CLXXXVI. THE INTERFERING ACTION OF GLUTATHIONE IN THE SILVER NITRATE TEST FOR ASCORBIC ACID.

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(Received May 2nd, 1935.)

SZENT-GYÖRGYI [1928] noticed that the cortical portion of ox suprarenals turned black when immersed in neutral 0.4 % silver nitrate solution. The compound, ascorbic acid, responsible for the reduction was later isolated in crystalline form from ox suprarenals [Szent-Györgyi, 1928], paprika [Svirbely and Szent-Györgyi, 1933] and lemons [King and Waugh, 1932]. Titration with 2:6-dibromo-, or 2:6-dichloro-phenolindophenol blue indicated that the ascorbic acid content of various organs of guinea-pigs decreased when these animals were maintained on a vitamin C-free diet [Svirbely and Szent-Györgyi, 1933; Harris and Ray, 1933, 1; Bassey and King, 1933].

Many observers attempted to utilise the reduction of silver nitrate by animal organs and extracts as a criterion for the ascorbic acid content, with varying degrees of success. Marked differences were noticed in staining the cut surfaces of the suprarenals in normal and scorbutic guinea-pigs by Moore and Ray [1932], Harris and Ray [1933, 1], Siehrs and Miller [1933; 1934], and Bessey *et al.* [1934]. Gough and Zilva [1933] and Harris and Ray [1933, 2] reported that the failure of suprarenal tissue to stain deeply with silver nitrate cannot be relied on as a test for the absence of ascorbic acid or vitamin C since the adrenals of guinea-pigs which showed no evidence of scurvy on autopsy did not stain with the reagent at all. Huszak [1933] showed that a substance or substances, precipitable by lead acetate, occurred in the suprarenal medulla which prevented the reduction of silver nitrate by the suprarenal medulla and cortex. Similar inhibitory substances occurred also in lemon and paprika juice and their function was probably a stabilising of the vitamin.

In contrast to the staining by silver nitrate of the suprarenal cortex, anterior and intermediate lobes of the pituitary and ovary from various animals, the liver does not stain at all with the reagent [Gough and Zilva, 1933] nor does the suprarenal medulla [Harris and Ray, 1933, 2]. The failure of liver, suprarenal medulla or trichloroacetic acid extracts of these organs to stain with this reagent, though they contain high amounts of ascorbic acid, was difficult to explain.

Birch and Dann [1933] noticed the close similarity in occurrence of ascorbic acid and glutathione. De Caro and Giana [1934] concluded that glutathione was the substance which caused stabilisation of ascorbic acid in tissue extracts. Mawson [1935] stated that protection by tissue extracts could not wholly be explained by glutathione, cysteine, cystine or H_2S since dialysed tissue extracts retained their anti-oxidative properties in full. These properties were also present, in reduced amount, in extracts that had been boiled or precipitated with mercuric acetate.

The work of Sato and Ohata [1931] indicated that the glutathione contents of suprarenals and liver were 0.075 and 0.24 % respectively and was little changed

in scorbutic guinea-pigs, with possibly a more or less definite increase in the suprarenals. Based on the above percentages, the glutathione content of liver was 2.4 mg./g. and of suprarenals 0.75 mg./g.

The high concentration of glutathione in liver and suprarenals suggested that the failure of the silver nitrate reduction might be connected with the relative amounts of glutathione and ascorbic acid actually present in the tissues. The object of this paper is to present experimental work in testing this relationship.

EXPERIMENTAL.

Reduction of aqueous solutions of ascorbic acid by silver nitrate in the presence of glutathione and cystine.

Preliminary work indicated that glutathione¹ was precipitated by normal lead acetate, mercuric acetate and silver nitrate, but that the precipitate dissolved readily in slight excess of the reagent added. Addition of ammonium hydroxide to the clear solution obtained by adding excess of lead acetate to glutathione caused a precipitate to form at about the same p_H range as that in which ascorbic acid is precipitated under similar conditions. Cystine was precipitated by mercuric but not by lead acetate.

The reduction of ascorbic acid alone by silver nitrate was estimated directly in test-tubes. The rapidity and character of the precipitation depended to a great extent on the acidity or alkalinity of the reagent. An immediate production of black, finely divided particles of silver was obtained when 1 ml. of slightly ammoniacal 5 or 0.5 % silver nitrate was added to 0.015 mg. ascorbic acid. Quantities as low as 0.003 mg. ascorbic acid could thus be detected. With neutral silver nitrate of like concentrations, the particles obtained by the reduction were of larger size and had a silvery sheen. The reduction took longer and was not as sensitive as before. Slightly acid silver nitrate (p_H about 3) gave no marked reduction in 15 minutes with 0.15 mg. ascorbic acid although 0.3 mg. gave a gradual reduction to silvery particles.

The concentration and alkalinity or acidity of silver nitrate were very important factors when mixed solutions of ascorbic acid and glutathione were considered. It made no difference whether or not glutathione and silver nitrate were first added, followed by ascorbic acid or ascorbic acid and glutathione and then silver nitrate. Excess silver nitrate had to be present since it seemed to form a stable salt or complex with the glutathione very readily, and the excess of silver nitrate was then reduced by the ascorbic acid. Care must be taken to see that the silver nitrate is not too ammoniacal as otherwise irregular results are obtained. Sufficient ammonium hydroxide is added just to dissolve the precipitate first obtained with silver nitrate.

The results of a series of tests are given in Table I.

With approximately equal amounts of ascorbic acid and glutathione, the gradual reduction with silver nitrate was not at all comparable with the immediate reduction obtained by using ascorbic acid alone. The depth and shade of the opalescent solutions in the above concentrations depend on the relative amounts of glutathione and ascorbic acid.

Addition of 1 ml. of 0.5 % ammoniacal silver nitrate to mixtures of 0.3 mg. cystine and 0.15 mg. ascorbic acid or 0.15 mg. cystine and 0.3–0.09 mg. ascorbic acid gave an immediate reduction to black finely divided silver with a brown

¹ I am greatly indebted to Dr H. L. Mason, Department of Biochemistry, Mayo Clinic, Rochester, Minn., for sending me the glutathione used in this work.

Table I. *Reduction of ascorbic acid-glutathione mixtures with silver nitrate.*

	mg. gluta- thione	mg. ascorbic acid	1 ml. silver nitrate			
			Ammoniacal		Acidic	
			5 %	0.5 %	5 %	0.5 %
		0.1	Intense reduction	—	Trace	—
		0.05	Reduction	—	No reduction	—
		0.015	Reduction	—	No reduction	—
<i>a</i>	2	0.45	Green-yellow to brown solution in 15 mins.	Red-brown solution	—	—
	2	0.30	Yellow solution in 15 mins.	Yellow-brown solution in 15 mins.	—	—
<i>b</i>	0.75	2.0	Intense black reduction	Intense black reduction	Very slight reduction in 20 mins.	—
	0.45	0.15	Yellow solution in 15 mins.	Very pale yellow solution in 10 mins.	—	—
	0.3	0.15	Yellow solution in 3 mins.	Slight yellow solution in 10 mins. No metallic Ag	—	—
<i>c</i>	0.15	0.6	Darkening in 1-2 mins., with reduction of black metallic Ag in 15 mins.	—	—	No reduction
	0.15	0.45	Darkening in 1-2 mins. Slight reduction metallic Ag	Brown solution in 4 mins.	—	—
	0.15	0.3	Darkening after 1 min. Slight reduction Ag in 10 mins.	Brown opalescent solution. No metallic Ag	—	Slight reduction 45 mins.
	0.15	0.15	Brown opalescent solution. Trace metallic Ag?	Yellow-brown opalescent solution	—	No reduction
	0.15	0.09	Brown opalescent solution. Trace Ag?	Brown opalescent solution	—	No reduction
	0.15	0.06	Pale brown solution	Pale brown solution	—	No reduction
	0.15	0.03	Pale brown solution	Pale brown solution	—	No reduction

a, Concentrations found in mouse liver/g.

b, Concentrations found in ox suprarenal cortex/g.

c, Concentrations found in suprarenals of guinea-pigs when fed a rich green diet and then placed on a vitamin C-free diet. The ascorbic acid values represent concentrations per g. while the glutathione probably corresponds to that of 0.2 g. of suprarenals.

solution if less than 0.09 mg. ascorbic acid was used. Cystine does not interfere to the same extent as glutathione with the silver nitrate test for ascorbic acid.

Adrenaline (0.025 mg.), glycogen, lactose, gelatin or starch did not interfere in the silver nitrate test with 0.15 mg. ascorbic acid.

The gold chloride test for ascorbic acid.

Gold sols are formed immediately when 3-15 mg. ascorbic acid are added to 1 ml. of 1 % gold chloride solution in 95 ml. distilled water. The sols range from purplish red to deep blood-red in colour according to the amount of ascorbic acid added. On heating no further change in colour occurs. As ordinarily prepared by the sodium citrate or formaldehyde method, the solutions must be heated for several minutes before reduction occurs.

If different concentrations of ascorbic acid are added to 1 ml. of dilute gold chloride (1 ml. 1 % gold chloride in 98 ml. H₂O), the limit of sensitivity is 0.045 mg. ascorbic acid as compared with 0.015 mg. with ammoniacal silver nitrate.

Addition of glutathione to gold chloride causes the yellow colour to disappear, indicating the formation of a salt or stable complex. A turbid white solution results when excess glutathione is added. Cystine and adrenaline cause no noticeable change in the colour of the gold chloride.

No sol formation results in 15–60 minutes when 1 ml. of diluted gold chloride is added to mixtures of 0.15 mg. glutathione with 0.3–0.015 mg. ascorbic acid. Cystine gives similar results. Apparently the glutathione and cystine form stable salts or complexes with the small quantity of gold chloride present and prevent reduction by ascorbic acid.

The silver nitrate test for liver extracts.

The mercuric acetate treatment of Emmerie and Van Eekelen [1934] and the lead acetate method of Huszak [1933] were employed to remove the greater portion of interfering substances before the silver nitrate test was tried.

Young mice, about two months old, were etherised and their livers removed, minced with sand and extracted with three times their weight of 10 % trichloroacetic acid. Calcium carbonate was added to remove any excess acid. The yellow solution gave a strong nitroprusside reaction for glutathione and a yellow-brown precipitate with 5 % ammoniacal silver nitrate. The solution was divided into two fractions—one part was treated with normal lead acetate and the other with mercuric acetate. The amount of reagent necessary to give maximum precipitation was first determined on a small sample and excess of the reagent carefully avoided. After filtration, H_2S was passed into the colourless turbid solution to remove the mercury and lead. The sulphides were filtered off and the clear solution was allowed to stand for one hour, saturated with the gas. The gas was then removed by distillation *in vacuo* for half an hour, the cold distilling flask being immersed in warm water to facilitate concentration and removal of H_2S . The resulting concentrated solution did not react with lead acetate, copper sulphate or mercuric acetate, indicating absence of H_2S . It gave no nitroprusside test. Addition of 5 % ammoniacal silver nitrate solution gave an immediate black reduction with the fraction treated with mercuric acetate and a reddish brown reduction with the fraction treated with lead acetate. Acidic 5 % silver nitrate gave no immediate reduction though several hours later a slight reduction had occurred. Trichloroacetic acid gives a white precipitate with silver nitrate.

DISCUSSION.

The marked interference of glutathione with the silver nitrate test for ascorbic acid explains to a great extent the differences reported by many investigators who attempted to utilise this reduction as a criterion for the presence of ascorbic acid in any organ or tissue extract. The observations of Siehrs and Miller [1933; 1934] who pointed out that the intensity of the reduction in the suprarenals decreased 24 hours after guinea-pigs were taken off a rich green diet and placed on a vitamin C-free diet agrees with the results in Table I. The colour ranged from black to dark brown on the first day to a reddish orange on the sixth day when it reached the minimum. Since colour gradations depend to a large extent on the relative amounts of glutathione and ascorbic acid associated with one another as well as on the p_H of the silver nitrate, this test must be used in a qualitative rather than a quantitative sense. Likewise a failure of the test does not indicate that there is no ascorbic acid present in the organ, since the ascorbic acid content, though sufficient to ensure absence of scurvy, may be too low in comparison with the glutathione content to give a positive reaction. The test is not as definite as titration with dichloro- or dibromo-phenolindophenol blue in an acid medium.

SUMMARY.

1. The silver nitrate test for ascorbic acid depends on the relative amounts of glutathione and ascorbic acid present in an organ or extract.
2. The precautions required in order to demonstrate the silver nitrate test in liver extracts are described.
3. A method for preparing gold sols with ascorbic acid in the absence of heat is discussed.

I wish to thank Dr L. J. Harris, Dunn Nutritional Laboratory, for reading the proof and for valuable suggestions.

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