CCLXIV. THE INDOPHENOL-REDUCING CAPACITY AND THE VITAMIN C CON- TENT OF EXTRACTS OF YOUNG GERMINATED PEAS.

By SYDNEY WALGATE JOHNSON.

From the Division of Nutrition, Lister Institute, London, S.W.1.

(Received November 6th, 1933.)

It has been known for a considerable period that though many dried seeds are devoid of antiscorbutic activity, they acquire this property very readily on germination. This observation, first made by Young [1782; quoted by Curtis, 1807], was demonstrated in the laboratory by Fürst [1912]. Quantitative data in this connection were first provided by Chick and her collaborators. In the case of peas (*Pisum sativum*) Chick and Delf [1919] found that young growing guinea-pigs could be fully protected from scurvy by adding as a supplement to their scurvy-producing diet 10 g., or in some cases 5 g., of peas which had been germinated for 2 days.

Recently the technique in which the capacity of antiscorbutic solutions for reducing indophenols, introduced by Zilva [1927], has been utilised by several workers in the quantitative assessment of vitamin C, since this vitamin (*l*-ascorbic acid) is known to reduce these indicators. This procedure is open to grave objections not only because substances other than ascorbic acid can reduce these indicators, but also on account of the fact that ascorbic acid is capable of existing in an oxidised form which possesses a very high antiscorbutic activity but which is incapable of reducing indophenols.

Harris and Ray [1933], on the basis of indophenol titrations of trichloroacetic acid extracts, record the following values for the ascorbic acid content of germinated peas.

	Ascorbic acid content	
	mg. per g.	mg. in single seed or seedling
Seed peas, before germination	0.00	0.00
„ soaked 24 hrs. (not germinated)	0.08	0.02
„ „ 48 „ (germinated)	0.69	0.21
„ „ 72 „ (germinated)	0.82	0.26
„ „ 96 „ (germinated)	0.86	0.27

Euler and Klusmann [1932; 1933, 1, 2] state that extracts obtained from dried fresh green peas and from pea seedlings varying in age from 7 to 32 days possess a remarkably low capacity for reducing indophenol. In the case of an extract of a 32-day old seedling this reducing capacity was increased ninefold by treatment with hydrogen sulphide, but even then its value was considerably less than that anticipated from its known biological activity. The spectrophotometric estimation of the ascorbic acid content of the extract before treatment with hydrogen sulphide appeared to show that it contained 100 times the amount of this substance indicated by the indophenol titration. On the basis of these

observations Euler suggests that the ascorbic acid in the germinating seedling is bound to "Hemmungstoffe," presumably in such a manner as to inhibit its reducing properties, from which it is liberated by hydrolysis in the animal body. So far, however, his experiments with various enzymes *in vitro* have failed to substantiate this opinion.

The experiments to be described here were undertaken in order to ascertain firstly whether a quantitative relationship exists between the indophenol-reducing capacity and antiscorbutic activity of extracts of peas in the early stages of germination and secondly, if no such relationship exists, whether this is due to the presence of an active oxidised form of the vitamin in the young seedling. Evidence will be produced which shows that the indophenol-reducing capacity of extracts is no true index of the antiscorbutic potency of the seedlings from which they were obtained and that the formation of the active oxidised form of the vitamin during the early stages of germination is unlikely.

EXPERIMENTAL.

Indophenol titrations of pea extracts.

In all cases, titrations were made with $N/1000$ indophenol (*p*-dimethylamino-phenylindophenol) which was standardised against titanous chloride. Titrations in acid solution were usually carried out by adding 1.0 cc. of 80 % acetic acid to 5 cc. of neutral extract.

I. *Dried peas (Pisum sativum).*

The dried peas were ground in a mill and then passed through a sieve to remove the skins. The sieved product was then milled to a fine flour. All the extracts described below were prepared from this flour, typical examples of each series being given below.

(a) *Aqueous extract.* 5 g. of pea flour were ground in a mortar for 10 minutes with 25 cc. of water and the mixture then left to stand for half an hour, after which it was centrifuged. The reaction of the supernatant liquid was adjusted to p_H 7.0 with dilute ammonia. The extract was turbid and yellow-green in colour.

1 g. equivalent of the pea flour reduced 8.0 cc. of $N/1000$ indophenol. The rate of reduction was relatively high at first but slowed down considerably towards the end. Titrations in acid solution were vitiated by the separation of a voluminous precipitate. A similar precipitate also separated on the addition of alkali. The extract did not reduce ammoniacal silver nitrate solution but it gave a marked nitroprusside reaction.

(b) *Phosphate extract.* 5 g. of pea flour were extracted with 25 cc. of 0.87 % K_2HPO_4 solution (Thunberg's solution) in precisely the same manner as the above. This extract was similar in appearance and properties to the aqueous extract.

1 g. equivalent of pea flour reduced 8.0 cc. of $N/1000$ indophenol in a similar manner to the previous extract.

(c) *Trichloroacetic acid extract.* 20 g. of pea flour were extracted in the above manner with 50 cc. of 5 % trichloroacetic acid. The deep green-coloured liquid was adjusted to neutrality with ammonia and centrifuged. A clear light yellow-green extract was finally obtained. 1 g. equivalent of the pea flour reduced 2.5 cc. of $N/1000$ indophenol quickly in acid solution and the same amount rather slowly in neutral solution. The extract gave a nitroprusside reaction.

It also deposited fine sharp-pointed needles on making alkaline with ammonia. It did not reduce ammoniacal silver nitrate solution.

(d) *N/10 sulphuric acid extract.* This extract was obtained from 20 g. of pea flour and 50 cc. of *N/10* sulphuric acid by the same procedure as above. The neutralised extract was similar in character and properties to the trichloroacetic acid extract. 1 g. equivalent reduced 2.5 cc. of *N/1000* indophenol in acid solution and 3.8 cc. at a slower rate in neutral solution.

(e) *Protein-free aqueous extract.* The rather low reducing capacity of the last two extracts seemed to show that this condition was associated with the absence of protein, indeed the removal of protein from aqueous extracts caused a diminution of their capacity for reducing indophenols, as will be seen from the following examples.

20 g. of pea flour were extracted with 50 cc. of water as previously described. In order to free it from protein the extract was acidified with 0.5 cc. 80 % acetic acid and centrifuged. The supernatant liquid was then decanted, heated to boiling-point, cooled and filtered. The filtrate was adjusted to neutrality with ammonia and filtered from the small amount of flocculent precipitate which separated. The final solution was clear and green-yellow in colour. 1 g. equivalent of pea flour reduced only 2.5 cc. of *N/1000* indophenol in both acid and neutral solution. The extract was also similar in properties to the acid extracts.

(f) *Protein-free phosphate extract.* This extract was prepared in the same manner as the previous extract from 20 g. of pea flour and 50 cc. of 0.87 % K_2HPO_4 solution. It had the same character and reducing properties as the aqueous extract and possessed the same capacity for reducing indophenol.

In all these extracts, the intensity of the nitroprusside colour appeared to be proportional to the capacity for reducing indophenol and the amount of the crystalline precipitate obtained with ammonia from the protein-free extracts was always greatest in those which gave the most intense nitroprusside reaction. The presence of a reducing substance other than ascorbic acid, which would reduce indophenol, was anticipated from the work of Kozlowski [1926; 1931] who isolated from peas a compound which contained cysteine and resembled glutathione. Whether the reducing substance met with here is identical with the one which Kozlowski isolated from peas has not been determined, but the crystalline material precipitated from the protein-free extracts by ammonia certainly resembles a similar substance which he found associated with his glutathione-like compound and which evidently can be obtained from the latter by hydrolysis.

II. *Germinated peas.*

The peas were soaked for 24 hours and then spread out on a layer of wet clean silver sand and lightly covered with drier sand so that they could respire freely. After 3 days, the seedlings were taken up, washed free from sand and the skins removed before extracts were prepared from them. The sand was well washed after each germination.

Taking the dry weight of the seed peas as 100 %, the average dry weights of some 30 batches of soaked and germinated seeds were 53 % and 43 % respectively. The average length of the radicles of the same batches of seedlings was 4.2 cm. The variations from these averages were small.

(a) *Aqueous extract.* 25 g. of germinated peas were finely ground in a mortar with 10 g. of clean sand and 50 cc. of water. The residue obtained by squeezing the mixture through muslin was again ground until it was uniformly fine. The watery extract was then added to it and the mixture well ground once more,

after which it was left to stand for half an hour. It was finally squeezed through muslin and the liquid centrifuged. The resulting extract, which was almost neutral (p_H 6.0) was yellow-green in colour and turbid. The addition of acids or alkalis caused the separation of a bulky precipitate. The titrations varied a great deal and only a few of the extracts gave a nitroprusside reaction. None of them reduced ammoniacal silver nitrate in the cold. The average titrations of 35 such extracts were 1.1 cc. and 0.9 cc. $N/1000$ indophenol at acid and neutral reaction respectively per g. equivalent of germinated pea.

(b) *Phosphate extract.* This extract was prepared in the same manner as the above, except that the germinated peas were extracted with 50 cc. of 0.87 % K_2HPO_4 solution. The extract, which was darker in colour than the aqueous extract, invariably gave a nitroprusside reaction and like that extract it did not reduce ammoniacal silver nitrate in the cold. It was also almost neutral in reaction and gave heavy precipitates with acids and alkalis. The indophenol titrations in this case were fairly uniform. The reduction of the indicator, however, was a composite effect which was due to at least two reducing substances. One of these substances, like ascorbic acid, reduced the indicator very quickly in acid and more slowly in neutral solution. The other, which was most probably the substance which gave the nitroprusside reaction (possibly Kozłowski's glutathione compound), reduced the indicator at a slower rate at both acid and neutral reaction. The average titrations due to the rapidly reducing substance or substances of 35 such extracts were 2.65 cc. and 2.5 cc. of $N/1000$ indophenol at acid and neutral reaction respectively per g. equivalent of germinated pea.

(c) *Trichloroacetic acid extract.* 50 g. of germinated peas were extracted with 75 cc. of 5 % trichloroacetic acid by the same procedure as above. After centrifuging, the extract was neutralised with ammonia and filtered. The final solution was light green-yellow in colour and slightly turbid, due probably to the starch which it contained. It invariably gave a nitroprusside reaction and deposited a crystalline substance (sharp-pointed needles) on making alkaline with ammonia, which was identical with that obtained from the dry pea flour. Its ability to reduce ammoniacal silver nitrate was doubtful, it inhibited the reduction of this reagent by decitrated lemon juice.

In the case of this extract, the extent of the reduction of indophenol by the substance or substances which reduced it rapidly was more easily ascertained than in the case of the phosphate extract. 1 g. equivalent of the extract had, thus, an average titration of 2.2 cc. of $N/1000$ indophenol at both acid and neutral reactions.

(d) *Sulphuric acid extract.* 50 g. of germinated peas were extracted with 100 cc. of $N/10$ H_2SO_4 in the same manner as in the case of the trichloroacetic acid extract. The final preparation was clear and contained no starch. Its properties were usually the same as those of the previous extract though it generally gave a more intense nitroprusside reaction than the trichloroacetic acid extract and deposited a greater abundance of the needle crystals on making alkaline with ammonia. 1 g. equivalent of the germinated peas reduced quickly 2.0 cc. $N/1000$ indophenol at both acid and neutral reactions.

(e) *Aqueous cyanide extracts.* It is now well known that the oxidation of ascorbic acid is greatly retarded by the presence of cyanides [Szent Györgyi, 1928; Euler, Myrbäck and Larsson, 1933]. It was, therefore, desirable to examine extracts prepared with solutions containing cyanides. The extract was obtained from 25 g. of germinated peas with 50 cc. of water containing 1 cc. of 5 % NaCN solution by the procedure already described for the simple aqueous

extract. The protein was removed from it with acetic acid in the same manner as in the case of the aqueous extract of dry pea flour. The final preparation was very similar in all its properties to the sulphuric acid extract described above. An average of several titrations showed that 1 g. equivalent of germinated peas rapidly reduced 2.4 cc. and 2.6 cc. *N*/1000 indophenol in acid and neutral solution respectively.

(f) *Cyanide-phosphate extracts*. The procedure followed here was precisely the same as in the previous case. A mixture of 1.0 cc. of 5 % NaCN and 8.5 cc. of 0.87 % K_2HPO_4 solutions diluted to 50 cc. was used to extract 60 g. of germinated peas. These preparations gave a more intense nitroprusside reaction, deposited more crystals on making alkaline with ammonia and had, consistently, a greater capacity for reducing indophenol quickly than the corresponding aqueous extract. The indophenol titrations were quite uniform. The averages for 42 such extracts per g. equivalent of germinated peas were 3.0 cc. and 3.4 cc. *N*/1000 indophenol in acid and neutral solution respectively.

The effect of hydrogen sulphide on various extracts of germinated peas. The presence in these extracts of the active oxidised form of the vitamin, which is incapable of reducing indophenol, was next considered. Attempts were made to convert any such form, if present, to the reduced form by treatment with hydrogen sulphide. Three extracts were used (1) trichloroacetic acid, (2) deproteinised aqueous extract, (3) deproteinised cyanide-phosphate extract. The treatment with hydrogen sulphide was carried out in the manner previously described [Johnson, 1933]. Table I shows the reducing capacities of these extracts before

Table I.

Extract	cc. <i>N</i> /1000 indophenol reduced by 1 g. equivalent			
	Before treatment with hydrogen sulphide		After treatment with hydrogen sulphide	
	Acid solution	Neutral solution	Acid solution	Neutral solution
Aqueous extract	0.0	0.0	0.3	0.3
Trichloroacetic acid extract	1.8	1.8	2.0	2.0
Cyanide-phosphate extract	2.3	2.3	2.5	2.5

and after treatment with hydrogen sulphide. The small increments shown cannot be considered significant.

Distribution of indophenol-reducing substances between cotyledons and radicles of germinated peas. 70 g. of germinated peas were divided into cotyledons and radicles. The former weighed 60 g. and the latter 10 g.

The cotyledons were extracted with 50 cc. of 5 % trichloroacetic acid in the manner already described. The neutralised extract, which was clarified with a small amount of alumina gel, gave a marked nitroprusside reaction and deposited needle crystals with ammonia in good yield.

1 g. equivalent reduced 3.3 cc. of *N*/1000 indophenol quickly in acid solution and 3.5 cc. in neutral solution.

The radicles were extracted with 20 cc. of 5 % trichloroacetic acid in the same manner as the cotyledons.

The final preparation, which was only faintly coloured, gave only a very doubtful nitroprusside reaction, and deposited an exceedingly small amount of needle crystals on adding ammonia. 1 g. equivalent reduced 4.3 cc. and 4.5 cc. *N*/1000 indophenol quickly at acid and neutral reaction respectively.

There appeared to be only one indophenol-reducing substance present in the radicles judging from the decisiveness of the end-points in the titrations and the absence of substances giving a nitroprusside reaction.

It will be seen that though the radicle has a greater indophenol-reducing capacity per g. than the cotyledons, the latter nevertheless possess 5/6 of the total amount of the reducing substances. Whether this ratio represents the distribution of ascorbic acid, can only be decided by future work. It may be that the reduction of indophenol in the case of the extract of the radicle is due only to ascorbic acid but this does not seem to be the case with the extract of the cotyledon.

Biological tests.

It was not possible to examine all the above preparations biologically for vitamin C content. Representative experiments were, therefore, performed which were calculated to shed some light on the quantitative relationship between the biological activity and the reducing capacity.

(1) *Dried peas.* To four guinea-pigs kept on the usual basal diet used in this laboratory, a daily dose of 2.5 g. of ungerminated peas was given in the form of flour. All the animals succumbed in the usual way to scurvy within the usual time.

(2) *Germinated peas.* Daily doses of 1.0, 2.5 and 5.0 g. were tested in this case and the results are given in Fig. 1. Under the conditions of germination

described above the peas were found to contain about 43 % of dry matter. There was no serious deviation from this figure.

(3) *Aqueous extract.* Three guinea-pigs were used in this experiment. A daily dose of an equivalent of 2.5 g. of germinated peas delayed somewhat the onset of scurvy, but all the animals died of the disease between 40 and 45 days. The daily dose of this extract reduced about 2.5 cc. of *N*/1000 indophenol. A dose

of ascorbic acid of an equivalent reducing capacity (about 0.25 mg.) would have afforded very much better protection [*cf.* Hirst and Zilva, 1933].

(4) *Phosphate extract.* Daily doses of an equivalent of 2.5 g. of germinated peas were tested on three guinea-pigs and as will be seen from Fig. 1, the protection was very much less than that obtained with 2.5 g. of germinated peas. Furthermore, the average dose reduced about 6.0 cc. of *N*/1000 indophenol which should be equivalent to 0.5 mg. of ascorbic acid. The antiscorbutic potency of this dose, however, falls somewhat but definitely short of that observed with 0.5 mg. of ascorbic acid [Hirst and Zilva, 1933]. The titration value of this extract gives, therefore, no quantitative index of the antiscorbutic potency of the germinated peas from which it was obtained since, in the first place, the vitamin is not thoroughly extracted and, secondly, the reducing capacity of the extract is apparently not entirely due to ascorbic acid. It should be pointed out that these extracts were prepared for the best part of the duration of the test from the germinated peas tested above.

(5) *Cyanide-phosphate extract.* These results are also given in Fig. 1. The average daily dose which was equivalent to 2 g. of the germinated peas reduced about 6.0 cc. of *N*/1000 indophenol. In this case also the equivalent extract was less potent than the germinated peas from which it was obtained and its capacity for reducing indophenol was rather higher than it would have been had the reduction been due to ascorbic acid alone.

DISCUSSION OF RESULTS.

Concerning the first aim of this investigation, namely, to ascertain whether a quantitative relationship exists between the indophenol-reducing capacity and the antiscorbutic activity of extracts of peas and the peas themselves, it may be said that the above results supply fairly conclusive evidence. As was seen, the phosphate and phosphate-cyanide extracts showed only half of the antiscorbutic activity of the peas from which they were obtained. Furthermore, these extracts, as well as the aqueous extract, were found to be less active antiscorbutically than would have been expected if their capacity for reducing indophenol was due solely to ascorbic acid. There was present in these extracts at least one other reducing substance which, though it reduced indophenol more slowly than ascorbic acid in the last stages of the titration, reduced this indicator fairly quickly at the beginning of the titration, particularly when its concentration was relatively high. It is evident, therefore, that the presence of such substances would seriously vitiate the end-point of an ascorbic acid estimation by indophenol.

It is highly probable that the other extracts (trichloroacetic acid and sulphuric acid), which possessed reducing capacities of a similar order and which showed similar chemical properties to the above, would also be less potent antiscorbutically than the peas from which they were obtained.

With regard to the second problem of this investigation, it seems certain from the evidence obtained that the activity of the peas is due entirely to the reduced form of the vitamin. It was seen that treatment with hydrogen sulphide did not increase the indophenol-reducing capacity of the extracts as would have been the case if the active oxidised form of the vitamin had been present. Further, the presence of the latter would have imparted to them a higher antiscorbutic activity than would have been anticipated from the indophenol titrations instead of a lower.

The lesser activity of the extracts than that of the peas from which they were prepared can best be explained on the grounds that it is not possible, by

the methods used, to extract the vitamin completely. This, incidentally, shows the ease with which misleading results may accrue from testing extracts instead of tissues.

If it should be that a part of the vitamin is bound to "Hemmungstoffe," as Euler suggests, it would seem most probable that such a complex would be insoluble or otherwise inextractable.

The stabilising influence of phosphate on the vitamin, which is at present inexplicable, has already been indicated by the work of Euler, Myrbäck and Larsson [1933].

SUMMARY.

1. A number of extracts have been prepared from peas germinated for 3 days and both their indophenol-reducing capacities and their antiscorbutic activities have been determined.

2. The extracts with even the highest indophenol-reducing capacity were found to possess only half the antiscorbutic activity of the germinated peas from which they were obtained.

3. Aqueous, phosphate and cyanide-phosphate extracts were found to be less active antiscorbutically than would have been expected if their indophenol-reducing capacity had been entirely due to ascorbic acid.

4. All extracts from the germinated peas contain at least one substance other than ascorbic acid which reduces indophenol.

5. No evidence was obtained which shows that the active oxidised form of the vitamin is present in the early stages of germination.

6. The ungerminated peas, which showed no antiscorbutic activity when tested in quantities of 2.5 g., gave extracts which also reduced indophenol, though at a rather slower rate than ascorbic acid.

My thanks are due to Dr S. S. Zilva for help and criticism and to the Medical Research Council for a whole time grant.

REFERENCES.

- Chick and Delf (1919). *Biochem. J.* **13**, 199.
Curtis (1807). Cited in Vitamins (Med. Res. Council), 189.
Euler and Klusmann (1932). *Svensk Kem. Tidskr.* **44**, 290.
— (1933, 1). *Ark. Kem. Min. Geol.* **11** B, 1.
— (1933, 2). *Z. physiol. Chem.* **219**, 215.
— Myrbäck and Larsson (1933). *Z. physiol. Chem.* **217**, 1.
Fürst (1912). *Z. Hyg.* **72**, 155.
Harris and Ray (1933). *Biochem. J.* **27**, 580.
Hirst and Zilva (1933). *Biochem. J.* **27**, 1271.
Johnson (1933). *Biochem. J.* **27**, 1287.
Kozłowski (1926). *Biochem. J.* **20**, 1346.
— (1931). *Biochem. Z.* **241**, 407.
Szent Györgyi (1928). *Biochem. J.* **22**, 1387.
Zilva (1927). *Biochem. J.* **21**, 689.