The Distribution of Ascorbic Acid between the Particulate and Non-particulate Components of Adrenal and Liver Cells

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(*Received* 10 *June* 1953)

For a number of years cytologists have been interested in the distribution of ascorbic acid within the cell. For the demonstration and localization of the acid a technique has been devised which depends on its ability to reduce silver nitrate to metallic silver, and convincing evidence has been presented for considering this technique specific for ascorbic acid (see Bourne, 1936). Deposition of metallic silver in the region of the Golgi apparatus and on mitochondria has been taken to indicate that the ascorbic acid may be present in these structures (Bourne, 1936, 1951).

Barnett & Fisher (1943) applied the silver nitrate technique to pieces of a gelatin medium in which ascorbic acid and olive oil had been thoroughly mixed prior to its setting. They observed aggregation of metallic silver particles at the interfaces between gelatin on the one hand and oil droplets or dust particles on the other; similar aggregation was seen on the surface of fragments of powdered glass that had been suspended in a gelatin medium containing ascorbic acid. Barnett & Fisher (1943) concluded from this that the concentration of metallic silver at the surfaces of mitochondria does not necessarily indicate that the ascorbic acid is concentrated in or on these bodies. Palade & Claude (1949*a*, *b*) have since brought forward weighty evidence in favour of the view that the Golgi apparatus is an artifact produced by the coalescence of intracellular phospholipids with the formation of characteristic patterns. If this view is accepted, the question of the association of ascorbic acid with Golgi material is no longer significant.

It was thought desirable to study the distribution of ascorbic acid in cytoplasmic fractions obtained by high-speed centrifugation of tissue dispersed in sucrose solutions at low temperatures. Most experiments were carried out with preparations obtained from ox adrenal medulla but a few observations on ox adrenal cortex and on dog liver are also reported.

METHODS

Preparation of tissue fractions. Ox adrenal glands from the abattoir were brought to the laboratory as soon as possible after the animal had been killed; the medulla and cortex were immediately dissected apart. Liver tissue was obtained fresh from dogs which had been bled under ether anaesthesia. The tissues were dispersed in ice-cold 0·25 M sucrose solution with a Potter-Elvehjem homogenizer. Sufficient sucrose solution was added to give a final concentration of 10 g. of tissue in a total volume of 100 ml. A Measuring and Scientific Equipment Ltd. (M.S.E.) refrigerated centrifuge set at $-1°$ was used in these experiments. The initial dispersion was centrifuged for 30 min. at 950 *g* (at the bottom of the tube) in order to throw down incompletely broken cells, cell nuclei, blood cells and any tissue which might have escaped disintegration. The supernatant fluid from this preliminary centrifugation served as the starting material for further fractionation. A portion was centrifuged at 22000 *g* for 30 min.; this produced an almost clear supernatant fluid, indicating complete sedimentation of particulate matter. This supernatant was poured off and an equal volume of 0·25 M sucrose solution was added, in which the sedimented particles were resuspended. Throughout these manipulations all vessels were kept in a water bath containing ice and every effort was made to maintain the temperature of the solutions below 4°. In one experiment with dog liver the tissue was dispersed in 0·88 M sucrose solution. The supernatant from the low-speed centrifugation and both supernatant and resuspended sediment from the high-speed centrifugation were assayed for their ascorbic acid content.

Estimation of ascorbic acid. A modification of the method described by Bessey (1938) was used. To 2 ml. of each fraction 23 ml. of 3% metaphosphoric acid were added; this solution was filtered and the pH of the filtrate was adjusted to 3·7 with citrate buffer. Equal quantities of the buffered extract and 1·6% (w/v) 2:6-dichlorophenol indophenol solution were mixed and the percentage transmission read at 518 mμ. in a Unicam spectrophotometer; the increase in transmission was proportional to the ascorbic acid concentration. In one experiment ascorbic acid was re-estimated after bubbling H_2S through the buffered extract for 15 min., followed by nitrogen for 1 hr., in order to obtain a measure of any dehydroascorbic acid present.

RESULTS

The ascorbic acid content of each fraction is shown in Table 1. The figures for the ascorbic acid content of the low-speed supernatants indicate that the bulk of the content of the tissues is present in this fraction. Data on the ascorbic acid content of the ox adrenal gland are given by Harris & Ray (1933). They found 1·1 to 1·2 mg./g. of tissue. The figures given in Table 1 for the suspension range from 0·47 to 1·17 mg. The figure of 1·17 mg. of ascorbic acid/g. of adrenal cortex is about two-thirds of the value

Table 1. *Distribution of ascorbic acid between the particulate and non-particulate fractions of adrenal and liver cells*

(For details see text)

Tissue	Ascorbic acid content: Supernatant from low-speed centrifugation (Calc. as mg./g. fresh tissue) (*a*)	Supernatant from high-speed centrifugation (Calc. as mg./g. fresh tissue) (*b*)	Supernatant from high-speed centrifugation (Calc. as % of value in *a*) (*c*)	Sediment from high-speed centrifugation (Calc. as mg./g. fresh tissue) (*d*)	Sediment from high-speed centrifugation (Calc. as % of value in *a*) (*e*)
Ox adrenal medulla	0·85	0·80	94	0	0
Ox adrenal medulla	0·47	0·41	87	0·06	14
Ox adrenal medulla	1·17	1·19	102	0	0
Ox adrenal medulla	0·77	0·64	84	0·07	10
Ox adrenal cortex	1·17	1·12	96	0	0
Dog liver					
Dispersed in 0·25 M sucrose	0·27	0·24	90	0·04	14
Dispersed in 0·88 M sucrose	0·43	0·39	91	0·05	11
Ox adrenal medulla					
Before treatment with H_2S	0·85	0·80	—	0	—
After treatment with H_2S	0·79	0·80	—	0·02	—

(1·85 mg.) given by Birch, Harris & Ray (1933). The finding that treatment with hydrogen sulphide did not increase the figures for the ascorbic acid content is in agreement with the findings of Bourne (1936) and Sayers & Sayers (1948) that in the adrenal gland all the ascorbic acid is in the reduced state.

In all the tissues examined the greater part of the ascorbic acid, 87 % to 100 %, was found in the supernatant from the high-speed centrifugation; the redispersed particles contained only 0 % to 14 % of the total amount of ascorbic acid.

In the procedure used in these experiments the use of 0·25 M sucrose resulted in the sedimentation of all the particulate components of the cytoplasm; on the other hand, it is known that in 0·88 M sucrose the smallest particles, the microsomes, remain in suspension. The 0·88 M sucrose was used in one experiment with dog liver; the conclusion that in this experiment the microsomes were not sedimented is supported by the observation that the supernatant fluid was opaque. There was no difference in the distribution of ascorbic acid in fractions obtained using either medium.

Some of the fractions obtained were treated with silver nitrate solution as described by Bourne (1936).

These fractions were: (1) the supernatant from the low-speed centrifugation, (2) the resuspended sediment from the high-speed centrifugation and (3) the same resuspended sediment with ascorbic acid (neutralized with NaOH) added in a final concentration of 0·1 mg./ml. To 2·0 ml. of each of the samples was added 0·1 ml. of 10 % (w/v) $AgNO_3$ in 10 % (v/v) acetic acid. A drop was placed on a microscope slide covered with a glass cover square and examined within 10–15 min.

Microscopic examination showed no staining of the resuspended mitochondria (2), but they stained brown when suspended in a medium containing sodium ascorbate (3). Particles were similarly stained in the sample (1) derived from low-speed centrifugation.

DISCUSSION

The findings show that in the dispersions used in these experiments little if any ascorbic acid was found in the cytoplasmic particles. The small amount sometimes found in the granular fraction can probably be attributed to contamination with small quantities of supernatant fluid. These findings raise the question whether in the living cell the ascorbic acid is present in the mitochondria or in the cytoplasmic sap. This question cannot be definitely answered on the basis of the experiments given here since the possibility remains that ascorbic acid associated with mitochondria could become detached in the preparation of the various fractions. However, other low-molecular weight components of the adrenal medulla are retained by the particles during this procedure (Welch & Blaschko, 1953).

Evidence that ascorbic acid is present in the granules of cells of adrenal medulla rests on the demonstration of precipitates of metallic silver in association with mitochondria when an acid silver nitrate solution is applied to medullary tissue (Bourne, 1936). However, a precipitate can occur on the surface of granules free from ascorbic acid when they are suspended in a medium containing ascorbic acid. This shows that the localization of the silver precipitate gives no indication of the distribution of ascorbic acid in the living cell.

SUMMARY

1. Estimations have been made of the ascorbic acid content of the particulate and non-particulate fractions of cytoplasm separated by high-speed centrifugation of sucrose dispersions of ox adrenal medulla and adrenal cortex, and of dog liver.

2. The ascorbic acid was found to be entirely or almost entirely in the non-particulate fraction.

3. These results are discussed and additional evidence is advanced for considering that the location of reduced silver particles does not necessarily indicate the localization of ascorbic acid within the cell.

I wish to express my thanks to Dr H. Blaschko for his advice and criticism throughout the course of this work, and to Prof. J. H. Burn for allowing me to work in his Department during the tenure of a C. J. Martin Fellowship of the Australian National Health and Medical Research Council.

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Biosynthesis of Fatty Acids by Slices and Cell-free Suspensions of Mammary Gland

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(*Received* 18 *June* 1953)

The experiments to be described were undertaken with the purpose of developing, if possible, cell-free enzyme preparations suitable for the study of the biosynthesis of fatty acids *in vitro*. The lack of even crude preparations of this kind until recently is probably responsible in a large measure for the paucity of detailed knowledge concerning the intermediary stages of fat metabolism.

While our work was in progress Gurin and his colleagues reported that 'homogenates' and some soluble enzyme preparations made from pigeon liver (Brady & Gurin, 1952*a*, *b*) and rat liver (Dituri & Gurin, 1953) synthesize long-chain fatty acids from [^{14}C]acetate. In our experiments, we have used the mammary gland of lactating animals, which is known to be very active in synthesizing fat (cf. reviews by Folley, 1952; Popják, 1952*a*, *b*). The mammary gland of lactating rats was used mostly, but some experiments were also carried out with sheep udder tissue. The results described here have already been communicated in a preliminary form (Popják & Tietz, 1953*a*).

MATERIAL AND METHODS

Lactating rats, 7–14 days after parturition, were anaesthetized with ether and killed by dislocation of the neck. The abdominal pair of the mammary glands was removed together with the overlying skin and packed in crushed ice. When the glands were thoroughly chilled, they were dissected and freed from connective tissue. One rat provided usually 5–6 g. of gland.

The sheep udder tissue, one portion packed in ice and another in solid CO_2, was transported from the slaughter house to the laboratory. The experiments with the cell-free suspensions prepared from the ice-cold tissue were set up 4 hr., and those with the slices 5 hr., after the killing of the sheep. The experiments with rat tissue were usually started 2–3 hr. after the death of the animals. (We are indebted to Dr S. J. Folley, F.R.S., for supplying the udder tissue from a lactating sheep.)

Preparation of tissue slices. Tissue slices were cut with a Stadie-Riggs (Stadie & Riggs, 1944) microtome and were kept in oxygenated ice-cold buffer until used. About 250 mg. of slices were incubated in 3 ml. of phosphate-saline (Krebs & Eggleston, 1940).

Preparation of suspensions. The tissues were minced finely with scissors and the mince was disintegrated for about 2 min. with 2 vol. of ice-cold buffer (0·154 M-KCl, 100 parts; 0·154 M-$MgCl_2$, 10 parts; 0·1 M-potassium phosphate buffer, pH 7·4, 35 parts) in a glass homogenizer tube provided with a loosely fitting pestle (difference between the diameter of the tube and that of the pestle, 0·40 mm.) which was driven by an electric motor. The suspension thus produced was filtered through four layers of gauze into another homogenizer tube with a tightly fitting pestle (difference between the diameter of the barrel and pestle, 0·10 mm.) and was ground further by hand. Large cell fragments and cell nuclei were removed by centrifugation at 400 *g* at 0° for 10 min. The fat which separated on the surface was removed and the supernatant fluid filtered through a small pad of cotton wool. This filtered supernatant, which contained 50±5 mg. dry

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