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Studies on the Binding of Ascorbic Acid in Animal Tissues.* (28452)

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Ascorbic acid is highly soluble in water and diffuses readily through tissue membranes. It has the physical characteristics of a monosaccharide but it is not distributed uniformly in tissues like glucose and there is no known polymerized storage form in tissues comparable to glycogen.

The concentration of ascorbic acid in the various tissues in the animal body varies widely. This is shown by Table I prepared from the data of Kuether, Telford and Roe (1). The tissue levels of ascorbic acid in guinea pigs upon a high intake of ascorbic acid (cabbage *ad lib*) were found to range from 3 to 157 times the blood level. These data indicate that there is some mechanism that maintains storage of ascorbic acid in animal tissues.

To account for the storage of ascorbic acid it has been assumed that this compound exists in tissues bound to protein. Evidence for this assumption was obtained by Sumerwell and Sealock(2). These authors homogenized liver under 95% ethyl alcohol, centrifuged the mixture, decanted the supernatant and washed the residue 5 times with 95% ethyl alcohol. The washed residue was hydrolyzed by boiling under 5% metaphosphoric acid for 30 minutes. Free ascorbic acid was always found in the hydrolysate. Sumerwell and Sealock found, per gram of pork liver, 80 μg of ascorbic acid in the supernatant and 18 μg in the washed residue. They concluded that the ascorbic acid obtained from the washed liver residue probably existed in a form bound to protein.

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Experimental. We repeated the procedure of Sumerwell and Sealock except that a high speed homogenizer was used instead of a Waring Blendor. Rat liver was ground under 95% ethyl alcohol in a Virtis homogenizer with homogenizing beads for 5 minutes at 45,000 rpm. The mixture was centrifuged, the supernatant was decanted and the procedure of grinding and washing was repeated upon the residue 4 times. The residue was suspended in 5% metaphosphoric acid solution and boiled for 30 minutes. The ascorbic acid in the hydrolysate was determined by the method of Roe and Kuether(3). Results are shown in Table II. After 5 homogenizations and washings, there still remained in

TABLE I. Distribution of Ascorbic Acid in Tissues of Guinea Pigs Upon a High Intake of Ascorbic Acid (Cabbage *ad lib*).

	Concentration of ascorbic acid	
	Mg per 100 g or ml	Tissue level/ Whole blood level
Whole blood	.75	—
Muscle	2.39	3.1
Heart	7.54	10.0
Kidney	9.14	12.1
Brain	18.60	24.6
Liver	23.77	31.5
Spleen	42.83	56.8
Adrenal	118.60	157.3

TABLE II. Ascorbic Acid in Residue of Liver Homogenate After 5 Homogenizations and Washings with 95% Ethyl Alcohol.

	Mg ascorbic acid per 100 g		
	Total	Residue after 5 washings	% of total in residue
Liver 1	37.0	.7	1.9
" 2	29.4	.7	2.5

TABLE III. Optical Density of Color Formed from Aliquots of Filtrates of Liver Homogenate in Different Fractions After Contrifugation at 100,000 × *g*. Fractions are numbered from bottom of tube.

pH of liver homogenate	Optical density of color formed in aliquot from fraction number 1	2	3	4	Concentration gradient % difference between samples 1 and 4
6.5	.308	.284	.259	.255	17
"	.256	.252	.226	.222	13
"	.252	.252	.240	.215	15
"	.260	.244	.235	.222	15
6.6	.325	.310	.284	.270	17
6.6	.325	.299	.268	.277	15
6.7*	.250	.235	.222	.215	14
6.9†	.150	.149	.137	.126	19

* In 3 N Urea.

† In 6 N Urea.

the residue ascorbic acid equivalent to 2% of the total. This recovery was lower than that obtained by Sumerwell and Sealock(2) but we attribute our low values to the greater solubilizing effect of the very vigorous homogenization we carried out. Our findings appear to confirm essentially the work of Sumerwell and Sealock.

Since the results obtained by Sealock's procedure cannot be considered as unequivocal proof of the presence of a bound form of ascorbic acid in tissues, a new approach to the problem, a study of the effect of high speed centrifugation on the distribution of ascorbic acid in a tissue homogenate, was made. The development of a concentration gradient in a tissue extract submitted to ultracentrifugation would be considered evidence in favor of the existence of ascorbic acid in a bound or complexed form.

Rat liver was homogenized under 10 volumes of distilled water in a Virtis homogenizer with homogenizing heads at 45,000 rpm for 10 minutes. H_2S was bubbled into the mixture to serve as a preservative of the ascorbic acid. The mixture was centrifuged at 2500 × *g* for 20 minutes and the residue was discarded. The pH of the supernatant was adjusted to a value ranging from 6.5 to 6.9 and the solution was centrifuged at 10,000 × *g* for 30 minutes. The supernatant from this centrifugation was placed in 10 ml plastic tubes and these tubes were centrifuged at a temperature of 4°C in a Spinco ultracentrifuge at 100,000 × *g* for 18 hours. The tubes were carefully removed, a hole was punched in the bottom of each tube, and 2.5 ml fractions were collected dropwise. Two ml portions of each fraction were analyzed for ascorbic acid by the method of Roe and Kuether(3).

Results. Results of the ultracentrifugation studies are shown in Tables III and IV. A concentration gradient of ascorbic acid was observed in each tube. The differences in concentration of ascorbic acid between the samples from the bottom and the top of the plastic tubes ranged from 13 to 19% and the increase was in every instance consistently towards the end of the tube. Two experiments were carried out in which urea in concentrations of 3 N and 6 N was added to the solution, since urea has the effect of breaking hydrogen bonds of proteins in solution. A density gradient similar to that in the other tubes was formed in the tubes containing urea.

In the control experiments (Table IV), carried out with pure ascorbic acid in a H_2S solution at pH values ranging from 5.6 to 7.35, the concentration differences between the first and fourth fractions were not over 5% and variations were plus and minus. The data obtained with the control tubes repre-

TABLE IV. Controls on Experiments in Table III, Using Ascorbic Acid in H_2S Solution.

pH of ascorbic acid solution	Optical density of color formed with aliquot from fraction number 1	2	3	4
5.6	.387	.403	.403	.409
6.0	.387	.387	.372	.387
6.5	.377	.377	.390	.398
7.0	.347	.357	.354	.354
7.4	.349	.347	.354	.367

sent the error inherent in the procedure. Since the variations in the experimental tubes were much greater than in the control tubes, and were always in the same direction, the results suggest that the ascorbic acid in the liver homogenate was bound to some other substance, forming a complex that gives rise to a density gradient under ultracentrifugation. The data are considered evidence favoring the assumption that ascorbic acid exists in tissues partly in a bound form. The pellets of mitochondria and microsomes removed after the second and third contrifugations were analyzed for ascorbic acid. They contained 1 to 5% of the total ascorbic acid, values that must be considered high as the particles were not washed.

Summary. 1. The procedure of Sumerwell and Sealock, which yields evidence in favor of the exsitence of ascorbic acid in liver tissue in a bound form, was repeated. Results were in essential agreement with the work of these authors. 2. A study of the behavior of ascorbic acid in an aqueous liver homogenate under ultracentrifugation was made. At a centrifugal force of 100,000 $\times$ g a significant density gradient of ascorbic acid was formed. 3. Addition of urea in 3 N and 6 N concentrations had no influence upon the density gradients of ascorbic acid formed in aqueous liver extracts submitted to high speed centrifugation. 4. The results obtained are interpreted as evidence in favor of the assumption that ascorbic acid exists in tissues in a bound form.

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Warburg Studies on Coccidioidin. (28453)

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In most studies of the mechanism of increased oxygen uptake associated with phagocytosis peritoneal exudate cells from guinea pigs and rabbits have been used(1-3).

Strauss and Stetson's Warburg studies on the effect of *in vitro* addition of macromolecular substances to lightly heparinized human blood have indicated a stimulation of oxygen uptake upon addition of bacterial endotoxins and soluble antigen-antibody complexes(4). The effect has been attributed to stimulation of respiratory activity of blood leucocytes.

In vitro addition of substances to whole blood samples provides an approach using leucocytes of peripheral blood under more physiological conditions than usual. Since skin sensitivity to coccidioidin has been passively transferred with leucocytes from sensitive donors(5) it seemed worthwhile to investigate *in vitro* addition of coccidioidin to whole blood samples from coccidioidin negative and positive skin reactors.

Materials and methods. Blood from apparently healthy individuals was drawn from the antecubital vein into a heparinized syringe so that the final concentration of heparin was 3 units (30 μg) per ml of whole blood.

Smith's lot 64D4 skin test antigen received from the San Fernando VAH was used throughout this study. Skin tests were performed by intradermal injection of 0.1 ml into the left forearm using a 1:1000 dilution. Those individuals with a negative reaction were tested similarly with a 1:100 dilution. Readings were taken at 48 hours.

Manometric determinations. Consumption of O_2 was measured by the direct method of Warburg. Upon withdrawal of blood by venipuncture, the blood was mixed by gently inverting the syringe several times. Aliquots of 2.8 ml were then measured directly into the reaction chambers of duplicate 15 ml single side arm Warburg flasks. The center well of each flask contained 0.2 ml of 20%