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Addendum

(Received 15 October 1943)

Since the submission of this manuscript it has come to our notice that Kagan, Latker & Zfasman (1942) have demonstrated phosphorylysis of sucrose with intact cells of *Leuconostoc mesenteroides*. Doudoroff, Kaplan & Hassid (1943) have shown the occurrence of a *Pseudomonas saccharophila* and further made the important discovery that the phosphorylytic breakdown of sucrose to glucose-1-phosphate and

fructose is reversible. Thus a mechanism for direct sucrose fermentation is disclosed, the suggested analogy as regards store of energy for polysaccharide synthesis between glucose-1-phosphate and sucrose is corroborated, and an enzyme system is revealed whereby levan and dextran can be formed from their constituent hexose units via sucrose.

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The Reduction of Dehydroascorbic Acid in Plant Extracts

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Pfankuch (1934) observed that when 0.3% cysteine was added to potato press juice in which the ascorbic acid (abbreviated to AA) had become oxidized, 10 mg./100 ml. of AA reappeared in 2–3 hr. at room temperature, after which time no more AA appeared. Further, he showed that this was probably an enzymic reaction, for it did not occur in heated

juice or in the filtrate from a protein precipitate. Hopkins & Morgan (1936) independently discovered a similar enzyme in cabbage and cauliflower juices, which accounted for the 'protection' of AA by reduced glutathione ('GSH'). Crook & Hopkins [1938] showed the dependence of this protective system upon pH and indicated that the activities of

ascorbic oxidase and the reducing enzyme ('dehydroascorbic acid reductase') could vary independently in different plants. Kohman & Sanborn (1937) found that the juice of peas, lima beans and navy beans catalyzed the reduction of dehydroascorbic acid (DHA) by GSH. They did not observe the effect with cabbage juice.

Until this date it was not certain that the reducing enzyme was different from ascorbic oxidase. Indeed, Stewart & Stewart (1936) and Fujita & Numata (1938) assumed them to be identical. But Crook (1941) was able to separate one from the other, and partially to purify each. He suggested that the Hopkins-Morgan system might possibly have a role as an 'end-system' in plants somewhat analogous to that of the cytochrome system in animals. In view of this, it seemed desirable to discover how widely the reducing enzyme was distributed in plants, and whether, in such plants as contained it in appropriate concentration, typical protection of AA by GSH could be obtained.

EXPERIMENTAL

Dehydroascorbic acid. This was prepared in solid form by a method kindly communicated to one of us by Prof. E. L. Hirst, who has consented to its publication here. AA is dissolved in excess absolute ethanol and treated with two equivalents of alcoholic I_2 . Pure $PbCO_3$ is then stirred in until all the I_2 has reacted with the AA and the HI has been removed by the $PbCO_3$. The neutral solution is evaporated to dryness at $30^\circ/12$ mm., and the product extracted with absolute ethanol. The filtered alcoholic solution on evaporation gives a slightly yellow frothy solid which usually contains 65–75% dehydroascorbic acid, about 25% other organic material (probably transformation products of the very labile dehydro- compound) and about 10% of ethanol. The method works very well, and the product is stable for at least 3 months in a desiccator.

Methods. DHA solutions were made up containing 15 mg./ml. of the material prepared by Hirst's method, equivalent to 9–10 mg./ml. of AA. Neutralization was not necessary since the pK of DHA is in the region of 9 (Borsook, Davenport, Jeffreys & Warner, 1937). GSH solution was made up to contain 25 mg./ml. in neutral solution. The plant juices were prepared by mincing in a tinned meat mincer, pressing out through cloth and centrifuging at 3500 r.p.m. for 10–20 min. They were always used on the day on which they were prepared.

All experiments were done at pH 6.0 in Sørensen phosphate buffer. Glass-distilled water was always used so as to avoid contamination of reagents with traces of copper. 1 ml. juice, 3 ml. M/5 buffer, 1 ml. DHA solution and 3 ml. glass-distilled water were placed in a large Thunberg tube, in the overhang of which were 2 ml. GSH solution. The final concentrations were thus 1 mg./ml. DHA, 5 mg./ml. GSH and M/15 buffer, which gave a molar ratio of DHA : GSH of 1 : 4, as in most of the earlier experiments of Hopkins & Morgan (1936). After the tube had been evacuated, the contents were mixed and the reaction was allowed to proceed for 9 min. This was found to be a convenient time for most juices. For the most active, e.g. broad bean and cauliflower, the reaction time used was 3 min. The reaction

was then stopped by the addition of a mixture of trichloroacetic and metaphosphoric acids, the volume was made up to 100 ml., and the AA in a filtered portion determined by the modified Tillmans titration (Birch, Harris & Ray, 1933; Harris & Olliver, 1942).

All experiments were done at room temperature. It was found more convenient to make no attempt to control the temperature, but simply to run a 'chemical control' with each experiment, i.e. a tube made up as above, but with no enzyme. The 'chemical reaction rate' consequently varies considerably. As a check on the stability of the DHA preparation and the other reagents, experiments were run with cauliflower juice from time to time. It was found possible to stabilize the DHA reductase in cauliflower juice by adding an equal volume of glycerol. Juice thus treated and kept in the refrigerator was found to retain its activity almost unimpaired for 4 weeks and is a convenient reagent for estimating DHA in extracts. Protection experiments followed the methods used by Hopkins & Morgan (1936) and Crook & Hopkins (1938).

Reaction rates and terminology. Three quantities were defined, all being expressed as mg. AA/min. formed during the initial stage of the reaction (usually the first 9 min.): (a) chemical reaction rate, i.e. the rate of reduction of DHA without added enzyme, and due to GSH alone; (b) total reaction rate, the total rate of AA formation, chemical + enzymic, in the presence of the enzyme; (c) enzymic reaction rate, this being defined as $b - a$. In addition, the term 'activity ratio' is used to denote the ratio of the velocities of the enzymic and chemical reactions; thus 'activity ratio' = $(b - a)/a$. If the activity ratio is positive, some acceleration of the rate of DHA reduction by GSH has resulted on the addition of the enzyme preparation. If the ratio is unity or greater, the rate of the reaction has been at least doubled by the addition of the enzyme-containing plant extract.

Where the term 'total oxidative rate' is used, this refers to the total rate of removal of AA by its oxidation to DHA, whether this is due to autooxidation, to the action of the ascorbic acid oxidase (Crook, 1941), or to any other oxidative systems which may be present in the plant juice.

RESULTS

In Table 1 are set out the results of tests of thirty species of easily obtainable vegetables and weeds. These are grouped to show their botanical relationships. The only examples of the monocotyledons are barley, wheat, and onion. In many cases, more than one part of the plant was tested, e.g. whenever the plant had a bulbous root or tuber, both this and the leafy part were tested separately, and in the case of legumes whose seeds were sufficiently large, these were also tested separately. Only in the case of turnip was it necessary to make any corrections for the natural ascorbic acid of the juice. In all other cases the concentration of AA was low enough, and the oxidizing power of the juice great enough, to make the residual AA negligible. It will be seen that except in nine cases, covering eight species, the addition of plant juice to the reaction mixture accelerated the reduction of DHA by GSH, but this acceleration was generally small. In thirteen cases,

Table 1. The influence of plant extracts on the rate of reduction of dehydroascorbic acid by reduced glutathione

Family	Species	Name	Part of plant	Date	Reaction rate expressed as ascorbic acid formed (mg./min.)				Activity ratio (b-a)/a
					Without added plant juice ('chemical reaction')	With added plant juice ('total reaction')	Increase due to addition of enzyme ('enzyme reaction')		
Graminae	<i>Hordeum vulgare</i>	Barley	Grain extract	17. xii. 41	0.122	0.685	0.573	4.7	
			Sprouted grain extract	2. i. 42	0.111	0.334	0.223	2.01	
	<i>Triticum vulgare</i>	Wheat	Sprouted grain extract	26. ii. 41	0.100	0.300	0.200	2.00	
Liliaceae	<i>Allium cepa</i>	Onion	Onion bulb	2. vii. 41	0.200	0.264	0.064	0.32	
			Onion tops	—	—	0.260	0.060	0.30	
Urticaceae	<i>Urtica dioica</i>	Nettle	Stalks and leaves	16. vi. 41	0.111	0.163	0.052	0.47	
	<i>Spinacea oleracea</i>	Spinach	Leaves	24. vi. 41	0.200	0.300	0.100	0.50	
Chenopodiaceae	<i>Beta vulgaris</i> var.	Mangold	Root and leaves	7. viii. 41	0.128	0.138	0.010	0.08	
	<i>Beta vulgaris</i> Rapa (?)	Sugar beet	Root and leaves	7. viii. 41	0.128	0.133	0.005	0.04	
Papaveraceae	<i>Papaver rhoeas</i> (?)	Wild poppy	Whole young plant with buds + roots	4. vii. 41	0.167	0.231	0.064	0.38	
Cruciferae	<i>Brassica oleracea</i>	Cauliflower	Florets	20. vi. 41	0.189	2.200	2.010	10.80	
	<i>Brassica campestris</i>	Cabbage	Leaves	20. vi. 41	0.189	1.004	0.815	4.32	
		Turnip	Root	10. xii. 41	0.128	0.150	0.020	0.17	
			Root	9. viii. 41	0.122	0.172	0.050	0.40	
			Top	—	—	0.540	0.418	3.42	
			Root	4. xii. 41	0.117	0.142	0.025	0.21	
		Swede	Root	4. xii. 41	0.117	0.149	0.032	0.30	
		Wild mustard	Leaves, flowers and stalk (less delay in preparation)	4. vii. 41	0.167	0.258	0.091	0.55	
Saxifragaceae	<i>S. alba</i>	White mustard	Seed extract	(5. vii. 41)	—	(0.340)	(0.170)	(1.00)	
	<i>Nasturtium officinale</i>	Watercress	Leaves and stalk	30. vi. 41	0.144	0.222	0.078	0.54	
Leguminosae	<i>Hydrangea</i>	Hydrangea	Leaves and stalk	24. vii. 41	0.144	0.138	0.006	0.04	
	<i>Vicia faba</i>	Broad bean	Beans only	11. vii. 41	0.211	2.382	2.171	10.3	
			Pods only	7. vii. 41	—	0.569	0.378	1.79	
			Extract dry beans	15. viii. 41	0.128	0.926	0.798	6.2	
	<i>Medicago sativa</i>	Lucerne	Leaves and stalks	18. i. 41	0.145	0.793	0.648	4.5	
	<i>Pisum sativum</i>	Pea	Peas	1. vii. 41	0.178	0.398	0.220	1.24	
Umbelliferae	<i>Phaseolus vulgaris</i>	French bean	Pods	—	—	0.209	0.031	0.17	
	<i>P. multiflorus</i>	Runner bean	Whole bean	31. vii. 41	0.139	0.162	0.023	0.16	
	<i>Aprum granulosus</i>	Celery	Stalks	1. viii. 41	0.133	0.156	0.023	0.17	
			Leaves	15. ix. 41	0.111	0.114	0.003	0.03	
	<i>Pastinaca sativa</i>	Parsnip	Root	—	—	0.133	0.022	0.20	
	<i>Daucus carota</i>	Carrot	Root	15. ix. 41	0.111	0.150	0.039	0.35	
			Top	23. vii. 41	0.150	0.158	0.008	0.05	
			Stalks and leaves	—	—	0.178	0.028	0.19	
Labiales	<i>Heracleum sphondylium</i>	Cow parsnip	Stalks and leaves	18. vi. 41	0.211	0.244	0.033	0.16	
	<i>Lamium album</i>	Dead nettle	Stalks and leaves	16. vi. 41	0.111	0.137	0.026	0.24	
Solanaceae	<i>Solanum tuberosum</i>	Potato	Old tubers	25. vi. 41	0.211	0.274	0.063	0.30	
			Haulm	30. vi. 41	0.144	0.556	0.412	2.86	
			New tubers	3. vii. 41	0.200	0.618	0.418	2.09	
			Shoots	6. viii. 41	0.128	0.271	0.143	1.12	
	<i>Lycopersicon esculentum</i>	Tomato	Ripe fruit juice	6. viii. 41	0.128	0.116	0.012	0.10	
			Sediment	—	—	0.133	0.005	0.04	
Cucurbitaceae	<i>Cucurbita Pepo</i> var.	Vegetable marrow	Skin	—	0.133	0.240	0.107	0.80	
			Flesh	1. viii. 41	—	0.151	0.018	0.14	
Compositae	<i>Cucumis sativus</i>	Cucumber	Fruit	23. v. 41	0.122	0.155	0.033	0.27	
	<i>Helianthus tuberosus</i>	Artichoke	Tubers	15. xii. 41	0.128	0.127	0.047	0.37	
	<i>Lactuca sativa</i>	Lettuce	Leaves	23. vi. 41	0.256	0.256	0.000	0.00	
	<i>Taraxacum officinale</i>	Dandelion	Leaves	27. vi. 41	0.200	0.200	0.000	0.00	

covering nine species, the activity ratio is greater than 1.0, and in an additional five, covering five more species, it is greater than 0.5. A few negative values occur, but, except in the case of *Sinapis alba*, these may be taken to be within the experimental error. But with this plant there seems, for some unknown reason, to be a slight but definite inhibition.

It is interesting to calculate the smallest activity ratio which would give protection in these circumstances with an oxidase system of about the same activity and concentration as in cauliflower. The total rate of reduction of DHA by GSH and cauliflower extract is four to five times the total rate of oxidation of AA by O_2 , as shown in Table 2, i.e. on

Table 2. *Relative total rates of oxidation and reduction of ascorbic acid in cauliflower extract at pH 6*

Date	Total oxidation rate (mg./min.)	Total reduction rate (mg./min.)	Ratio: Reduction/ Oxidation
4. v. 38	0.44	2.14	4.9
5. v. 38	0.43	2.10	4.9
20. vi. 38	0.80	2.90	3.7
30. vi. 38	0.51	2.20	4.3
4. vii. 38	0.33	2.00	6.0
8. vii. 38	0.36	1.70	4.7
10. vii. 38	0.29	1.25	4.3
23. ix. 38	0.18	0.97	5.4
4. x. 38	0.31	1.02	3.3
5. x. 38	0.22	0.84	3.8
10. x. 38	0.24	1.05	4.4
14. i. 39	0.34	1.84	5.4
19. i. 39	0.36	1.50	4.2
23. i. 39	0.47	1.88	4.0
24. i. 39	0.27	0.82	3.0
17. ii. 39	0.26	1.80	6.9
10. iii. 39	0.39	1.50	3.8
17. iii. 39	0.42	1.50	3.6
		Average	4.5

the basis of the figures given in Tables 1 and 2, about $2.200/4.5 = 0.49$ mg./min. Thus for protection of AA, the least total rate of reduction would have to be approximately 0.5 mg./min., of which 0.19 could be chemical, leaving 0.31 mg./min. for the enzymic rate, i.e. an activity ratio of approximately 1.6.

Two sets of protection curves (Figs. 1, 2) show that the extent of protection of AA is in fact that which would be predicted from the above considerations, at least in the two species examined. Both the pea and the broad bean (excluding the pods) have an active ascorbic oxidation system, the average total rates of oxidation of AA being quite close to that obtained with cauliflower. Whether the system consists of the specific ascorbic oxidase alone was not fully investigated, but neither juice gives a direct guaiacol reaction, although an active peroxidase is present. On the other hand, their activity ratio for reduction is quite different, as

seen from Table 1, i.e. 10.3 for broad beans, and only 1.24 for peas. It would therefore be expected

Fig. 1. 'Protection' of ascorbic acid by glutathione in the presence of broad bean juice. pH 6.0, phosphate buffer, 1 mg./ml. of ascorbic acid, 3 mg./ml. of glutathione, final concentration of broad bean juice, 1 in 10. Upper curve C (dots), course of oxidation of ascorbic acid in presence of glutathione. Lower curve B (circles), course of oxidation of glutathione. Lower curve A (crosses), course of oxidation of ascorbic acid alone.

Fig. 2. Failure of 'protection' of ascorbic acid by glutathione in presence of pea juice. Conditions as for Fig. 1. Upper curve (dots), course of oxidation of ascorbic acid in the presence of glutathione. Lower curve (circles), course of oxidation of glutathione. Lower curve (crosses), course of oxidation of ascorbic acid alone.

that GSH would protect AA in broad bean juice, but not in that from peas. Fig. 1 shows that this expectation is borne out for broad beans, which

give a 'classical' protection diagram where the rate of disappearance of GSH in the presence of AA (curve *B*) is the same as the rate of disappearance of AA alone (curve *A*), whilst AA in the presence of GSH is completely protected until all the GSH is oxidized (curve *C*). The situation is quite different with pea juice (Fig. 2). Here there is no protection, as was predicted, the AA being oxidized from the beginning even in the presence of GSH. The rate in the presence of GSH, however, is only about one-half that with AA alone, showing that considerable reduction is occurring, as would be expected from the fact that the activity ratio of the DHA reductase is only a little below that necessary for protection.

Similarly, a classical protection diagram might have been anticipated with the juice of new potato tubers, which also oxidizes AA at about the same total rate as does cauliflower juice, and has an activity ratio for reduction of about 2. However, an unexpected result was obtained. Ascorbic acid was certainly protected, but at the same time GSH was oxidized at only about one-tenth the rate at which AA alone was oxidized, i.e. the system became almost inert, having many of the characters of the system Cu-AA-GSH described by Hopkins & Morgan [1936]. This is interesting in view of the fact that the oxidase system of the potato is known to be of the catechol oxidase type, i.e. the AA is not oxidized directly. The explanation may be that GSH had inhibited the oxidase, or combined with the quinones responsible for the oxidation of AA. Although no mention is made of this in the literature, it may well be true, as catechol oxidase is known to be a Cu-protein (Keilin & Mann, 1938). Unfortunately it has not been possible to follow this up.

In the plants examined, there does not seem to be any marked grouping of the activity. On the whole, the Graminae, Cruciferae and Leguminosae seem to be more favoured than the other families so far as this enzyme is concerned, although the number of species tested is not great enough for this conclusion to have any great weight. The same might be said for the distribution among plant parts. The activity of seeds and storage tissues is often high, but on the other hand turnip leaves contain more than the root, and potato haulm as much as the tuber, whilst in the vegetable marrow the skin is richer than the flesh. The seeds of the latter were not tested.

No attempt was made, in compiling Table 1, to make any allowance for possible seasonal variations in concentration of the enzyme concerned. That these may be considerable is shown by the data available for cauliflower, covering the period April 1938 to March 1939, respectively (Fig. 3). In Fig. 3, upper curve, is shown the variation in the total

reducing activity at pH 6 and with the concentrations of reagents used above. It is seen that, in general, the activity is a maximum in April, and a minimum in September, the ratio of the maximum and minimum being approximately 2 : 1. The lower curve in Fig. 3 shows the variation of the oxidase

Fig. 3. Seasonal variation of strength of ascorbic oxidase (lower curve) and dehydroascorbic acid reductase (upper curve) in cauliflower juice, covering the period April 1938 to March 1939. Conditions as for Fig. 1.

activity over the same period. It is seen that the maximum and minimum points in this curve roughly coincide with those for the reductase and their ratio is about the same. This similar behaviour of the activity curves removes the possibility of a seasonal failure in protection; indeed, from the data available it appears that protection is actually better when reducing activity is minimal, for here the ratio of reduction velocity to oxidation velocity is nearly 5 : 1, whereas when reducing activity is maximal the ratio is only approximately 3.5 : 1. This, of course, does not necessarily hold for other plants, and it may easily be the case, particularly in those plants which have an activity ratio little greater than 1.6, that such a seasonal failure in protection may occur.

In view of the instability of the reducing enzyme in plant extracts noted by Crook & Hopkins (1938) and Crook (1941), it is interesting that the dry broad bean seeds should give such potent extracts. This agrees with the observations of Kohman & Sanborn (1937), who found reductase in dried peas.

DISCUSSION

The primary aim of this investigation was to discover whether the cauliflower and cabbage were unique in possessing a high concentration of Hopkins & Morgan's DHA reductase. At least one other plant, the broad bean, has been found of a similar activity and with this plant it was possible to demonstrate a 'classical' protection system. Three or four more plants have been found in which the reductase is present in considerable amount.

How far this system represents a physiological hydrogen transfer system (Crook, 1941) is difficult to decide. The possibility of this depends upon the ability of plant tissues to reduce GSSG. Ganapathy (1938) has shown that in the latex of *Carica papaya*, —S—S— linkages produced by aeration or by oxidation with I_2 or H_2O_2 are reduced enzymically (the reaction does not occur with heated latex) to —SH. It is interesting to note that of the three latex-bearing plants included in the present survey—lettuce, dandelion and poppy—the activities of the first two are among the lowest tested, while that of poppy, although not insignificant, is not very high. Presumably then, this system could not be expected to play a very great part in such plants. But reduction of GSSG is not confined to latex-bearers. Kohman & Sanborn (1937) have shown that it occurs in extracts from peas, lima beans and navy beans, and that GSSG formed by the oxidation of GSH by DHA is later reduced by other systems in the juice.

Further indication of the close association between AA and GSH is afforded by the well-known observation that, in germinating seeds and sprouting potato tubers, AA and GSH appear at the same time (e.g. Pett, 1936; Hopkins & Morgan, 1943). The observation of Hageman, Hodge & McHargue (1942), that the addition of small amounts of KI to the soil in which tomato plants are grown increases the iodine content and greatly reduces the AA content of the plants, is compatible with the idea that reduction of DHA by GSH is physiological, since Crook (1941) has shown that DHA reductase is inhibited by quite small concentrations of KI.

James & Cragg (1941) have shown that in barley sap there is an active ascorbic oxidase, and that the oxygen uptake of the sap is greatly increased by lactic acid in the presence, but not in the absence, of AA. The AA remains reduced, and the lactic is oxidized to pyruvic acid. Since it has been demonstrated here that barley has a highly active DHA

reductase, efforts were made to demonstrate the reduction of G-SS-G by lactate in extract of sprouted or unsprouted barley, but without success. Whether this was due to the use of Cu-oxidized GSSG, or whether some other system is normally responsible for reduction of DHA in barley, was not investigated.

That other reducing mechanisms for DHA exist in the plant is probable from the experiments of Rubin, Syssakian & Lutikova (1937), who showed that when DHA is injected into living leaves it is quickly reduced. They list the most actively reducing plants as cabbage and hydrangea. According to the present experiments, cabbage possesses an active DHA reductase, whereas hydrangea has none.

Thus, although the enzyme catalyzing the reduction of DHA by GSH is moderately widely distributed, we can still say very little about its physiological significance.

SUMMARY

1. Thirty species of plants have been examined for the presence of the enzyme catalyzing the reduction of dehydroascorbic acid by reduced glutathione.

2. In eight of these species the enzyme was absent. Addition of the juice from nine species at least doubled the rate of reduction of dehydroascorbic acid caused by GSH alone without added enzyme.

3. The most active species were cauliflower and broad bean, in both of which glutathione will protect ascorbic acid from oxidation by atmospheric O_2 .

4. The physiological role of the enzyme is discussed.

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