2. In all cases the results indicate that the total alkaloids have an effect similar to, or even better than, that of quinine.

3. Industrial and laboratory preparations are of equal value.

4. The total alkaloids from which the quinine has been extracted have a poor therapeutic effect, and the difference between these and the results with the total alkaloids may be due to the synergistic action of small amounts of quinine and other alkaloids.

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The Reducing Properties of Fibrinogen

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The protein, fibrinogen, is characterized by (1) its low solubility in moderately strong salt solutions, (2) its conversion to fibrin by thrombin and (3) its marked instability and ease of denaturation. The last property has made difficult its purification and study but it is now possible [Florkin, 1930] to prepare fibrinogen free from other proteins and without any evidence of denaturation. Study of the clotting of this purified fibrinogen by thrombin [Jaques, 1938] could not be continued until a method was found to stop the reaction quickly in order to obtain samples for analysis. The methods commonly used in enzyme chemistry for this purpose (heat, etc.) are inapplicable because they affect the solubilities of fibrinogen and fibrin. Dilute iodine, potassium permanganate and potassium dichromate, tested for this purpose, were found to give a heavy precipitate with fibrinogen. As this phenomenon was not found with the serum proteins and does not appear to have been reported before, it was investigated. Secondary reactions took place with $K_2Cr_2O_7$ and $KMnO_4$, as shown by re-solution of the precipitate. The study was therefore limited to the action of iodine and H_2O_2.

EXPERIMENTAL

Materials

Fibrinogen. Preparation by repeated precipitation with concentrated salt solutions is not very satisfactory in practice. The principle of Florkin [1930], who found it necessary for preparation of homogeneous solutions of the protein that all operations should be made at 2° and at *p*H 6·0–7·0, has been followed. Fibrinogen is rapidly denatured by acid even at low temperatures [Fay & Hendrix, 1931].

Florkin used NaCl as the precipitant, but the resulting 'salt of fibrinogen' tends to form gels and it is difficult to

control the rates of stirring and of addition of the salt, so as to obtain precipitates which dissolve readily. Also, the NaCl used must be carefully decalcified or the fibrinogen will clot after the first precipitation. Fibrinogen precipitated with ammonium and potassium sulphates or phosphates flocculates and has not the same tendency to form gels [cf. Ferguson & Ericksòn, 1939]. Instead of $(NH_4)_2SO_4$ [Ferguson & Erickson, 1939], phosphate buffer has the advantages that the *p*H is exactly controlled at all stages and the protein content of the solutions can be easily determined by nitrogen analyses. Butler & Montgomery [1932; 1935] have drawn attention to the particularly valuable properties of the equimolecular mixture of K_2HPO_4 and KH_2PO_4, *p*H 6·6, used by Florkin and other associates of Cohn. The method of Florkin has been found by the author to be facilitated if this phosphate buffer is substituted for NaCl. The 3 *M* phosphate buffer of Butler & Montgomery is diluted to 2 *M* before use, and the fibrinogen is precipitated by adding 1 vol. of this solution to 1 vol. plasma. The concentration of phosphate (*M*) appears to correspond to ½ sat. NaCl solution in its precipitating power. The fibrinogen is washed with *M* phosphate buffer and redissolved in a vol. of *M*/4 phosphate buffer slightly less than that of the original plasma. Three such successive precipitations are made. All operations are conducted in the cold room at 2°. All solutions and glassware are chilled before use and it is advisable to rinse the glassware with a weak solution of Na citrate. The salt solution is added slowly to the continuously stirred solution exactly as directed by Florkin. The use of a centrifuge or a Sharples Super Centrifuge installed in the cold room will be found much preferable to filtration. With these modifications, the whole preparation can be readily carried out in 24 hr. The fibrinogen used in this study was prepared from citrated horse plasma, kindly supplied by Dr E. G. Kerslake of the Connaught Laboratories, University of Toronto.

A simple test to demonstrate the complete absence of other plasma proteins in the fibrinogen solution is the lack of any antithrombin action of heparin when tested with the fibrinogen [Jaques & Mustard, 1940]. Mere traces of plasma are sufficient to restore the antithrombin action of heparin.

Methods

The fibrinogen was dissolved in *M*/4 K phosphate buffer (*p*H 6·6). The reaction vessels were 25 ml. glass-stoppered Erlenmeyer flasks rocked in a thermostat at 25°. 1 ml. fibrinogen solution (0·5 mg. N) + 4 ml. of H_2O were taken and 1 ml. *N*/75 I_2 in KI or H_2O_2 added. The consumption of I_2 by the blank was very small but a control with phosphate buffer instead of the fibrinogen solution was always made. The value reported for the I_2 or H_2O_2 consumption represents the difference between the titre of the control and the titre with fibrinogen added.

The reaction was stopped by adding 1 ml. of 60% KI. The residual I_2 was then titrated with standard *N*/200 $Na_2S_2O_3$ using a 2 ml. microburette and starch as an internal indicator. Residual H_2O_2 was estimated by titrating the equivalent I_2 liberated from the KI. For this, 1 ml. of 4*N* H_2SO_4 was added at the same time as the KI, and after 5 min. the contents were titrated. Clotting was carried out as by Jaques [1938]. The thrombin had been purified by the methods of Seegers and was kindly supplied by Parke, Davis & Co., Detroit.

RESULTS AND DISCUSSION

Kinetics of the oxidation of fibrinogen by H_2O_2 or I_2

The value of *K*, the velocity constant of a bimolecular reaction, was calculated from the data of Table 1 by the equation:

$$K = \frac{1}{t\,(C_A - C_B)} \log_e \frac{(C_A - C_x).C_B}{(C_B - C_x).C_A}.$$

The values of *K* for the reaction with H_2O_2 show very good agreement. This indicates that the reaction is bimolecular and shows that probably a specific reacting group is involved. The values with I_2 do not show such good agreement. This was

Table 1. *Reaction of fibrinogen with H_2O_2 and I_2*

H_2O_2: 1 ml. *N*/100 H_2O_2 + 2 ml. fibrinogen (0·890 mg. N)			I_2: 1 ml. *N*/75 I_2 + 1 ml. fibrinogen (0·500 mg. N)		
Reaction time hr.	H_2O_2 consumed ml.	*K*	Reaction time min.	I_2 consumed ml.	*K*
0·5	0·164	0·00430	8	0·511	0·0179
1·0	0·263	0·00398	25	1·044	0·0179
2·0	0·443	0·00428	48	1·250	0·0141
3·0	0·535	0·00414	75	1·381	0·0153
5·0	0·629	0·00378	79	1·435	0·0132
6·6	0·762	—	120	1·562	0·0126
8·0	0·763	—	300	1·713	—
22·0	0·763	—	720	1·711	—
C_A = 1·953 C_B = 0·763			C_A = 2·750 C_B = 1·712		
K (average) = 0·00410			*K* (average) = 0·0152		

(All concentrations are expressed as equivalent ml. of *N*/200 $Na_2S_2O_3$.)

partly due to technical difficulties but, as indicated later, the reaction with I_2 probably involves several groups. The results with I_2 indicate, however, that here also the reaction involves specific groups of the protein. The velocity of the reaction is fairly high, e.g. with I_2 equilibrium was reached in 5 hr. This was in the presence of 0·06% KI from the standard I_2 solution, and KI strongly inhibits the reaction. At equilibrium, 2·17 mg. I_2 and 0·146 mg. H_2O_2 were reduced per mg. fibrinogen. The same equilibrium values were obtained when the relative concentrations of fibrinogen and oxidizing agent were varied. Iodine evidently takes the oxidation further than H_2O_2: the I_2 consumed is just 4 times the H_2O_2—at equilibrium 3·42 ml. *N*/200 I_2 compared with 0·856 ml. *N*/200 H_2O_2/mg. N.

The effect of various factors on the reaction is shown in Fig. 1: (*a*) varying amounts of KI, followed by 1 ml. *N*/75 I_2, were added to a series of tubes and the tubes allowed to stand 7 hr. KI strongly suppresses the reaction, a final concentration of 6% being sufficient to inhibit it completely.

(*b*) Fig. 1*b* shows the effect of *p*H. The amount of I_2 reduced in 1 hr., and of H_2O_2 in 5 hr., was determined. The *p*H level was established by adding before the oxidizing agent either $N/2$ H_3PO_4 or $N/2$ KOH to the fibrinogen solution. From zero reduction at *p*H 2·3, the amount of I_2 reduced rose rapidly with increasing *p*H. Beyond *p*H 7·5 the 'blank' consumption of I_2 became so great that it interfered with the titrations, but there was no evidence of a decreased consumption by fibrinogen. The effect of *p*H on the reduction of H_2O_2 is similar to but less than the effect on I_2. There appears to be a definite break in the *p*H-curve at the isoelectric point of fibrinogen, the H_2O_2 consumption increasing only relatively slowly on its alkaline side. Isoelectric precipitation of fibrinogen occurred from *p*H 4·4 to 5·4. With I_2, precipitation occurred from *p*H 3·1 to 7·5. With H_2O_2, precipitation was observed only in the region of the isoelectric point of fibrinogen. (*c*) Fig. 1*c* shows the I_2 consumption found with varying amounts of fibrinogen, the phosphate concentration being always $M/12$. The I_2 consumption was determined after 1 hr. The resulting curve approximates to a straight line. The approximately linear relationship and the close agreement observed with duplicates (Fig. 1*c*) suggest that the reaction might be used for the determination of fibrinogen. When the oxidation proceeded for only ½ hr., the graph tended to become parabolic.

The results obtained with I_2 (inhibition by KI, rapid increase in the amount of I_2 reduced, with rise in *p*H, etc.) closely resemble those obtained in the iodimetric determination of cysteine and related compounds [Lucas & King, 1932] and suggest that sulphydryl group is responsible. Fibrinogen does not contain a free SH group, however, for it gives a negative nitroprusside reaction. Very little iodine substitution has occurred in the protein, as shown by analysis. It appears likely, therefore, that the reduction of I_2 and H_2O_2 by fibrinogen is due to a group other than sulphydryl.

The reaction of the serum proteins with I_2

A qualitative difference between the reactions with serum proteins and with fibrinogen is evident from the fact that the former do not give any precipitate with I_2. The I_2 consumption of a sample of horse plasma was therefore determined as usual and was corrected for the reducing power of the fibrinogen present. 0·10 ml. plasma (1·10 mg. N) gave an I_2 uptake for 1 hr. of 1·964 ml. Total fibrinogen N of the sample was found to be 0·08 mg. equivalent to 0·211 ml. Therefore the I_2 uptake of the serum proteins = 1·72 ml./mg. N, which is a little lower than the value for fibrinogen (2·64 ml./mg. N).

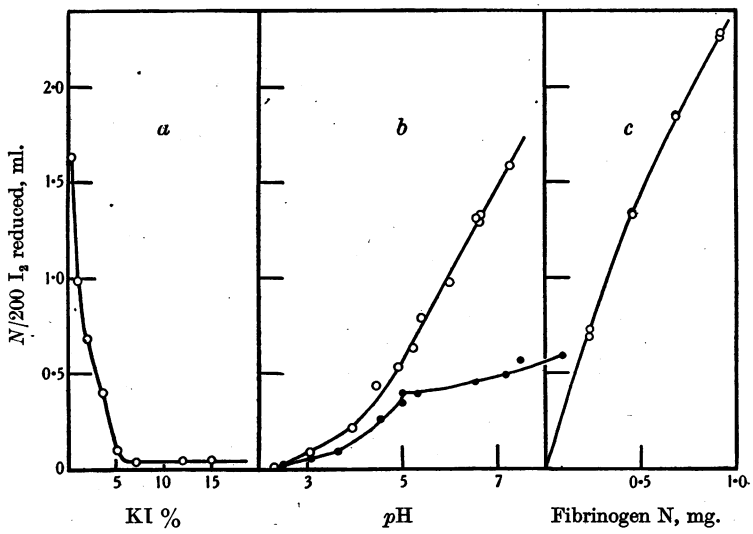

Fig. 1. Effect of various factors on the reaction: *a*, effect of potassium iodide; *b*, effect of *p*H; *c*, effect of fibrinogen concentration. o—o $N/200$ I_2 reduced; •—• $N/200$ H_2O_2 reduced. Fibrinogen present, as mg. N: *a*, 0·500; *b* (I_2), 0·425, *b* (H_2O_2), 0·850. Iodine or peroxide present, 1 ml. of *ca.* $N/100$. Total volume, 6 ml., and phosphate concentration $M/24$, in all.

Effect of denaturation on the reducing properties of fibrinogen

Mirsky & Anson [1936] found that denaturation of proteins increased their reduction of ferricyanide. This was not found with the I_2 reduction by fibrinogen (Table 2). The fibrinogen was denatured in a water-bath at 56°, when an increase in the reducing

Table 2

A. *Effect of denaturation on the reducing properties of fibrinogen*

Incubated at 56° min.	Insol. N mg.	$N/200$ I_2 reduced ml.	$N/200$ H_2O_2 reduced ml.
0	0·000	1·167	0·372
0·5	0·022	1·174	—
2	0·252	1·195	0·366
5	0·325	1·194	0·355
10	—	1·143	0·322
36	0·322	1·094	—
60	—	1·038	0·200
240	—	0·847	—

(H_2O_2 consumption for 5 hr. and for 2 ml. fibrinogen solution (1·0 mg. fibrinogen N). I_2 uptake determined at 25° for 1 hr. for 1 ml. fibrinogen solution (0·5 mg. fibrinogen N).)

B. *Effect of denaturation on the reducing properties of plasma*

Temp. ° C.	$N/200$ I_2 consumed ml.
25	0·955
40	0·956
60	0·957
80	1·113
100	1·179

(Plasma diluted 1 : 25 with H_2O and exposed to the raised temperature for 5 min. I_2 consumption in 1 hr. at 25° was determined for 1 ml. of the diluted plasma.)

value of not more than 2 % occurs in the first 5 min. After prolonged heating, the reducing value falls rapidly, but this was due to a decrease in the rate of reaction, since no difference between the denatured and native fibrinogen was found when the I_2 uptake at equilibrium (12 hr. oxidation) was determined. Similar results were obtained when H_2O_2 was used, but there was no evidence of any increase in the reducing value, which shows a gradual but definite decrease from that of native fibrinogen. In view of the unstable nature of fibrinogen, it was of interest to see if atmospheric oxygen had any effect on the reducing properties of the protein. No difference between the reducing properties of samples of fibrinogen kept in N_2 or in O_2 could be detected, even after prolonged incubation at 56°.

The effect of alkali and acid, to which fibrinogen is very sensitive, was tested on the reducing properties of the protein. Samples of the protein were kept 24 hr. at 25° at various *p*H levels, and their I_2 consumption at *p*H 6·6 was then determined. There was very little effect, however, so that this lability is not associated with or accompanied by a change in the reducing groups.

While denaturation has little effect on the reducing properties of fibrinogen, it definitely increases the reducing power of plasma, as shown in Table 2B. 3 ml. of water were added to 1 ml. of a 1 : 25 dilution of plasma and the tube was placed in a water-bath at the desired temperature for 5 min. The tube was then cooled, 1 ml. phosphate buffer and 1 ml. I_2 added, and the I_2 consumption after 1 hr. determined. There is no increase in the I_2 consumption after heating at temperatures below 80°, i.e. the denaturation of fibrinogen causes no increase in the I_2 consumption. On heating to 100°, where denaturation of the serum proteins will occur, there is a definite increase (20 %) in the I_2 consumption.

Changes in the reducing power of fibrinogen during clotting

To 5 ml. solution (0·880 mg. fibrinogen N in $M/10$ phosphate buffer) was added 0·1 ml. of a crude thrombin solution (0·021 mg. N). After 10 min., when a firm clot had formed (fibrin N = 0·564 mg.), 1 ml. $N/75$ I_2 was added and the amount of I_2 reduced after 1 hr. determined. For the clot this was 1·18 ml., while for the fibrinogen and thrombin it was 2·24 + 0·065 = 2·30 ml. This result indicates less reduction by the fibrin but does not distinguish a slower rate of reaction from a lower reducing power. Therefore, the amounts of H_2O_2 and I_2 reduced by the time equilibrium had been attained (30 hr.) were determined. To 5 ml. solution containing 1·0 mg. fibrinogen N was added 0·1 ml. thrombin solution (0·01 mg. Parke, Davis thrombin). After 3 hr. (fibrin N = 0·762 mg.) 1 ml. $N/50$ H_2O_2 was added. 0·903 ml. $N/200$ H_2O_2 was reduced after clotting, compared with 0·819 ml. for the fibrinogen control. This represents an increase of 0·110 ml./mg. of fibrin N, i.e. 13 %. In a similar experiment with I_2, the amount of I_2 reduced after clotting (fibrin N = 0·362 mg., original fibrinogen N = 0·478 mg.) was 1·818 ml., that of the fibrinogen control, 1·812 ml.

The reducing power of fibrin at equilibrium was more accurately determined with commercial beef fibrin (Table 3). 10 mg. samples were weighed out

Table 3. *The reduction of H_2O_2 and I_2 by fibrin*

Time hr.	$N/200$ H_2O_2 reduced ml.	Time hr.	$N/200$ I_2 reduced ml.
6	1·390	1·3	3·32
12	1·792	3	3·83
17	1·810	3·5	3·86
20	1·808	7·5	5·13
24	1·898	19	5·57
28	1·695	24	5·26
36	1·841	27	5·95
40	1·870	29	5·95

(10 mg. fibrin = 1·401 mg. N.)

on the micro-balance and 5 ml. $M/20$ phosphate buffer and 1 ml. H_2O_2 or I_2 solution added. The

average values for the amount of $N/200$ H_2O_2 and I_2 reduced at equilibrium were 1·823 and 5·57 ml. This gives values of 1·301 and 3·98 ml./mg. N. The previous values found for fibrinogen were 0·855 and 3·42 ml., so that the increase in reducing power is equivalent to 0·446 ml. H_2O_2 and 0·56 ml. I_2. The increase in reducing power is approximately the same with the two reagents but, owing to the more extensive oxidation by I_2, the increase with this reagent is a much smaller percentage of the total titration and hence the experimental error is increased accordingly. The increase is equivalent to half the reducing power of fibrinogen for H_2O_2.

These high concentrations of I_2 and H_2O_2 appear to have little effect on the activity of thrombin. Further, after oxidation of fibrinogen with H_2O_2, the addition of thrombin still results in a normal clot. The product resulting from the action of I_2 on the protein, however, is not clotted by thrombin, although thrombin still attacks it. The more extensive oxidation by I_2 evidently interferes with the gel properties of fibrin, since no clot is formed by the thrombin and the altered fibrin is merely precipitated.

The reduction of H_2O_2 by non-protein substances under the conditions tested

As a possible indication of the nature of the reducing groups of fibrinogen, 20 mg. of various substances were weighed into flasks, and 1 ml. of $M/4$ phosphate buffer and 4 ml. water added; 1 ml. of $N/75$ H_2O_2 was then added, the flask shaken for 28 hr., and the excess H_2O_2 titrated (Table 4).

Table 4. *Reduction of H_2O_2 by various substances*

Substance (20 mg. unless otherwise indicated)	$N/200$ H_2O_2 reduced ml.
l-Tyrosine	0·000
l-Tryptophan	0·246
2-Methyl-1:4-naphthoquinone	0·161
2-Methyl-1:4-naphthohydroquinone	0·247
Glucose	0·603
p-Dimethylamino-benzaldehyde	0·950
Casein	0·726
Fibrinogen	2·402
Cysteine HCl (1·0 mg.)	0·822
Ascorbic acid (0·1 mg.)	0·451

Tyrosine did not reduce H_2O_2. Tryptophan, glucose and vitamin K showed slight activity but this was very small compared with that of fibrinogen. Cysteine was more active than fibrinogen, but on the basis of these reduction figures fibrinogen would contain 15% cysteine and fibrin 22%: values reported for the cysteine content of fibrin are from 1·5 to 3·7% [Jordan Lloyd & Shore, 1938]. This suggests that cysteine is not responsible for the reduction by fibrinogen, and it seems reasonable to assume that the reducing group will not be more than 5% of the protein. The high reducing power of the active group is shown by the fact that only ascorbic acid gave a reduction of this order.

DISCUSSION

The characteristic reaction of fibrinogen with iodine appears to indicate a further example of the non-sulphydryl reducing groups of proteins studied by Mirsky & Anson [1936] and more recently by Bowman [1941 *a*, *b*] and ascribed by them to the tyrosine and tryptophan residues of the protein. Mirsky & Anson studied the reduction of ferricyanide by edestin, serum globulin, egg albumin and gelatin. There appear to be marked differences between the reaction studied by them and that reported here, although the same group appears to be involved. They found that their reaction was not clearly defined, for even when the ferricyanide concentration was 30 and 50 times that reduced by the protein, the amount reduced still depended on the ferricyanide concentration. This is in marked contrast to the present results, which show a clearly defined bimolecular reaction. Also, they found little reduction of the ferricyanide unless the protein had been denatured. Fibrinogen reduces no more H_2O_2 or I_2 after denaturation than before, although denatured serum proteins do show an increase in reduction. Bowman observed the reduction of I_2 by the chorionic gonadotropic hormone, and found that the reaction was inhibited by KI, and that the presence of phosphate markedly and specifically accelerates the reaction. The fibrinogen used in the above experiments was dissolved in phosphate buffer. Bowman found that in the presence of phosphate, tyrosine reduces I_2 very rapidly at room temperature, the reaction being influenced by KI and *p*H in the same way as the reduction by protein. But tryptophan may be equally responsible for the reducing power, since under the same conditions tryptophan shows equally strong reducing properties.

The results with H_2O_2 indicate, however, that fibrinogen contains another reducing group with much stronger reducing properties than tyrosine or tryptophan. This group reduces H_2O_2 at *p*H 5–7. Further, there is an apparent increase in the number of such groups in the protein on conversion of the fibrinogen to fibrin. The nature of this group is unknown, cysteine and ascorbic acid being the only simple substances tested which show similar reducing power; but the negative nitroprusside reaction and the quantitative relationships found suggest that the former is not responsible.

SUMMARY

The action of H_2O_2 and I_2 on fibrinogen has been studied. Fibrinogen in phosphate buffer solution reduces these substances rapidly at 25° and *p*H 6·6. The reaction with I_2 is associated with the aromatic residues of the protein. The reducing power of fibrinogen is not changed by denaturation, but the reducing power towards H_2O_2 is increased on conversion to fibrin.

The substance of this paper was presented before the meeting of the Division of Biological Chemistry of the American Chemical Society at Detroit, Michigan, 13 April 1943.

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The Vitamin B_1 Content of Potatoes

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The use of the mould *Phycomyces blakesleeanus* as a test organism for estimating vitamin B_1 was first worked out by Schopfer & Jung [1937*b*]. The fungus responds to such small quantities of the vitamin that the method is the most sensitive known; on this account it was adapted for estimating the vitamin B_1 content of human blood by A. P. Meiklejohn [1937] and Sinclair [1938; 1939]. The method has been used to estimate vitamin B_1 in plant materials by several American workers [Burkholder & McVeigh, 1940; Robbins, 1939; Bonner & Erickson, 1938]. It has the advantage, as compared with other methods, of requiring such small samples of plant material that it can be used to study the distribution of the vitamin in a single plant or part of a plant.

This paper deals with the use of the *Phycomyces* method for estimating the vitamin B_1 content of potato tubers, and with variations in the vitamin content and its distribution in the potato plant.

METHODS

The method employed was an adaptation, for use with plant material, of the method for blood estimations worked out by Meiklejohn and Sinclair. It consisted essentially in the comparison of the growth of *Phycomyces* in cultures containing potato samples with a dose-response curve obtained from a series of control cultures containing known amounts of the vitamin.

Medium. The *Phycomyces* cultures were grown in 50 ml. conical flasks; each flask contained 4 ml. of a mixture of equal parts of the following solutions in distilled water:

Glucose (A.R.)	Equal weights of solid and water
Asparagine (recrystallized)	4 g./100 ml.
KH_2PO_4 (A.R.)	1·5 g./100 ml.
$MgSO_4$, $7H_2O$ (A.R.)	0·5 g./100 ml.

The volume of liquid in each flask was made up to 10 ml. by the addition of vitamin B_1 solution and distilled water, or distilled water only.

Control cultures. A range of 12 control cultures was set up in every experiment, containing the following amounts of vitamin B_1 in 10 ml. liquid: none, 0·05, 0·1*, 0·15, 0·2*, 0·25, 0·3* μg., and excess (2·5 or 5 μg.) (quantities marked * in duplicate). A solution of vitamin B_1 containing 1 μg./ml. was freshly prepared for each experiment from a stock solution in 75% ethanol (*p*H 4·5) kept in a refrigerator. The required volume of the dilute solution was added to medium and water in each flask, immediately before the adjustment of the *p*H.

Potato samples. The size of sample was designed to give a reading in the central part of the dose-response curve. Preliminary experiments showed that, for potato, samples of between 0·1 and 0·2 g. were correct. According to the amount of vitamin expected, samples were taken of 0·1, 0·15, or 0·2 g. (±10 mg.), in triplicate from each tuber, or part of a tuber, to be tested, by taking out cores with a small cork borer and cutting off the required length. Skin samples were taken by removing the corky skin and cutting a thin slice off the exposed surface, and sprout and leaf samples by cutting off a small piece. All samples were