CCLXIV. A NOTE ON THE IDENTITY OF THE INDOPHENOL-REDUCING SUBSTANCES IN BRAIN TISSUE.

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HARRIS [1933] concluded from the results of biological tests that only about one-third of the material in Jensen rat sarcoma which reduces 2:6-dichlorophenolindophenol is ascorbic acid. Later Young & Mitolo [1934] and Mitolo [1934] brought forward evidence which they believed to indicate that brain tissue also contains an indophenol-reducing factor (I.F.) which is not ascorbic acid. Recent investigations have cast doubt on the conclusion that tumour tissue contains an I.F. other than ascorbic acid [Woodward *et al.* 1936; Watson, 1936; Kellie & Zilva, 1936]. The experiments described in this paper suggest further that the evidence previously believed to demonstrate the existence of a non-ascorbic acid I.F. in brain tissue is inconclusive.

The evidence previously presented that brain tissue contains such a substance [Young & Mitolo, 1934; Mitolo, 1934] was as follows:

Substance in crude brain tissue extracts	Ascorbic acid
Readily reduces acid ammonium molybdate at room temperature	Does not readily do so
Does not reduce ammoniacal silver nitrate at room temperature	Reduction at room temperature instantaneous
Insoluble in acetone	Freely soluble in absolute acetone
Precipitated by mercuric acetate	Not so precipitated
No antiscorbutic activity	Antiscorbutic

The validity of the non-biological evidence given above that the substance present in crude brain extracts is not ascorbic acid is undermined by the following observation. Prolonged aeration, in alkaline solution, of a crude brain extract results in the complete disappearance of the I.F. If the reaction is then adjusted to its previous value (about pH 6) and pure ascorbic acid added in amount calculated to be equal to the I.F. destroyed, the properties of the resulting solution are similar to those of a crude brain extract. In other words, if ascorbic acid is added to crude brain extracts, it assumes the chemical and physical properties of the unknown I.F. The question of the biological evidence, which is unsatisfactory, will not be considered in this paper. It may be stated, however, that the results have been inconsistent. The crude brain extracts used in the biological tests necessarily had a high solid content because of the low concentration of the I.F. in ox brain. They were therefore somewhat unpalatable to the guinea-pigs receiving a scorbutic diet. Some indication was obtained of a definitely deleterious effect of these extracts on the growth of the animal before symptoms of scurvy appeared. It is clear that, when such highly concentrated crude tissue extracts are used, interpretation of the results must be cautious.

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EXPERIMENTAL.

Ox brains obtained from the slaughter-house within a short time of the death of the animal were used in the majority of these experiments. In many cases confirmatory results were obtained with brain tissue from other species.

Ascorbic acid and the I.F. in brain were estimated by the reduction of 2:6-dichlorophenolindophenol, using the technique of Birch *et al.* [1933]. The indicator was standardized against pure ascorbic acid (Hoffmann La Roche and Co.) which was itself checked against standard iodine solution. The figures for I.F. are expressed as mg. equivalent of ascorbic acid.

Crude brain extract was prepared by rapidly mincing the brain tissue into 1.5 volumes of absolute alcohol and extracting for 24 hours at 0° with occasional stirring. The extract was then filtered off and the residue extracted twice with 70% alcohol. The alcoholic extracts were combined and evaporated *in vacuo* at an outside temperature of 30–40° to such a volume that 1 ml. contained about

Table I.

Reagent (5 ml. in all cases)	1 mg. equivalent crude extract	Equivalent inactivated extract	1 mg. pure ascorbic acid	"Reconstituted extract"
8% ammonium molybdate	Rapid reduction	0	0?	Rapid reduction
8% ammonium molybdate + 1 ml. 1% KH_2PO_4	Rapid reduction	0	Rapid reduction	Rapid reduction
Ammoniacal silver nitrate	Very slow blackening	0?	Instantaneous reduction	Very slow blackening
Ammoniacal silver nitrate + 10 mg. glutathione	Very slow blackening	0?	Very slow blackening	Very slow blackening
Acid (acetic) silver nitrate	Fairly rapid reduction	0	Rapid reduction	Fairly rapid reduction

1 mg. equivalent of I.F. "Inactivated crude extract" was prepared by adjusting the pH of the crude extract to about 10 and bubbling air through briskly until no reduction of indophenol reagent could be detected. The reaction was then adjusted to its original value. "Reconstituted crude extract" was prepared by adding pure ascorbic acid to "inactivated crude extract" in amount equivalent to the I.F. removed by aeration.

Reducing tests are summarized in Table I.

Acetone-solubility. To 10 ml. of crude extract, containing 15.6 mg. equivalent of I.F., 95 ml. of acetone were added. The precipitate was redissolved and the precipitation repeated twice. The final precipitate contained 11.9 mg. equivalent of I.F. The combined filtrates were freed from acetone by distillation *in vacuo* and found to contain 0.82 mg. equivalent of I.F.

A repetition of this experiment using "reconstituted crude extract" gave the following results: precipitate contains 11.4 mg.; filtrate contains 0.35 mg. ascorbic acid.

Mercuric acetate precipitation. To 10 ml. of neutral crude extract were added 18.75 ml. of 20% mercuric acetate solution. Precipitate and filtrate were separately freed from mercury by H_2S and the H_2S removed by evacuation combined with a stream of nitrogen. The precipitate contained 2.4 mg. equivalent of I.F.; the filtrate contained 5.4 mg. equivalent of I.F.

A repetition of this experiment with "reconstituted crude extract" gave the following results: precipitate contained 3.0 mg.; filtrate contained 5.5 mg. ascorbic acid.

Biological tests. A comparison of the antiscorbutic activity of ascorbic acid or orange juice with that of the I.F. in crude brain extracts has been made by determining the ability of these substances to prevent the appearance of scurvy in guinea-pigs receiving a scorbutic diet. The symptoms of scurvy were assessed on the growth curve and on the general post-mortem appearance. In one experiment the daily administration of 6 mg. was ineffective in preventing the appearance of scorbutic symptoms, in another the feeding of 3 mg. equivalent was effective. In general six animals were used in each group, and as far as possible the conditions were similar in all cases, but over a considerable number of experiments the results were inconsistent.

Absorption spectrum. "Crude extract" at pH 7 gave an absorption band with a (somewhat broad) maximum at 255 m μ . This might have been considered additional evidence that the I.F. differed from ascorbic acid, which in neutral aqueous solution has a well-defined peak at 263 m μ . Fractionation of "crude extract" with lead acetate followed by removal of lead from the separated fractions by H₂S showed that 71 % of the recovered I.F. had been precipitated by basic lead acetate, and now exhibited a band at 263 m μ , whereas the filtrate from this precipitation contained only 14 % of the recovered I.F. but possessed a strong band with maximum absorption at 247 m μ . That the original band at 255 m μ was constituted largely, if not entirely, of two bands, one at 247 m μ and the other at 263 m μ received support from the observation that "inactivated crude extract" exhibited a strong band with maximum at 247 m μ .

DISCUSSION.

The simulation of the properties of the I.F. in crude brain extracts by added ascorbic acid definitely invalidates the chemical evidence on which the existence of a non-ascorbic acid I.F. was deduced. It is now known that the reducing properties of ascorbic acid are greatly modified by the presence of glutathione [Emmerie, 1934; de Caro & Giani, 1934] and other substances present in tissue extracts [Mawson, 1935].

The facts presented in this note cannot be taken as evidence that the I.F. in brain tissue is entirely ascorbic acid. Nevertheless the chemical evidence is such that there is no reason to believe that an indophenol-reducing factor other than ascorbic acid exists in brain tissue.

SUMMARY.

1. The properties of the indophenol-reducing substance in crude brain extracts differ in certain respects from those of pure ascorbic acid.
2. If pure ascorbic acid is added to an "inactivated" crude brain extract the properties of the resulting solution resemble those of the crude brain extract.
3. There is therefore no reason to believe that the indophenol-reducing substance in crude brain extracts is other than ascorbic acid.

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