APPEARANCE OF GLUTATHIONE DURING THE EARLY STAGES OF THE GERMINATION OF SEEDS

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TWO interesting papers have been published containing studies of the phenomena with which the present research is concerned. Firket and Comhaire[1] state that while absent in the dry pea, glutathione, or at least –SH compounds, rapidly appear after these are placed under water. These sulphydryl compounds are distributed throughout the cotyledons, seeming to prepare the conditions for the growth of the embryo rather than being produced under its influence. After reaching a maximum they diminish during the growth of the embryo, more slowly when growth proceeds in the dark. Vivario and Lecloux[2] confirm the early appearance of glutathione during hydration. It reaches a maximum after 4–6 hr.; this increase is more rapid at 38°. The authors found that formation is quicker in powdered peas owing to the more rapid uptake of water.

The facts revealed by the above authors are of much interest but the numerical data they present require revision. In each case it was, of course, realized that other reducing substances besides thiol groups might be present. They therefore carried out two titrations with iodine, one with nitroprusside as indicator and the other with starch. The latter gave somewhat higher figures than the former, and the difference was assumed to be due to other reducing substances. It is now known, however, that the use of nitroprusside as an indicator gives unreliable results. Moreover, these results were calculated as glutathione when this was still supposed to be a dipeptide. In any event, although with animal tissues it is usually safe to assume that no other thiol compounds need to be considered, this assumption was not justified in the case of germinating seeds, where protein changes are so active. Free cysteine might well be present, and, indeed, we have found this to be the case.

We have ourselves succeeded in isolating glutathione from germinating peas by methods which will be described.

Experimental

The early appearance of –SH groups during germination is clearly general among plants, and appears universal. On qualitative lines we have proved their presence in the seeds of the following: Gramineæ (endosperm): rye, barley, wheat, maize; Leguminosæ (cotyledons): sanfoin, vetch; Cruciferæ (cotyledons): cabbage, mustard, wallflower; Chenopodiaceæ (endosperm): spinach; Caryophyllaceæ (perisperm): silene.

In each case, 5 gm. was extracted with trichloroacetic acid, the extract saturated with ammonium sulphate and centrifuged, and to the solution one drop of 5 per cent nitroprusside and then ammonia were added. This was usually on about the second or third day of germination. A positive reaction was always obtained varying somewhat in intensity, the strongest being in the Leguminosæ and the weakest in the Gramineæ. Kozlowski[3] obtained qualitative reactions for –SH in a variety of seeds. He also found that the Leguminosæ gave the strongest reaction.

Isolation of Glutathione. We found greater difficulty in this task than is offered in the case of yeast and animal tissues. Our first attempts, however, were made on lines that have been customary, but these early experiences may be very briefly described. We worked chiefly with different strains of the garden pea, and we used trichloroacetic acid as an extractive. Such extracts have the fundamental fault that, in them, all metallic precipitants produce precipitates which are apt to be colloidal in character. We tried cadmium acetate but found that this gave so bulky a precipitate that it could have little selective value. We therefore used the familiar mercuric sulphate reagent. Its precipitates tend to be colloidal, but with careful adjustment of *p*H we found we could centrifuge and wash them, though at each stage with some loss.

In one experiment 3 kgm. of peas, on the fifth day of germination, was ground up in trichloroacetic acid by use of sand in a mechanical pestle and mortar. In the extract, after some preliminary treatment, we used mercuric sulphate as a fractional precipitant, and from the three fractions, after the usual treatment, we obtained three separate products. Of these only one (the most soluble) yielded the typical copper compound of glutathione on adding cuprous oxide to its solution in 0·5 *N* sulphuric acid. The product obtained from this acted characteristically as a coagent to glyoxalase activity. Moreover, when heated in aqueous solution, it gave, after evaporation on the open water bath, typical crystals of the diketopiperazine of cysteinyl-glycine. The product evidently contained a large proportion of glutathione, but its melting point was low, and it gave analytical data which showed it to be impure. It is noteworthy that the other two of the three products referred to, after oxidation, showed typical hexagonal crystals of cystine.

Trichloroacetic acid is clearly not very satisfactory for the extraction of plant tissues. Moreover, at this time it became difficult for us to work on a large scale. We therefore worked out another process applicable to relatively small quantities of material; this gave satisfactory results. The seeds are first ground up by hand in a mortar with 0·1 *N* sulphuric acid and a little washed sand, the extract centrifuged and the solution half saturated with solid ammonium sulphate. The filtrate from the precipitate thus produced (350 ml.) is brought to 0·5 *N* sulphuric acid. To this, cuprous oxide is added in small quantities with frequent stirring. It is remarkable that no precipitate appears at once, but after standing for twenty-four hours a quite typical precipitate of the copper compound separates. It would seem that some unstable association between the cuprous compound and the ammonium sulphate is first formed (? a co-ordination compound) which slowly decomposes. These facts apply no less to pure glutathione.

The only drawback to this simple procedure is that, owing to the delay in the separation, it is difficult to estimate with exactness the amount of cuprous oxide which is necessary. Slight excess is added, and after standing for twenty-four hours in an open beaker, the precipitate, which may be mixed with a small amount of copper oxide, is centrifuged off and the fluid allowed to stand for a further twenty-four hours. If more of the copper compound separates, this is centrifuged off and added to the first precipitate. After forty-eight hours no further precipitate has ever been observed.

Preparations 1 and 1a. 10 gm. of peas was soaked

for twenty-four hours in water and then treated as above. The crystalline copper precipitate, after washing free from sulphate, was decomposed as usual, and the filtrate from the sulphide freed from hydrogen sulphide by a stream of hydrogen. One half was evaporated to dryness in a vacuum desiccator and weighed 25·5 mgm. (51·0 mgm. per cent). This residue titrated with 0·01 *N* iodine used 7·3 ml., equal to 22·41 mgm. (44·82 mgm. per cent) of glutathione. A quantitative estimation was made by Woodward's manometric method[4]. For the latter a four-point reference curve was first obtained to establish accurate relations between the tripeptide and the carbon dioxide measured. The glyoxalase was made from rats' red corpuscles on the lines described by Jowett and Quastel[5]. The amount of the solution used for the estimation contained 0·127 mgm. of the preparation, and the amount of carbon dioxide evolved in 20 min. (99·54 µl.) was equivalent to 23·0 mgm. (46·0 mgm. per cent) of glutathione. As in this experiment the whole solution was taken directly to dryness and not crystallized out from a mother liquor, it will be understood that the slight excess in the weight of the residue might be due to a trace of impurity. But it would seem that the product was practically pure.

100 gm. of the peas, after soaking for twenty-four hours in water, was transferred to a funnel (which permits of an adequate oxygen supply) and allowed to germinate for three days. The peas were then treated as above. The total residue of the final product was 14·6 mgm. per cent, by iodine 11·05 mgm. per cent, and by Woodward's method 11·0 mgm. per cent. These results confirm the statements of Firket and Comhaire[1], also those of Vivario and Lecloux[2], that the sulphydryl groups diminish once actual growth is established.

Preparation 2. The preparation obtained in the last experiment, although giving satisfactory evidence for approximate purity, was not crystalline. We therefore decided to work on a larger scale in order to obtain a crystalline product. We still found it convenient to work on small quantities of 100 gm. each, accumulating the copper compound from eleven such extractions. This, washed free from sulphate and decomposed in the usual manner, yielded 0·346 gm. of a crystalline product. This was undoubtedly pure glutathione. On rapid heating it melted sharply at 190°, and the product mixed with pure glutathione also melted at 190°. It gave the following analytical results, the mean of two closely agreeing duplicates: found C, 39·03; H, 5·52; N, 13·68; S, 10·36; glutathione, $C_{10}H_{17}O_6N_3S$, requires C, 39·09; H, 5·54; N, 13·68; S, 10·42.

Kozlowski[3] was the first to attempt the isolation of glutathione from peas. So far back as 1926, working in the Biochemical Laboratory at Cambridge, he spent much labour on the problem, employing as much as 25 kgm. of material for each extraction. He succeeded in proving the presence of non-protein cysteine but obtained no product identical with glutathione. After Hopkins[6] had described the use of cuprous oxide as a precipitant, he returned to the problem[7], but again was not successful in isolating a pure substance.

It will be remembered that Vivario and Lecloux[2] found that the production of –SH groups proceeded in powdered peas when this material was placed under water. We have confirmed and extended this observation.

Thunberg vacuum tubes were employed. Two series of –SH determinations were made. In one the powder had stood under an aqueous buffered solution; in the second it had stood under a similar solution to which the disulphide form of glutathione had been added. The following are the details of our procedure.

5 gm. of the pea powder was weighed into each of a series of large Thunberg tubes. 16 ml. of water and 2 ml. of *M*/5 Sørensen's phosphate buffer *p*H 6·0 were then added to each tube; to one series of tubes, 2 ml. of water, and to the others, 2 ml. of a solution of the disulphide form of glutathione containing 60 mgm. (neutralized to *p*H 6·0) were then added. The tubes were then evacuated, refilled with nitrogen and again evacuated; the procedure being repeated three times. The tubes were allowed to stand, with an occasional shake, at the room temperature of 17°. At intervals the reaction was stopped by sucking in through the side tube 10 ml. of acid (8 per cent trichloroacetic and 2 per cent metaphosphoric acids) as used by Musulin and King[8]. The precipitate was centrifuged off, successively washed with 5 per cent acid and made up to 100 ml. Half was titrated with 0·01 *N* iodine in the presence of potassium iodide, and the other half with Tillman's reagent using the micro method of Birch *et al.*[9] The iodine value of the very small amount of ascorbic acid as determined by the indophenol titration found under the conditions of our experiment at 18 hr. and 24 hr. has been deducted in the following table.

Hours	0·01 *N* iodine per 5 gm. peas (ml.)		Calculated for glutathione (mgm./100 gm. peas)	
	No GSSG*	+ GSSG	No GSSG	+ GSSG
0	1·4	—	85·96	—
4	2·0	6·2	122·80	380·68
12	2·0	7·6	122·80	466·64
18	2·0	8·8	122·80	540·32
24	2·3	9·1	141·12	558·74
24†	1·5	1·5	92·10	92·10

* GSSG = Disulphide form of glutathione.
† Heated for 15 min. at 70°.

To judge from many known cases, the production of sulphydryl groups is likely to depend on the transference of hydrogen from donators to acceptors capable of yielding –SH groups. In the present case these are likely to be the oxidized form of glutathione together with cystine. We do not yet know whether the former is present ready formed in the seed stores, or whether it may be synthesized rapidly when the necessary amino-acids are made available by proteolysis.

Thunberg, in a number of papers, has shown that dehydrogenases of various kinds are present in seeds, but we do not yet know which of these, if any, are concerned with the –SH production.

Our experiments with the powdered material show that the –SH groups are rapidly formed. Referring first to the preparations containing none of the disulphide form of glutathione, it will be seen from the above table that at the fourth hour their concentration has already reached a maximum.

It will be seen from the table that there was a considerable concentration of the –SH groups present in the pea powder we used for the experiment. This experiment was made at a time of the year when natural germination would occur. There is no doubt that when the atmosphere is warm and moist, reactions may begin in the intact pea.

We were led to try the effect of adding the oxidized tripeptide owing to a suspicion that the hydrogen acceptor groups were saturated before the hydrogen

supply was exhausted. It will be seen that, in consequence of this addition, a large increase in the titratable –SH groups was found.

In order to convey an idea of the extent of the reactions, we have converted the whole of the iodine values as though due to the reduction of the disulphide form of glutathione. It will be seen that the powder by itself reaches the relatively high figure of more than 140 mgm. per 100 gm., while with the addition of the disulphide form of glutathione it becomes no less than 540 mgm.

There is no doubt that the –SH groups start disappearing as soon as visible growth begins (see preparations 1 and 1*a*). We have made no attempt to ascertain the nature of their fate.

Summary

(1) When dry seeds are exposed to water, their contents come to display a high concentration of thiol groups, which rapidly reach a maximum. Later, when growth proceeds, there is a steady fall in the concentration of these groups.

(2) A method is described which has enabled us to isolate (from peas) pure crystalline glutathione.

(3) We have also worked with peas in the powdered form, and have found the same rapid production of thiol groups. The powder further displays a high reducing power towards the oxidized form of glutathione.

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PRINCIPLES OF THE USE OF NON-REFLECTING FILMS IN OPTICAL INSTRUMENTS*

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WHEN light from an object passes through an optical system to form an image, a small fraction is reflected at each air-glass surface instead of being transmitted. For normal incidence, the reflectivity is usually between 4 and 7 per cent, depending on the refractive index of the glass. This affects the image in three ways. First, the brightness of the image is reduced. Secondly, the reflected light, eventually reaching the image plane after two or more reflexions at the lens surfaces, invades the dark parts of the image and gives rise to a haze called 'veiling glare'. This reduces the contrast in the image. Thirdly, the reflected light may concentrate near the image plane to form 'glare spots' and 'ghost images'.

Optical systems are therefore only able to function at their best when unwanted surface reflexions are reduced to a minimum, and that is the purpose of non-reflecting films. The property of light on which they depend is that of interference, by means of which the distribution of the radiation is altered in such a way that the reflexion is reduced and the transmitted light increased. Nothing is sacrificed in reducing the reflexion.

Interference at a glass surface is brought about by coating it with a thin transparent film. As we are concerned with getting a reduction of reflected light, it is necessary, as will be shown later, that the film shall be of lower refractive index than the glass. Suppose, then, that a pencil of monochromatic light from a distant point source falls on a glass surface which has been coated with such a film (see diagram).

Consider the composition of the reflected ray R. As will be seen from the diagram, it consists of the reflected portion of the ray A, augmented by contributions from other rays such as B and C which emerge at A' in the direction $A'R$ after multiple reflexions within the film. Similarly, the reflected fraction of the multiple reflexions arriving at A' is added to the directly transmitted ray AT. The directly transmitted components of B, C, etc., and their reflexions at the first surface each become part of separate interference systems identical with the one being considered, so that whatever happens to the rays R and T applies equally to similar rays from any other point on the film.

The calculation of the intensity of the transmitted and reflected beams is tedious and is to be found in the text-books. As an introduction, however, a simplified calculation of the thickness and refractive index of a film giving no reflexion can be made by taking into account only the incident rays A and B. This is permissible because although a large number of incident rays contribute to the reflexion R, the amplitude of their contributions is very much attenuated by the multiple reflexions which they undergo before reaching A'.

The composite reflected ray R will have zero intensity when the components from A and B are in opposite phase and equal in amplitude. For the first condition, the optical path difference must be an odd number of half wave-lengths. For a film of thickness t and refractive index μ, the path difference is $2\mu t \cos r$, where r is the angle of refraction at the air-film boundary.

Therefore for minimum reflexion of light of wave-length λ

$$2\mu t \cos r = (2n+1)\frac{\lambda}{2},$$

n being an integer, whence

$$\mu t = (2n+1) \cdot \frac{\lambda}{4} \cdot \frac{1}{\cos r} \quad \ldots\ldots (1)$$

The condition of equality of amplitudes is met by equating the Fresnel formulæ (which hold for normal incidence and are almost correct for angles of incidence up to 20°) for the amplitudes of the rays reflected at the air-film and film-glass surfaces (refractive index of the glass, μ_g).

$$\frac{\mu - 1}{\mu + 1} = \frac{\mu_g - \mu}{\mu_g + \mu},$$

which gives

$$\mu = \sqrt{\mu_g} \quad \ldots\ldots (2)$$

It is not possible to calculate the exact effect on the transmitted beam of a film defined by equations (1) and (2) without taking into account all the multiple reflexions. It is clear, however, that with

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