XXXVIII. INTERACTION OF VITAMIN C AND TISSUE PHOSPHATASES

By K. V. GIRI¹

*From the Nutrition Research Laboratories, I.R.F.A., Coonoor,
South India*

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In previous publications [Giri, 1938, 1 and 2] the results of experiments on the interaction of plant phosphatases, vitamin C and glutathione were reported. A similar investigation on animal tissue phosphatases has been undertaken with a view to completing a general survey of the effect of vitamin C upon the activity of phosphatases of both plant and animal origin.

EXPERIMENTAL

Preparation of material. The tissues used in the present investigation were those of sheep kidney, liver and brain. Dry powders were prepared from the tissues by treatment with acetone and ether as described by Giri & Datta [1936]. Active extracts were obtained from the acetone-treated tissue materials, as described below.

The method of determining the activity of the phosphatase and the other procedures were similar to those already described [Giri, 1938, 2].

I. KIDNEY PHOSPHATASE

Influence of vitamin C on the activity of kidney phosphatase. 10 g. acetone-treated kidney powder were extracted with 100 ml. water, saturated with

Table I. *Influence of vitamin C on the activity of kidney phosphatases
without purification by dialysis*

Reaction mixture. 10 ml. glycine buffer *M*/5 (pH 8.9) (for alkaline phosphatase) and 10 ml. *M*/5 acetate buffer (pH 5.0); 10 ml. 2% sodium-β-glycerophosphate (Merck); 4 ml. 0.1 *M* MgCl₂, 6H₂O (only for alkaline phosphatase); 5 ml. enzyme extract. Total volume, 40 ml.; vitamin C, 5 mg.; Cu as CuSO₄, 5H₂O, 0.062 mg.

Time of hydrolysis in min. ...	Activity in mg. in 10 ml. reaction mixture	
	Alkaline phosphatase	Acid phosphatase
	60	120
Crude phosphatase	0.642	0.130
Crude phosphatase + vitamin C	0.642	0.130
Crude phosphatase + vitamin C + Cu	0.620	0.130
Crude phosphatase + Cu	0.642	0.130

Oxidation of vitamin C

	mg. of vitamin C in the total volume of reaction mixture	
Crude phosphatase + vitamin C	4.6	4.4
Crude phosphatase + vitamin C + Cu	2.0	3.5

¹ Lady Tata Memorial Scholar.

toluene for 24 hr. and filtered. A portion of the filtrate (30 ml.) was dialysed for 2 days in collodion bags and centrifuged to remove the suspended impurities.

The results show that the vitamin C, Cu and the vitamin C-Cu complex have no influence on the activity of the alkaline and acid phosphatases of kidney when crude aqueous extracts without purification by dialysis are used (Table I).

Table II A. *Influence of vitamin C on the activity of kidney phosphatase purified by dialysis*

Reaction mixture. 10 ml. glycine buffer *M*/5 (pH 8.9) for alkaline phosphatase and 10 ml. acetate buffer *M*/5 (pH 5.0) for acid phosphatase; 10 ml. 2% Na- β -glycerophosphate; 4 ml. enzyme extract; total volume, 40 ml.; vitamin C, 5 mg.; Cu as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.062 mg.).

Alkaline phosphatase				
Activity in mg. P in 10 ml. reaction mixture				
Time of hydrolysis in min. ...	15	30	60	
1. Purified phosphatase	0.470	0.800	1.50	
2. Purified phosphatase + vitamin C	0.470	0.800	1.50	
3. Purified phosphatase + vitamin C + Cu	0.300	0.500	1.08	
4. Purified phosphatase + Cu	0.470	0.800	1.50	

Oxidation of vitamin C				
mg. vitamin C in the total volume of reaction mixture				
Time in min. ...	0	15	30	60
2. Purified phosphatase + vitamin C	5.0	4.9	4.8	4.5
3. Purified phosphatase + vitamin C + Cu	5.0	2.8	2.4	1.3

Table II B.

Acid phosphatase				
Activity in mg. P in 10 ml. reaction mixture				
Time of hydrolysis in min. ...	60	120		
1. Purified phosphatase	0.083	0.154		
2. Purified phosphatase + vitamin C	0.083	0.150		
3. Purified phosphatase + vitamin C + Cu	0.066	0.100		
4. Purified phosphatase + Cu	0.083	0.150		

Oxidation of vitamin C				
mg. vitamin C in the total volume of reaction mixture				
Time in min. ...	0	30	60	120
1. Purified phosphatase + vitamin C	5.0	4.9	4.7	4.7
3. Purified phosphatase + vitamin C + Cu	5.0	4.8	2.6	1.3

It can be seen from the figures in Tables II A and B, that the vitamin C-Cu complex inhibits the activity of the purified phosphatase, while vitamin C or copper alone has no influence. Thus the behaviour of the phosphatase present in dialysed aqueous extracts of kidney towards the vitamin C-Cu complex is different from that of the enzyme present in crude aqueous extracts of the tissue. Some factors present in the latter appear to interfere with the inhibition of the activity of the phosphatase by the vitamin C-Cu complex. In the light of the previous observations made on the influence of vitamin C on plant phosphatases, it was thought that the factors which interfere with the inhibition of the activity of the phosphatase by the vitamin C-Cu complex, protect the vitamin against oxidation, thereby preventing the inhibition. The following experiment

was therefore undertaken with a view to finding out whether the protective action of kidney extracts against the oxidation of the vitamin is responsible for the differences observed in the behaviour of crude aqueous extracts and dialysed extracts of kidney towards the vitamin C-Cu complex (Table III).

Table III. *Protective action of dialysed and undialysed kidney extracts against the oxidation of vitamin C*

Reaction mixture. 20 ml. acetate buffer *M*/5 (pH 5.0); 5 ml. extract; 5 mg. vitamin C; Cu as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.062 mg.); total volume, 40 ml.

Time in min. ...	mg. vitamin C in the total volume of reaction mixture			
	0	15	30	60
1. Vitamin C	5.0	3.4	3.0	2.3
2. Vitamin C + dialysed extract	5.0	3.8	3.4	3.0
3. Vitamin C + undialysed extract	5.0	5.0	5.0	5.0
4. Vitamin C + Cu	5.0	2.7	2.5	1.4
5. Vitamin C + Cu + dialysed extract	5.0	2.5	2.4	1.0
6. Vitamin C + Cu + undialysed extract	5.0	4.5	4.1	3.6

It is seen from these results that undialysed extracts contain some substances which protect the vitamin against catalytic oxidation by Cu, while the dialysed extract does not exert any such protective action, although a slight protection in the absence of Cu is noticed. Further, the dialysed extracts contain certain substances which accelerate the oxidation of vitamin C by Cu. Such constituents, which are probably colloidal in nature, are found to be present in the dialysed extracts of acetone-treated liver and brain. Further investigation of the nature of the constituents and their relation to the oxidation of the vitamin is in progress.

The results so far obtained on the influence of the vitamin C-Cu complex on the activity of kidney phosphatase show that the activity is in some way related to the oxidation of the vitamin.

II. LIVER PHOSPHATASE

The influence of vitamin C on the activity of liver phosphatase

Preparation of active extract. 20 g. acetone-treated liver powder were extracted with 100 ml. water saturated with toluene for 48 hr. at room temperature and filtered. The filtrate, which was coloured yellow, was used directly. The dialysed extract was prepared by dialysing the crude aqueous extract (60 ml.) for 3 days and centrifuging to remove the suspended impurities.

The results are similar to those obtained with kidney phosphatase. The liver phosphatase activity is inhibited by the vitamin C-Cu complex when dialysed extracts are used as the source of the enzyme, while the activity of the phosphatase present in crude aqueous extracts is not affected by vitamin C-Cu complex. Neither vitamin C nor copper alone has any influence on the activity (Table IV).

Protective action of liver extracts against the oxidation of vitamin C. Table V shows the difference in the protective properties of the dialysed and undialysed extracts of acetone-treated liver powder.

It can be seen from the results that undialysed extracts of liver protect the vitamin against catalytic oxidation, while the dialysed extracts lack such protective properties. The difference in behaviour of liver phosphatase contained in dialysed and undialysed extracts towards the vitamin C-Cu complex

Table IV. *Influence of vitamin C on the phosphatase activity of dialysed and undialysed liver extracts*

Reaction mixture. 10 ml. glycine buffer *M*/5 (*pH* 8.9); 10 ml. 2% Na- β -glycerophosphate; 4 ml. 0.1 *M* MgCl₂, 6H₂O (only for alkaline phosphatase); 5 ml. enzyme extract; total volume, 40 ml. Vitamin C, 5 mg.; Cu as CuSO₄, 5H₂O (0.062 mg.). Temperature, 35 $\pm$ 0.1°.

Time of hydrolysis ...	Activity in mg. P in 10 ml. reaction mixture		
	Undialysed extract	Dialysed extract	
	1 hr.	3 hr.	5 hr.
1. Phosphatase	0.160	0.690	1.00
2. Phosphatase + vitamin C	0.160	0.680	0.950
3. Phosphatase + vitamin C + Cu	0.160	0.520	0.714
4. Phosphatase + Cu	0.160	0.690	1.00

Oxidation of vitamin C

Time in min. ...	mg. vitamin C in the total of reaction mixture							
	Undialysed extract			Dialysed extract				
	0	30	60	0	30	60	120	
2. Phosphatase + vitamin C	5.0	4.5	4.1	5.0	4.6	4.3	3.3	
3. Phosphatase + vitamin C + Cu	5.0	3.8	3.3	5.0	2.2	1.5	0.2	

Table V. *Protective action of dialysed and undialysed liver extracts against the oxidation of vitamin C*

Reaction mixture. 10 ml. glycine buffer *M*/5 (*pH* 8.9); Cu as CuSO₄, 5H₂O (0.062 mg.); 5 ml. 0-100% vitamin C; 5 ml. liver extract; total volume, 40 ml. Temperature, 35 $\pm$ 0.1°.

Time in min. ...	mg. vitamin C in total volume of reaction mixture						
	Undialysed liver extract			Dialysed liver extract			
	0	30	60	0	30	60	
1. Vitamin C	5.0	4.3	4.0	5.0	4.4	3.8	
2. Vitamin C + Cu	5.0	2.2	1.6	5.0	2.1	1.4	
3. Vitamin C + liver extract	5.0	4.8	4.6	5.0	4.4	4.0	
4. Vitamin C + Cu + liver extract	5.0	4.0	3.4	5.0	1.7	0.8	

is due to the presence of certain substances in the undialysed extract, which protect the vitamin against catalytic oxidation, so that the activity of the phosphatase is unaffected in the presence of vitamin C and copper. When these substances are removed by dialysis, the extract loses its protective properties, and the phosphatase is inhibited by the vitamin C-Cu complex because under such conditions the vitamin is oxidized. Further, the results show that, as in the case of kidney extracts, the dialysed extracts of liver contain some factors which accelerate the oxidation of vitamin C by copper.

The influence of glutathione (GSH) on the activities of the alkaline and acid phosphatases of kidney and liver in the presence and absence of vitamin C-Cu complex

The results of experiments on the effect of glutathione on the alkaline and acid phosphatases of kidney and liver in the presence and absence of the vitamin C-Cu complex are presented in Table VI.

The results show that glutathione (GSH) protects the acid phosphatase of kidney from inhibition by the vitamin C-Cu complex, while the inhibition of the activity of the alkaline phosphatases of kidney and liver is not greatly

Table VI. *Effect of glutathione on the activities of the alkaline and acid phosphatases of kidney and liver in the presence and absence of vitamin C-Cu complex.*

Reaction mixture. (Kidney phosphatases): 20 ml. glycine buffer *M*/5 (*pH* 8.9), for alkaline phosphatase and 20 ml. acetate buffer *M*/5 (*pH* 5.0) for acid phosphatase; 10 ml. 2% Na- β -glycerophosphate; 4 ml. $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 *M* (only for alkaline phosphatase); 5 ml. enzyme (dialysed extract); vitamin C, 5 mg.; glutathione, 5 mg.; Cu as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.062 mg.); total volume, 50 ml.

(Liver phosphatase): 10 ml. glycine buffer *M*/5 (*pH* 8.9); 10 ml. 2% Na- β -glycerophosphate; 4 ml. 0.1 *M* $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$; 5 ml. enzyme (dialysed extract); vitamin C, 5 mg.; Cu as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.062 mg.); glutathione, 5 mg.; total volume, 40 ml. Temperature, $35 \pm 0.1^\circ$.

	Activity in mg. P in 10 ml. reaction mixture					
	Kidney alkaline phosphatase		Kidney acid phosphatase 12 hr.	Liver alkaline phosphatase		
	0.5 hr.	1 hr.		2 hr.	4 hr.	
1. Purified phosphatase	0.400	0.640	0.200	0.457	0.800	
2. Purified phosphatase + vitamin C	0.400	0.640	0.200	0.450	0.789	
3. Purified phosphatase + vitamin C + Cu	0.310	0.490	0.040	0.333	0.580	
4. Purified phosphatase + vitamin C + Cu + glutathione (GSH)	0.310	0.500	0.200	0.350	0.640	
5. Purified phosphatase + glutathione (GSH)	0.400	0.571	0.182	0.444	0.762	

Oxidation of vitamin C

	Vitamin C in mg. in the total volume of reaction mixture								
	Kidney alkaline phosphatase			Kidney acid phosphatase			Liver alkaline phosphatase		
	0	30	60	0	30	60	0	30	60
2. Purified phosphatase + vitamin C	5.0	3.6	3.0	5.0	4.8	4.8	5.0	4.5	4.1
3. Purified phosphatase + vitamin C + Cu	5.0	1.4	0.4	5.0	2.2	1.3	5.0	2.2	1.4
4. Purified phosphatase + vitamin C + Cu + glutathione	5.0	3.0	1.4	5.0	4.9	4.9	5.0	4.4	2.9

influenced in its presence. This difference in behaviour of glutathione is perhaps due to the fact that the vitamin is protected completely from oxidation at *pH* 5.0, which is the optimum for the acid phosphatase, while at *pH* 8.9, which is the optimum for the alkaline phosphatase, it has very little protective action. It is found that glutathione itself has a slight inhibiting action on the activity of the phosphatase.

III. BRAIN PHOSPHATASE

The influence of vitamin C on the activity of brain phosphatase

Preparation of extract. *Extract No. 1.* 20 g. acetone-treated brain powder were extracted with 100 ml. water saturated with toluene for 48 hr. at room temperature and filtered.

Extract No. 2. Purified by isoelectric precipitation of the inert material at *pH* 4.8. The extract was prepared by adding an equal volume of *M*/5 acetate buffer *pH* 4.8 to the aqueous extract, and centrifuging to remove the suspended impurities.

Extract No. 3. Prepared by dialysing the crude aqueous extract of acetone-treated brain.

Table VII. *Influence of vitamin C on the phosphatase activities of dialysed and undialysed extracts of brain*

Reaction mixture. The composition was the same as that of the mixture used for kidney and liver phosphatases.

	Activity in mg. P in 10 ml. reaction mixture after 4 hr. hydrolysis	
	Undialysed extract	Dialysed extract
1. Phosphatase	0.158	0.110
2. Phosphatase + vitamin C	0.158	0.110
3. Phosphatase + vitamin C + Cu	0.090	0.060
4. Phosphatase + Cu	0.158	0.110

Oxidation of vitamin C

		mg. vitamin C in the total volume of reaction mixture					
		Undialysed extract			Dialysed extract		
Time in min.	...	0	30	60	0	30	60
2. Phosphatase + vitamin C		5.0	4.2	3.8	5.0	4.1	3.8
3. Phosphatase + vitamin C + Cu		5.0	2.6	1.6	5.0	2.5	1.4

The results show that, unlike kidney and liver phosphatases, the alkaline phosphatase of brain is inhibited by vitamin C-Cu complex irrespective of the purity of the enzyme. This is probably due to the absence of protective substances from the aqueous extracts of acetone-treated brain powder.

The results of the experiments on the protective action of brain extracts against the oxidation of the vitamin are presented in Table VIII.

Table VIII. *Influence of brain extracts on the oxidation of vitamin C*

Reaction mixture. 10 ml. glycine buffer *M*/5 (*pH* 8.9); 5 ml. 0.100% vitamin C; Cu as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.062 mg.); total volume, 40 ml.

		Oxidation of vitamin C mg. of vitamin C in the total volume of reaction mixture							
		Undialysed extract				Dialysed extract			
Time in min.	...	0	15	30	90	0	15	30	60
1. Vitamin C		5.0	4.8	4.6	3.8	5.0	4.6	4.3	4.0
2. Vitamin C + brain extract		5.0	4.8	4.8	4.4	5.0	4.6	4.3	4.0
3. Vitamin C + Cu + brain extract		5.0	3.2	2.6	0.3	5.0	2.8	2.2	1.1
4. Vitamin C + Cu		5.0	3.2	2.6	0.5	5.0	3.0	2.6	1.5

The results show that the protective action of the undialysed extracts of brain is very little compared with those of kidney and liver. The phosphatase is therefore inhibited by the vitamin C-Cu complex, because the vitamin is oxidized by copper under such conditions. Further, just as in the case of dialysed extracts of kidney and liver, brain extracts also accelerate the catalytic oxidations of vitamin C by copper. Experiments on the influence of the vitamin C-Cu complex on the activity of brain acid phosphatase have shown that, like the alkaline phosphatase, the acid phosphatase also is inhibited by the complex.

The influence of glutathione on the vitamin C-Cu inhibition of the activities of the alkaline and acid phosphatases of brain

The results of experiments on the influence of glutathione on the vitamin C-Cu inhibition of the activities of the alkaline and acid phosphatases of brain are presented in Table IX.

Table IX. *Effect of glutathione on the vitamin C-Cu inhibition of brain phosphatase*

Reaction mixture. 10 ml. glycine buffer *M*/5 (*pH* 8.9); 10 ml. 2% Na- β -glycerophosphate; 4 ml. 0.1 *M* MgCl₂, 6H₂O; Cu as CuSO₄, 5H₂O (0.062 mg.); vitamin C, 5 mg.; glutathione, 5 mg.; total volume, 41 ml.

	Activity in mg. P in 10 ml. reaction mixture after 4 hr. hydrolysis	
	Alkaline phosphatase	Acid phosphatase
1. Phosphatase	0.148	0.284
2. Phosphatase + vitamin C + Cu	0.121	0.172
3. Phosphatase + vitamin C + Cu + glutathione (GSH)	0.121	0.284
4. Phosphatase + glutathione (GSH)	0.138	0.284

Oxidation of vitamin C								
mg. vitamin C in the total volume of reaction mixture								
	Alkaline phosphatase				Acid phosphatase			
	0	15	30	60	0	30	60	120
2. Phosphatase + vitamin C + Cu	5.0	3.2	2.3	1.0	5.0	3.4	2.7	1.8
3. Phosphatase + vitamin C + Cu + glutathione (GSH)	5.0	4.4	4.2	3.3	5.0	4.8	4.7	4.7

The results show that glutathione (GSH) entirely prevents both the oxidation of vitamin C at *pH* 5.0 and the inhibition of the activity of the acid phosphatase by the vitamin C-Cu complex. The inhibition of the alkaline phosphatase activity is, however, not influenced by glutathione.

The influence of cysteine, cystine, cyanide and sodium hydrosulphite on the inhibiting action of vitamin C-Cu complex

It is well known that the catalytic influence of Cu on the oxidation of vitamin C is entirely or partly removed by glutathione (GSH), cysteine, cystine and cyanide [Hopkins & Morgan, 1936; De Caro & Giani, 1934]. Glutathione, which prevents the oxidation of the vitamin by metal catalysts by virtue of its ability to form a complex with Cu, is found to annul the inhibiting action of the vitamin C-Cu complex on the activity of the phosphatase. It was thought that the inhibition of the activity of the phosphatase by vitamin C might also be annulled by other substances which prevent the oxidation of the vitamin in the presence of Cu. It was found that such compounds as cysteine, cystine and cyanide, when added to the reaction mixture, either diminished or entirely eliminated the inhibiting action of the vitamin C-Cu complex. Sodium hydrosulphite, which is a reducing agent, was also found to protect the phosphatase from the inhibiting action of the complex.

The results are presented in Table X.

Table X. *Influence of cysteine, cystine, cyanide and sodium hydrosulphite on the inhibiting action of vitamin C-Cu complex*

Reaction mixture. 20 ml. acetate buffer $M/5$ (pH 5.0); 10 ml. 2% Na- β -glycerophosphate; 10 ml. enzyme (purified by isoelectric precipitation of the impurities at pH 4.8); vitamin C, 5 mg.; Cu as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.062 mg.); total volume, 51 ml. Cysteine, 5 mg.; cystine, 5 mg.; NaCN, 0.001 M ; $\text{Na}_2\text{S}_2\text{O}_4$, 0.004 M .

Preparation	Substance tested	Reaction mixture	Activity in mg. P in 10 ml. of reaction mixture after 4 hr. hydrolysis
Brain No. 2	Cysteine	Phosphatase	0.231
		Phosphatase + vitamin C + Cu	0.122
		Phosphatase + vitamin C + Cu + cysteine	0.228
		Phosphatase + cysteine	0.220
Brain No. 3	Sodium cyanide	Phosphatase	0.308
		Phosphatase + vitamin C + Cu	0.160
		Phosphatase + vitamin C + Cu + NaCN	0.308
		Phosphatase + NaCN	0.297
Brain No. 4	Cystine	Phosphatase	0.320
		Phosphatase + vitamin C + Cu	0.173
		Phosphatase + vitamin C + Cu + cystine	0.280
		Phosphatase + cystine	0.320
Brain No. 4	$\text{Na}_2\text{S}_2\text{O}_4$	Phosphatase	0.320
		Phosphatase + vitamin C + Cu	0.173
		Phosphatase + vitamin C + Cu + $\text{Na}_2\text{S}_2\text{O}_4$	0.292
		Phosphatase + $\text{Na}_2\text{S}_2\text{O}_4$	0.310

DISCUSSION

The results obtained in the present investigation on the interaction of vitamin C and tissue phosphatases (kidney, liver and brain) have confirmed the view-point developed by the author previously [Giri, 1938, 1 and 2], that the activity of the phosphatase is related to the oxidation of the vitamin. The phosphatases of all tissues are inhibited by the vitamin C-Cu complex, while the vitamin or copper alone has no effect on the activity. Further, it was found that whereas the phosphatase present in purified extracts of tissues is inhibited by the vitamin C-Cu complex, this is not true of the phosphatases present in the crude aqueous extracts under similar experimental conditions. These results are of particular interest since they suggest that the influence of vitamin C on the activity of tissue phosphatases is associated with certain factors present in the tissues, which protect the vitamin against oxidation.

These experiments have an interesting physiological significance. It seems likely that normal tissues always contain sufficient protecting substances to enable the enzyme to act at its full capacity within the cell in the presence of vitamin C and Cu, and that when these substances are removed or their effective concentration decreased by some means, the activity of the enzyme is adversely affected by the vitamin in association with Cu. Thus the interaction of vitamin C, Cu and protective substances like glutathione, which occur together in all tissues, may play an important role in the regulation of the activity of tissue phosphatases.

SUMMARY

1. The activities of the alkaline and acid phosphatases of kidney, liver and brain are inhibited by the vitamin C-Cu complex. Vitamin C or Cu alone has no effect on their activity.

2. The phosphatase activity of crude aqueous extracts of tissues, with the exception of that of brain, is not influenced by the vitamin C-Cu complex, while the activity of extracts purified by dialysis is inhibited by the complex. This difference in the behaviour of the extracts was found to be due to the presence of factors in crude extracts which protect the vitamin against oxidation.

3. The inhibition by vitamin C-Cu complex was entirely or partly annulled by glutathione, cysteine, cystine and reducing agents like NaCN and $\text{Na}_2\text{S}_2\text{O}_4$.

4. It is suggested that vitamin C, Cu and protective substances like glutathione, which occur together in all tissues, play an important role in the regulation of the activity of tissue phosphatases.

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REFERENCES

- De Caro & Giani (1934). *Hoppe-Seyl. Z.* **228**, 13.
Giri (1938, 1). *Nature, Lond.*, **141**, 119.
— (1938, 2). *Hoppe-Seyl. Z.* **254**, 126.
Giri & Datta (1936). *Biochem. J.* **30**, 1009.
Hopkins & Morgan (1936). *Biochem. J.* **30**, 1446.