caused by *D*-cycloserine; however, this amino acid (25 μg/ml) is able to compete in the protection afforded by *D*-alanine (from 25 to 500 μg/ml) against *D*-cycloserine (20 μg/ml). A competition for a common extracellular site between these 2 diastereoisomers is not enough to explain this effect, since high concentrations of *L*-alanine do not inhibit the protection afforded by *D*-alanine. Moreover, it would be difficult to explain why 500 μg/ml of *D*-alanine were required to protect against the effect of 25 μg/ml of *L*-alanine as well as against the effect of 500 μg/ml of *L*-alanine.

Mora and Snell(5) have shown that in spite of the fact that extracellular *L*-alanine is able to slow down the penetration of *D*-alanine in protoplasts of *Streptococcus faecalis*, pyridoxal was only able to stimulate the transport of *L*-alanine; this probably indicates that more than a simple competition is involved at the receptor site in the membrane. Studies on the uptake of radioactive amino acids and peptides by *Pediococcus cerevisiae* lead Shankman *et al*(6) to postulate the existence of 2 physically different sites in the membrane interacting at a distance.

In regard to the accumulation of a mucopeptide by the resistant strain of *M. acapulcensis* to *D*-cycloserine, the results are in accordance with data obtained by Park(7) in cells of *Staphylococcus aureus* resistant to penicillin.

Summary. Growth of *Mycobacterium acapulcensis* is inhibited by *D*-cycloserine. This effect is completely reversed by equimolecular concentrations of *D*-alanine. *L*-alanine is totally inactive while *DL*-alanine is only effective at higher concentrations. *L*-alanine antagonized the protection afforded by *D*-alanine, a fact that cannot be explained by a simple competition between these diastereoisomers at the cell membrane level. In the presence of *D*-cycloserine a mucopeptide accumulates in the sensitive strain and also in the resistant mutant when allowed to grow at high antibiotic concentrations.

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Histochemistry LXXVIII. Ascorbic Acid in Normal Mast Cells and Macrophages and in Neoplastic Mast Cells.* (30097)

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The role of ascorbic acid in the mast cell remains to be delineated, but some interesting observations of possible influences have appeared. Relative depletion of mast cells from guinea pig skin due to ascorbic acid deficiency was reported by von Numers(1), and Pettersson(2) who confirmed this also observed a similar effect in aortic adventitia. The possibilities indicated are that ascorbic acid is necessary, either directly or indirectly, for formation of mast cells, or for their maintenance once formed, or both. Another point of interest is whether ascorbic acid plays a role in hydroxylation of tryptophan (TP) as the first step in the biosynthesis of 5-hydroxytryptamine (serotonin, 5HT), since the pos-

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sible role of ascorbic acid in hydroxylation of aromatic compounds(3-7), proline(8-10), etc., has been stressed. 5HT is found in normal(11-13) as well as neoplastic(14,15) mouse and rat mast cells. It was not the aim of this report to deal with the role of ascorbic acid in mast cells, but, because of the interest in this subject, to obtain some fundamental data on concentrations and content of ascorbic acid in normal rat and neoplastic mouse mast cells isolated from peritoneal fluid, data hitherto unobtained. The macrophage fraction separated from normal mast cells was also analyzed. A control check was made of the influence of the isolation of the cells by the density-gradient method employed. Rats were used to provide the normal mast cells since this species has been most commonly employed for studies on the isolated cells. Mice were used to furnish the tumor cells since the cell line has been maintained in this species. Along with the determination of ascorbic acid, its first products of oxidation, dehydroascorbic and diketogulonic acids, were also measured. Protein-nitrogen was estimated to provide a reference quantity for the expression of concentration. This investigation follows an unpublished preliminary study of ascorbic acid in normal rat mast cells begun with Dr. A. C. Parekh at the University of Minnesota in the laboratory of one of the authors (D.G.).

Animals and cells. Male albino Sprague-Dawley rats weighing 350-400 g were used to provide normal mast cells, and the neoplastic cells were obtained from strain BALB/c × DBA mice, line P-815, developed by Dunn and Potter(16). The tumor cells were maintained by injection of 0.1 ml of peritoneal cell suspension into the peritoneal cavities of each of a group of normal female, 2 month old, DBA/2 mice. Transplantation to new mice was made every 7 days. The tumor cells used in this work, taken 7 days after transplantation, were from generation 443 of the original colony; the cells first obtained from Dr. Michael Potter were from generation 429.

All animals were housed in a constant climate room (25°C, controlled illumination, lights on 6 a. m and off 6 p. m.) for about a week prior to use. They received Purina Fox Chow and tap water *ad libitum,* and were killed instantly by decapitation between 11 a. m. and 1 p. m.

Isolation of cells. Isolation of mast cell fractions in a sucrose density gradient was originally described by Glick *et al*(17), and later Holter and Møller(18) showed the value of employing the dextran polymer, Ficoll (Pharmacia, Uppsala, Sweden) for such gradients. Uvnäs and Thon(19) used a Ficoll gradient for isolation of mast cells, and the procedure used in the present work for normal mast cells follows from these previous experiences with certain small changes found to be advantageous: 1. Decapitate animal and drain blood. 2. Remove hair from abdomen with clippers. 3. Wet abdomen to keep stray hairs from contaminating peritoneal fluid later. 4. Expose abdominal muscle wall and open peritoneal cavity with a mid-ventral incision using an electric cautery knife. (A careful operator can use scissors and still avoid contamination by blood of the peritoneal fluid.) 5. Place 6 ml of heparinized (100 μg/ml) Hanks' balanced salt solution (without phenol red) into the peritoneal cavity and gently massage abdomen for 1.5 minutes. 6. Gently hold the intestines to one side and remove 5 ml of fluid. 7. Carefully place the fluid on top of a density gradient prepared by layering successively 1 ml volumes of 40% and 30% Ficoll in a Pyrex tube (13 × 100 mm, I.D. 11 mm) at room temperature and letting stand for 3-4 hours for stabilization. 8. Gently stir fluid with top Ficoll layer using a thin glass rod, and let cells settle for 20-30 minutes. 9. Spin tube in a horizontal centrifuge (International, Size 1, Model SVB, with tachometer) by advancing rheostat 1 division every 5 seconds until a speed of 2100 rpm (800 × *g*) is attained. Then after 1.5 minutes reduce the speed 1 division every 5 seconds to zero. 10. Carefully withdraw most of the Hanks' solution from the top and pipette the macrophage layer remaining over the uppermost Ficoll layer into another tube. 11. Lower a polyethylene tube (I.D. 0.045 in., O.D. 0.062 in., *e.g.,* No. PE160, Intramedic, Clay Adams Inc., New York), attached to a No. 18 hypo-

dermic needle on a 2 ml syringe, slowly through the upper 2 Ficoll layers and remove the mast cell fraction from the bottom layer, including most of the intermediate (30%) layer with the cells. Transfer this fraction into another tube. 12. To have sufficient material for cell counting and the chemical determinations, pool the corresponding fractions from several animals (about 10 ml total for each fraction from 5 animals) and wash 3 times by centrifugation and resuspension, using 5 ml of Hanks' solution for each wash in each tube, and sedimenting the cells at 800 × *g* for about 5 minutes the first time and 2 minutes the last 2 times. For the unfractioned total cell mixture, spin down the cells and wash them 3 times in the same way. 13. After the last sedimentation resuspend the cells in each tube in 200 μl of Hanks' solution.

The following procedure was used to isolate the tumor cells which composed about 98% of the cell population in the peritoneal fluid of the mice: 1. Collect the peritoneal fluid 7 days after inoculation (about 3 ml/mouse) following steps 1-4 in the procedure for normal cells. Pool the fluid from several mice. 2. Sediment the cells at 2000 rpm (700 × *g*) and wash them 3 times with Hanks' solution in a manner similar to that used for the normal cells (step 12), but with all centrifugations at 2000 rpm. 3. After the last centrifugation resuspend the cells in Hanks' solution to the original volume.

Cell counting. For cell counting in a hemocytometer, 10 μl of final cell suspension from the isolation procedures was diluted with 40 μl of 0.05% toluidene blue. The percentage of different cells in a mast cell fraction was obtained by counting a Giemsa stained smear of the cells.

Chemical determinations. Protein-nitrogen was determined on 10 μl aliquots of the cell suspensions dried *in vacuo* over silica gel. The bromosulfalein-binding method of Nayyar and Glick(20,21) was used. The protein was precipitated by adding 0.3 ml of 40% trichloroacetic acid to 1 ml of sample, and the analytical factors (μg protein-nitrogen/1.0 change in absorbance) found for the fractions of normal rat mast cells and macrophages, and for neoplastic mouse mast cells were 5.5, 5.8 and 5.6 respectively. Determinations of ascorbic acid and the sum of ascorbic, dehydroascorbic and diketogulonic acids by the method of Glick *et al*(22) were carried out using 200 μl of total cell mixture, 180 μl of mast cell fraction, and 60 μl of macrophage or tumor cell fraction.

Results. From the cell counts (Table I) it is evident that mast cells comprised 6 to 7% of the total population in the original peritoneal cell suspension, not quite 3% of the cells in the macrophage fraction, and about 92% of the cells in the mast cell fraction. Smears of the latter gave counts of about 88% mast cells, 9% macrophages and 3% eosinophiles. The macrophage fraction contained about 97% macrophages and less than 3% mast cells. The tumor cell suspension contained about 98% mast cells.

Ascorbic acid and protein-nitrogen determinations (Table II) showed that the mast cell fraction had about 2.6 times more ascorbic acid than that in the same number of cells of the macrophage fraction, while the

TABLE I. Cell Population of Fractions from Peritoneal Washings of Normal Rats.

No. rats	Mean cell count × 10^4/5 ml: Original suspension Mast	Original suspension Macrophage*	Macrophage fraction Mast	Macrophage fraction Macrophage	Mast cell fraction Mast	Mast cell fraction Macrophage	% cells in mast cell fraction: Mast	Macrophage	Eosinophile
5	70	1674	19	628	13	1.4	90	8.6	1.0
6	83	1770	23	975	17	—	91	8.3	1.0
5	125	1210	13	567	16	2.0	89	8.3	2.0
5	100	1290	20	590	16	1.6	87	9.3	4.0
5	90	1374	18	751	20	—	84	10.6	5.3
5	135	1152	19	416	31	—	89	8.3	3.0
Mean	100	1423	19	655	19	1.7	88	8.9	2.7
S.D.	25	254	3	190	6	.3	3	.9	1.7
S.E.	10	104	1.4	77	2.6	.2	1.1	.4	.6

*About 5% eosinophiles present.

TABLE II. Ascorbic Acid and Protein-Nitrogen in Cell Fractions from Peritoneal Washings of Normal Rats.

No. rats (pooled washings)	mμg ascorbic acid/ 10^6 cells		μg protein-nitrogen/ 10^6 cells		mμg ascorbic acid/ μg protein-nitrogen	
	Macrophage	Mast cell	Macrophage	Mast cell	Macrophage	Mast cell
5	215	521	14	40	15	13
6	192	516	12	39	16	13
5	184	525	10	45	18	12
5	166	528	10	45	17	12
5	209	546	14	56	15	10
5	205	446	12	43	17	10
Mean	196	514	12	45	16	12
S.D.	33	32	1.7	6.0	1.2	1.4
S.E.	14	13	.7	2.5	.5	.6

protein-nitrogen was about 3.7 times as great to give a concentration of ascorbic acid per unit protein-nitrogen for cells of the mast fraction of 75% that of the macrophage fraction.

In a comparison of ascorbic acid and the sum of ascorbic, dehydroascorbic and diketogulonic acids in normal rat and neoplastic mouse mast cells (Table III), ascorbic acid represented 87% of the sum in the normal cells and 97% in the neoplastic cells. The normal cells had about 1.5 times the protein-nitrogen of the tumor cells. The concentration of ascorbic acid per unit protein-nitrogen in the normal cells was 1.3 times that of the tumor cells, and the corresponding value for the sum of the ascorbic, dehydroascorbic and diketogulonic acids was about 1.5 times.

Discussion. Although the differences in ascorbic acid and protein-nitrogen content of the normal mast cells and macrophages were appreciable (Table II), the difference in ascorbic acid concentration was of no great significance. The greater content in the mast cell depended largely on its greater size, indicated by the higher protein-nitrogen content. Similarly, the smaller mouse tumor cell with less ascorbic acid than the normal rat mast cell had a concentration only a little different from the latter (Table III). Although the concentration of ascorbic acid alone was slightly less than the sum of ascorbic, dehydroascorbic and diketogulonic acids in the normal cell, no difference was demonstrable in the tumor cell.

Relatively little comparative data of ascorbic acid and the sum of ascorbic, dehydroascorbic and diketogulonic acids in leucocytes have appeared, but recently Hendry *et al* (23) gave respective mean values of 140 and 150 mμg per 10^6 cells in human blood (age of individuals not given). Considering the error of the measurements, these values are only slightly lower than the ascorbic acid content of the rat macrophages but considerably lower than the content in the normal rat mast cell or even in the mouse neoplastic mast cell. The variability of the data in the literature on the ascorbic acid content of human leucocytes is apparent from data such as those of Denson and Bowers(24) who found a mean of 350 mμg per 10^6 cells from blood of a group of normal adults, 18 to 45 years old, and of Denson and Richards(25)

TABLE III. Ascorbic Acid and Sum of Ascorbic, Dehydroascorbic and Diketogulonic Acids in Normal Rat and Neoplastic Mouse Mast Cells.

Normal cell		Tumor cell		Normal cell	Tumor cell	Normal cell		Tumor cell	
Ascorbic acid	Sum	Ascorbic acid	Sum	Protein-nitrogen		Ascorbic acid	Sum	Ascorbic acid	Sum
(mμg/10^6 cells)				(μg/10^6 cells)		(mμg/μg protein-nitrogen)			
551*	631*	271†	279†	43	28	13	15	10	10

* Pooled cells from 5 rats.

† Mean of individual determinations on cells from each of 3 mice (S.D. 18, 29; S.E. 10, 16, respectively, for ascorbic acid and sum).

who reported values ranging from 156 to 643 in the blood from random patients (ages not stated).

Summary. Ascorbic acid and the sum of ascorbic, dehydroascorbic and diketogulonic acids in the mast cell fraction (about 92% mast cells) from peritoneal washings of normal rats, and in mouse ascites tumor mast cells (about 98% mast cells), were determined per 10^6 cells and per μg protein-nitrogen. Ascorbic acid (551 mμg/10^6 cells) comprised about 87% of the sum of the acids in the normal cells and about 98% (271 mμg/10^6 cells) of the sum in the tumor cells. The concentrations of ascorbic acid in the normal and tumor cells were 13 and 10 mμg/μg protein-nitrogen, respectively. The ascorbic acid per 10^6 cells in the rat macrophage cell fraction (96% macrophages) was 38% of that in the mast cell fraction, while the ascorbic acid per μg protein-nitrogen in the former was 1⅓ times that in the latter.

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Pathogenesis of Acute Inflammation. VI. Influence of Osmolarity and Certain Metabolic Antagonists Upon Phagocytosis and Adhesiveness by Leucocytes Recovered from Man.* (30098)

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A clear understanding of the process responsible for the adhesion of polymorphonuclear granulocytes (PMNG) to vascular endothelium during acute inflammation has not been obtained. It is recognized that this event represents a prime step in the mobiliza-

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