ₃ formed	Urease units (mg. urea split per g. dry weight of material per hour)	
				Before defatting	After defatting
1	<i>Canavalia ensiformis</i> , jack bean	8.758	6.44	8822	—
		8.646	6.59	—	9139
2	<i>C. obtusifolia</i>	8.974	4.36	5826	—
		8.971	4.55	—	6088
3	<i>Cucurbita maxima</i> , common gourd (squash)	9.454	1.08	1376	—
		8.748	2.24	—	3073
4	<i>Citrullus vulgaris</i> , water melon	9.583	0.92	1152	—
		8.650	1.69	—	2351
5	<i>Trichosanthes anguina</i> , snake gourd	9.557	0.58	734	—
		8.818	1.17	—	1592
6	<i>Soja hispida</i> , soya bean	8.892	0.49	657	—
		8.063	0.92	—	1374
7	<i>S. hispida</i> , soya bean (B.D.H.)	9.170	0.68	892	—
		8.598	0.88	—	1223
8	<i>Citrullus colocynthis</i> , colocynth	9.574	0.49	610	—
		8.482	0.95	—	1337
9	<i>Dolichos biflorus</i> , horse-gram	8.674	0.29	404	—
		8.654	0.49	—	675
10	<i>Momordica charantia</i> , bitter gourd	9.537	0.18	232	—
11	<i>Benincasa cerifera</i> , white gourd	9.560	0.05	63	—
12	<i>Lagenaria vulgaris</i> , bottle gourd	—	—	—	—
13	<i>Cucumis sativus</i> , cucumber	—	—	—	—
14	<i>C. melo</i> , melon	—	—	—	—
15	<i>Luffa acutangula</i> , luffa	—	—	—	—
16	<i>Coccinia indica</i> , scarlet gourd	—	—	—	—

of a molar solution of urea (in $M/4$ phosphate buffer) in a Van Slyke & Cullen aeration apparatus. The reaction mixture was allowed to stand at room temperature (30°) for 15 min., at the end of which time 2 ml. of 10% NaOH were added and the ammonia determined by aeration. The results in Table I are typical of those obtained in numerous experiments carried out with different batches of enzyme preparations. It is apparent from the values that three seeds belonging to the Cucurbitaceae have a higher urease content than soya bean and that the potency of these seeds is more than doubled by preliminary treatment with light petroleum.

Urea formation from urease preparations in presence of ox blood

Oxalated ox blood obtained fresh from the slaughter-house and immediately brought to the laboratory in ice was used in all the experiments. Urea was determined (i) gravimetrically by the xanthidrol method [Fosse *et al.* 1914], and (ii) using different urease preparations, the difference giving the "extra urea" formed during the reaction by the action of blood enzymes on substrates in the urease. For the first method it was found convenient to work with fairly large amounts of blood. 50 ml. were usually taken, mixed with an equal volume of water and deproteinized using Folin's tungstic acid reagent. The precipitate was thoroughly washed, the filtrate and washings evaporated to a small volume and then treated in the usual way with glacial acetic acid and a 10% methyl alcoholic solution of xanthidrol. After standing overnight the precipitate was washed thoroughly with water and methyl alcohol by centrifuging, then at the pump in a weighed Jena glass filter, dried at 100° and weighed. Trial experiments showed that added urea could be quantitatively recovered by this procedure. For determination of total urea (blood urea plus urea from the urease preparation) by the urease method 5 ml. of the oxalated blood were used. Determinations were made not only under the standard conditions but also using a much larger concentration of urease (50% suspension) as well as with longer reaction periods (30 min. and 2 hr. respectively). Preformed ammonia was in every case determined in a blank experiment and allowed for.

Table II. *Blood urea determinations with different urease preparations*

Blood sample	Sources of urease	Quantity of urease used for each determination	Reaction time	mg. urea/100 ml. blood		
				Xanthidrol method	Urease method	Excess urea
1	Jack bean	2 ml. of 10% suspension	15 min.	26.90	27.48	0.58
	<i>Canavalia obtusifolia</i>	"	"	26.90	27.48	0.58
	Water melon	"	"	26.90	26.88	-0.02
	Common gourd	"	"	26.90	26.88	-0.02
2	Colocynth	"	"	62.14	62.14	0.00
	Snake gourd	"	"	62.14	62.60	0.46
	Soya bean	"	"	62.14	64.28	2.14
	Horse-gram	"	"	62.14	64.70	2.56
3	Water melon	"	2 hr.	10.95	10.92	-0.03
	Jack bean	"	"	10.95	15.20	4.25
4	Soya bean	"	"	35.00	40.62	5.62
	<i>C. obtusifolia</i>	"	"	35.00	38.98	3.98
5	Jack bean	1 ml. of 50% suspension	30 min.	31.00	31.72	0.72
	Soya bean	"	"	31.00	39.40	8.40
6	Water melon	"	"	12.24	12.42	0.18
	<i>C. obtusifolia</i>	"	"	12.24	17.14	4.90

The results of Table II are self-explanatory. Using for each determination 2 ml. of a 10% suspension of the enzyme with a reaction time of 15 min. at 30° both jack bean and soya bean give rise to appreciable amounts of "extra urea" in presence of ox blood, the quantity of urea formed being greater with soya bean than with jack bean. The use of larger concentrations of enzyme or of longer periods of reaction considerably increases the urea production, but the two effects do not run parallel in the two preparations. On the other hand, urease from the seeds of the water melon, of the common gourd, and of the snake gourd give blood urea values which are in close agreement with those obtained by gravimetric determination as dixanthylurea. Increase in the quantity of enzyme preparation or prolonging the period of action to 2 hr. does not give rise to appreciable error.

Urea formation from urease preparations in presence of minced liver

Fresh ox liver was washed free from blood, minced finely and a weighed amount ground up thoroughly with distilled water. The material was squeezed through muslin and the uniform suspension made up to volume. Aliquots were removed for urea determination by the urease and the xanthydrol methods. The results, after allowing for the blank determinations for free ammonia, are given in Table III. Both jack bean and soya bean give rise to abnormally high

Table III. *Liver urea determinations with different urease preparations*

Liver sample	Sources of urease	Quantity of urease used for each determination	Reaction time	mg. urea/100 g. of liver tissue		
				Xanthydrol method	Urease method	Excess urea
1	Jack bean	2 ml. of 10% suspension	15 min.	12.42	54.52	42.10
	<i>Canavalia obtusifolia</i>	"	"	12.42	42.28	29.86
	Water melon	"	"	12.42	12.22	-0.20
	Common gourd	"	"	12.42	13.16	0.74
	Snake gourd	"	"	12.42	14.10	1.68
	Colocynth	"	"	12.42	12.68	0.26
2	Soya bean	"	"	10.36	10.92	0.56
	Horse-gram	"	"	10.36	10.92	0.56
3	Jack bean	"	2 hr.	8.60	59.76	51.16
	Soya bean	"	"	8.60	15.96	7.36
4	Water melon	"	"	22.72	22.74	0.02
	<i>C. obtusifolia</i>	"	"	22.72	66.60	43.88
5	Jack bean	1 ml. of 50% suspension	30 min.	18.23	142.46	124.23
	<i>C. obtusifolia</i>	"	"	18.23	137.71	119.48
	Water melon	"	"	18.23	19.00	0.77
	Soya bean	"	"	18.23	24.69	6.46

values. Jack bean and *Canavalia obtusifolia* give values which are many times the preformed urea even under the standard conditions of urea concentration and time of reaction; soya bean gives rise to slightly raised values under these conditions and to more flagrant error on prolonging the time of incubation. With the preparation from water melon seeds, the correctness of the results is unaffected by prolonging the reaction period to 2 hr., and only very slightly affected by using five times the usual quantity of enzyme.

SUMMARY

1. The seed of the water melon (*Citrullus vulgaris*) is shown to be a potent source of urease suitable for use in urea determinations.

2. Water melon urease is, for this purpose, distinctly superior to soya bean and jack bean preparations because it determines urea quantitatively even in such materials as whole blood and liver which give abnormally high values when the latter sources of urease are used.

3. Using jack bean or soya bean with ox blood and liver it is shown that abnormal values are obtained even under standard experimental conditions, while on increasing either the time of action or the concentration of the enzyme, the errors are considerably aggravated. Such effects are found to be negligible with water melon seeds.

We are indebted to the kindness of Prof. T. Ekambaram of the Presidency College, Madras, for the botanical identification of many of the seeds examined.

REFERENCES

- Addis (1928). *Proc. Soc. exp. Biol., N.Y.*, **25**, 365.
Anderson & Tompsett (1936). *Biochem. J.* **30**, 1572.
Annett (1914). *Biochem. J.* **8**, 449.
Behre (1923). *J. biol. Chem.* **56**, 395.
Folin & Wu (1919). *J. biol. Chem.* **38**, 81.
Fosse, Robyn & Francois (1914). *C.R. Acad. Sci., Paris*, **159**, 367.
Kirk (1933). *J. biol. Chem.* **102**, 683.
Kitagawa & Yamada (1932). *J. Biochem., Tokyo*, **16**, 339.
Klein (1933). *Handbuch der Pflanzenanalyse*, **4**, (2), 889. (Wien.)
Peters & Van Slyke (1932). *Quantitative clinical Chemistry*, **2**, 545. (Baltimore.)
Rehberg & Blem (1932). *Pflug. Arch. ges. Physiol.* **230**, 689.
Van Slyke & Cullen (1914). *J. biol. Chem.* **19**, 211.