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Depressed Growth of Morris Hepatomas and Altered Lysosomal Hydrolases during Altitudinal Hypoxia[1] (38193)

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Warburg *et al.* originally reported that tumors transplanted into rats breathing a low oxygen gas mixture ceased dividing and underwent necrosis (1). Subsequently, a number of investigators (2–19) examined the effects of various types of hypoxia on neoplastic growth, and their consensus is that exposure of tumor-bearing hosts to a hypoxic environment does indeed reduce the overall incidence of various tumors. However, because of the varied environmental conditions and the different malignancies and oncogenicities of the tumors employed, the mechanisms underlying hypoxic retardation of tumorigenesis remain unclear.

The present study was undertaken to evaluate the effects of controlled hypoxia on the growth of 2 lines of Morris transplanted hepatomas. These tumor transplants were selected because they not only have the same genetic origin, but also different degrees of malignancy. Furthermore, these hepatomas, as well as their common normal cells of origin, i.e., hepatocytes, have been extensively studied so that a broad base of reference methods and growth parameters already exists.

Additionally, since it has been shown that hepatic cytoplasm regresses during acute exposure to altitude by lysosomal autophagy (20, 21), and since lysosomes are abundant in tumors (22–31), the activities of representative lysosomal enzymes in the hepatomas were measured, in order to determine whether these might be implicated in the reported growth alterations of hypoxic tumorous tissue. For purposes of comparison, the hepatic lysosomal hydrolase activities of both control and tumor-bearing rats exposed to altitudinal hypoxia also were determined, since these data were not available for altitude-acclimated rats.

Materials and Methods. Animals and tumors. Twenty-four male, 86-day old, and 24 female, 78-day old, Buffalo strain rats were inoculated bilaterally into the femoral musculature with Morris hepatomas 5123C (Generation 76) and 7793 (Generation 31), respectively. The injections were performed at the Cancer Research Unit, Howard University, by Dr. H. P. Morris, and the animals shipped to Memphis, TN, within the week after transplantation. Twenty-four nontumorous male, 92-day old, Buffalo strain rats, obtained from Simonson Laboratories, Inc., Gilroy, CA, were used as controls. After habituation to our handling procedures, the respective tumorous and nontumorous rats were subdivided into three groups. Each group of animals was exposed for 8 weeks to the following environments, respectively: Group I: to 4500 m simulated altitude in a hypobaric chamber, described previously (32);

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Group II: to 450 m simulated altitude in another, identical hypobaric chamber; and Group III: to sea level under the conditions normally prevailing in our animal room (mean ambient temperature = 24.4 ± 1.0°, alternating light and darkness [with lights from 0600–1800 hrs]), in a cage of the same floor dimensions as the altitude chambers. All the animals in a group were housed together; they were fed regular Purina Rat Chow and water *ad libitum*. The rats were weighed weekly, and their tumors were sized by the method described by Morris and Wagner (33), viz., the sum of the length and width of the tumor at right angles to each other, using a Glogan vernier caliper No. 12. The altitude-exposed animals were returned briefly to sea level for these measurements. At the conclusion of the eighth week of exposure to their respective environments, i.e., when the tumors grown in the sea level hosts were fully developed, all the rats were anesthetized with ether. Sufficient blood was then withdrawn by cardiac puncture for subsequent enzyme assays. After exsanguination to death, tumors and livers were quickly excised and all attached connective tissue was dissected away. The hepatoma tissue was patted dry and weighed on a Mettler Model P1200 Top Loader Balance. Tumors and livers were then frozen at −20° until assayed for lysosomal hydrolase activity.

Determination of enzyme activities. Frozen samples of livers or hepatomas were thawed and prepared as 10% homogenates in 0.25 *M* sucrose using a modification of the Potter-Elvehjem procedure (34). Acid phosphatase (EC 3.1.3.2), beta-glucuronidase (EC 3.2.1.31), acid ribonuclease (EC 2.7.7.16), and cathepsin D (EC 3.4.4.23) were assayed as total hydrolase activities. Total determinations were achieved by incorporating 0.01% Triton X-100 into the substrate; this has been amply shown to release hydrolases with minimal undesirable side-effects of denaturation or enzyme inhibition (35).

Acid hydrolase measurements. Acid phosphatase was determined by the method of Gianetto and deDuve (36) at pH 5.0, with beta-glycerophosphate as the substrate. Released P_i was measured by the Fiske-Subbarow method (37). Beta-glucuronidase was determined by the method of Gianetto and deDuve (36) at pH 5.0, with phenolphthalein monoglucosiduronic acid as the substrate. Released phenolphthalein was colorimetrically analyzed in pH 10.6–11.0 glycine buffer. Acid ribonuclease was measured by the method of deDuve *et al.* (38) at pH 5.0, with yeast ribonucleic acid as substrate. Split nucleotide fragments were analyzed at 260 mμ in a Beckman DU spectrophotometer. Cathepsin D activity was determined at pH 3.6 according to the method of Gianetto and deDuve (36), with denatured hemoglobin as substrate. The released fragments were analyzed using the colorimetric method of Lowry *et al.* (39). All substrates were purchased from Sigma Chemical Company, St. Louis, MO. Tissue proteins were determined by the method of Lowry, *et al.* (39). Sera were assayed for acid phosphatase and beta-glucuronidase by the methods described above except that the incubation time was 60 min.

Data expression and analyses. In this study all tissue enzymatic activities were expressed as units of substrate released per gram of liver or hepatoma protein, as follows:

acid phosphatase—mg of inorganic phosphorus/10 min incubation/g protein.
beta glucuronidase—mg of phenolphthalein/10 min incubation/g protein.
acid ribonuclease—μmoles of mixed nucleotides/10 min incubation/g protein.
cathepsin D—μEq of tyrosine/10 min incubation/g protein.

Serum enzymatic data were expressed as mg of substrate released/1 hr incubation/100 ml serum.

Both morphometric and biochemical data were summarized as the means ± standard errors. Significant differences between the responses of the nontumor- and tumor-bearing animals were determined by the Student *t* test, based on a 95% level of confidence.

Results. Body mass changes. The rates of body mass gain of the nontumorous and tumorous rats exposed to either sea level or

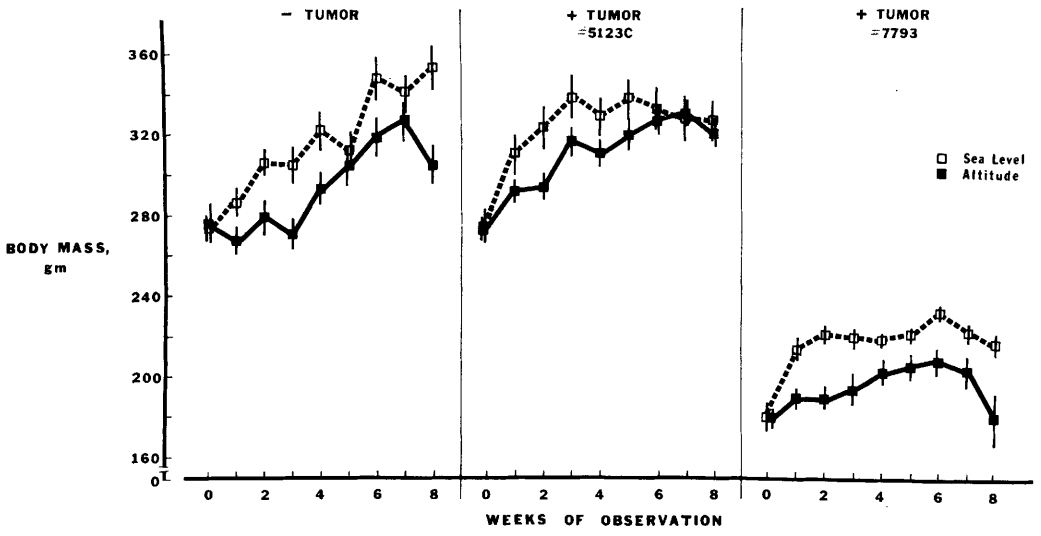

FIG. 1. The influence of 8 weeks' exposure to sea level or 4500 m simulated altitude on the body mass of nontumorous and Morris hepatomas 5123C- and 7793-bearing Buffalo strain rats. Each point represents the mean body mass of 8 rats; the bars indicate ± S.E.

4500 m simulated altitude are plotted in Fig. 1. The body masses of the nontumorous rats (left panel) were reduced at altitude in comparison with sea level. The decrement, however, was not continuous throughout the 8 weeks of altitude exposure, but lasted only for the first 3 weeks. Thereafter, the animals gained weight at an increased rate. But after the seventh week of exposure, a significant weight loss was again observed.

The rats bearing Morris hepatoma 5123C (middle panel), both at sea level and at altitude, increased in body mass more rapidly during the first 4 weeks than the corresponding controls. But during the subsequent weeks, they displayed only slight further gains in both environments. Again, the mean weekly weights of the altitude-exposed hosts were generally smaller than those of the tumorous rats maintained at sea level.

Morris hepatoma 7793 greatly depressed the growth of their hosts (right panel). When compared with the rats bearing the 5123C, altitude exposure further retarded the rates of weight gain of these animals, so that the body mass of the altitude hosts remained significantly lower than that of their sea level counterparts for the duration of this hypoxic exposure.

Tumor growth. Figure 2 illustrates the rates of growth of Morris hepatomas 5123C and 7793 in 8 rats maintained at sea level and altitude, respectively. The first measurements of palpable tumors were obtained after 4 weeks of exposure (5 weeks after inoculation). The values shown at 2 weeks represent, in effect, the width of the rats' thighs before the tumors were palpable. At 4 weeks, the dimensions of the 5123C tumors (left panel) were not significantly different in the two environments. It should be noted that, according to accepted physiological criteria, the altitude-exposed rats were by this time acclimated to their environment. During the subsequent weeks the tumors at sea level grew steadily. But at altitude, they did not further increase in size until after the sixth week of exposure; thereafter, however, they grew again, and as rapidly as the hepatomas in the rats kept at sea level. But overall, the altitude hepatomas were, from the fifth week until sacrifice, smaller than the sea level tumors. As shown in the insert, the final mass of the tumors from the altitude hosts was only 50% that from the sea level rats. At 4500 m

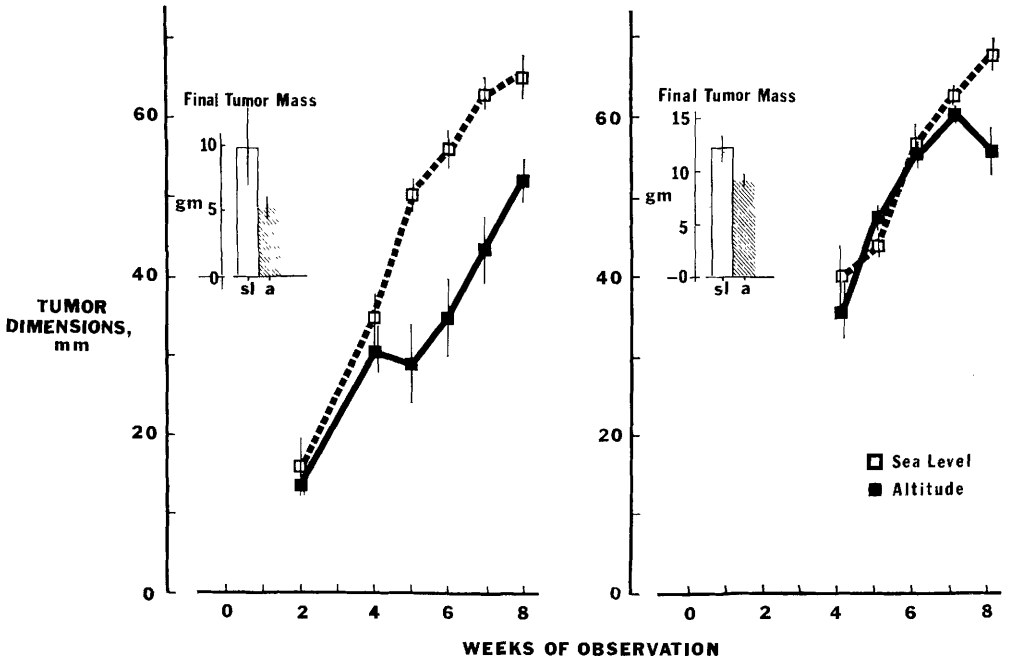

FIG. 2. The influence of 8 weeks' exposure to sea level or 4500 m simulated altitude on the rate of growth and the mass (at sacrifice) of Morris hepatomas 5123C (left) and 7793 (right). The values are the weekly means of the sums of the tumor length and width; the bars indicate ± S.E.

the hepatomas accounted for 1.55% of the mass of their hosts, as contrasted to 2.95% at sea level. Thus, a marked diminution in the size of the 5123C hepatoma was induced by exposure of the hosts to altitude, of the level and duration studied.

The growth of hepatoma 7793 (right panel) also was stunted by altitude, although to a lesser degree than that of hepatoma 5123C. Its rate of growth, however, was not different at the two altitudes until the last week, when it evidently ceased to develop and abruptly decreased in size at 4500 m, in conjunction with the fall in body weight noted earlier; but it continued to grow unhinderedly at sea level. Nevertheless, at sacrifice, the weight of this hepatoma constituted an insignificantly different fraction of the mass of its respective hosts at the two altitudes, viz., 5.60% at sea level and 4.54% at altitude. This lack of difference was evidently due to the smaller body weights of the rats bearing this tumor at altitude, in whom, therefore, it loomed as a relatively larger tumor than at sea level. Hepatoma 7793, moreover, appeared to be very toxic to its hosts at both altitudes. Attention is called, in this connection, to the observation that the maximum size of this tumor was attained after 2.1 mo of growth, in contrast to 8.9 mo reported for earlier generations.

Lysosomal hydrolase activities. Figure 3 shows the activities of the 4 representative hydrolases in the nontumor-bearing and the 5123C-bearing rats, under the conditions of these experiments. Exposure to 4500 m resulted in a slight, but significant, decrease of the acid phosphatase activity in the livers from the nontumorous rats (upper left panel). Altitude had, however, no significant effect on the serum activity of this enzyme (middle left panel). At sea level, the hepatic acid phosphate activity of the tumorous rats was increased markedly (69.4%), while at altitude it was raised significantly less. Serum acid phosphatase activity also was significantly elevated in the tumorous rats, both at sea level and at altitude. On the other hand, the activity of

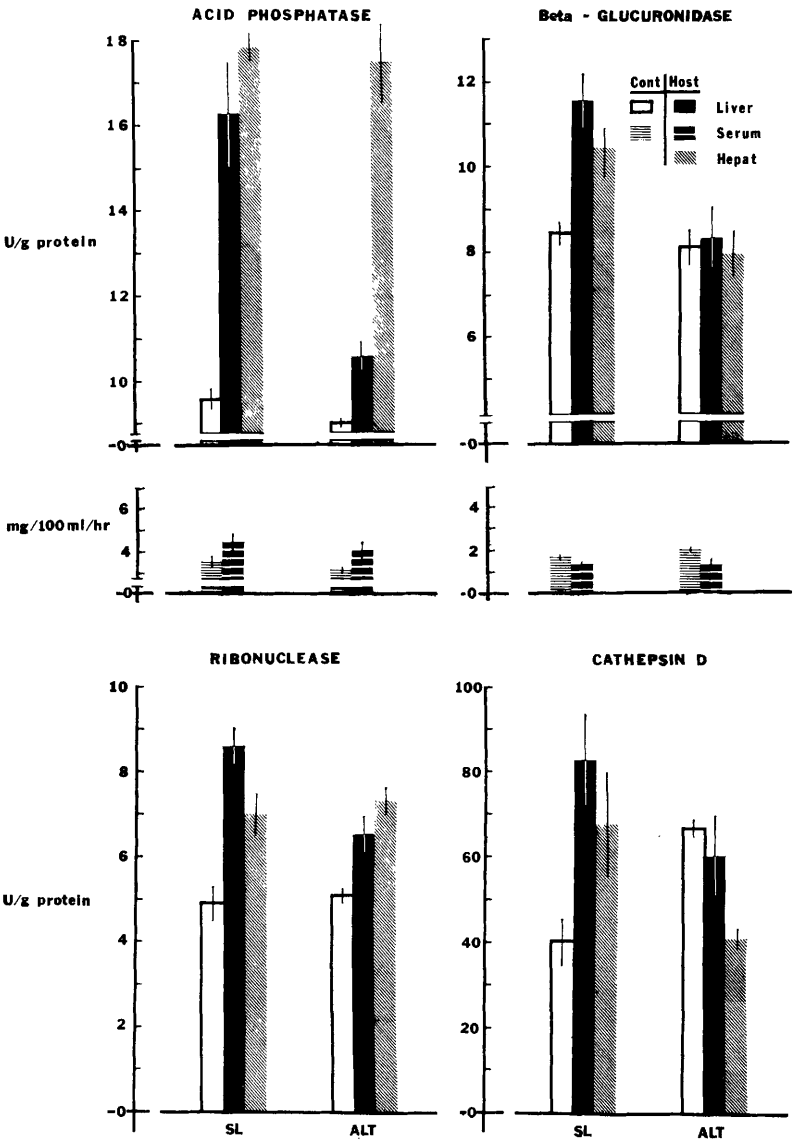

FIG. 3. Lysosomal hydrolase changes (means ± S.E.) in liver, serum, and Morris hepatoma 5123C from rats exposed for 8 weeks to sea level or 4500 m simulated altitude.

this hydrolase in the hepatomas was not different in the two environments. It was, however, increased by comparison to that in the livers from both the hosts and the nontumorous controls, especially at altitude.

Altitude exposure had no significant effect on the activity of beta-glucuronidase in the livers of the nontumorous rats (upper right panel); but it increased this activity slightly in the serum (middle right panel) of these animals. The hepatoma, on the other hand, induced elevations in the hepatic activity of this enzyme (35.4%) in the rats kept at sea level, but not in those exposed to altitude; and it depressed this activity in the serum at altitude, but not at sea level. The beta-glucuronidase activity was significantly lower in the hepatomas from the altitude than in those from the sea level animals. At sea level, hepatoma

beta-glucuronidase activity was decreased relative to host livers, and increased relative to normal livers. But at altitude, it was not altered relative to both of these.

Altitude exposure did not significantly affect the activity of acid ribonuclease in the livers (lower left panel) of the nontumorous animals. But the livers of the tumorous rats maintained at sea level displayed marked increases in the activity of this enzyme (73.7%). In contrast, this activity was much less augmented in the livers from hosts exposed to 4500 m. The activity of acid ribonuclease in the hepatomas was not different in the 2 groups. But it was altered in comparison to the activity in both the relevant host and normal livers. Serum activities of this enzyme were not determined.

Contrary to its effect on the activity of the previous lysosomal enzymes, altitude increased cathepsin D activity in the livers (lower right panel) from the nontumorous rats (67.5%). At sea level, the hepatic activity of this enzyme was greatly increased in the tumor-bearing animals (104.8%), while at altitude it was unchanged. In the hepatomas, cathepsin D activity was lower at altitude than at sea level, and lower too at 4500 m in relation to the host livers. This activity was, however, greater at sea level and lower at altitude in relation to the corresponding normal livers.

The effects of the present experimental conditions on the activities of acid phosphatase in the rats bearing hepatomas 7793 are illustrated in Fig. 4. This tumor produced significant increases in both the hepatic and serum activities of this hydrolase, in the two environments. But, in contrast to the responses of this lysosomal enzyme in the animals inoculated with hepatoma 5123C, these increases were significantly smaller in the livers of the tumorous rats kept at sea level than in those exposed to 4500 m; serum acid phosphatase activity was elevated equally in the two groups. The activity of this enzyme was significantly smaller in the hepatomas from the altitude than in those from the sea level animals. Relative to host livers, hepatoma acid phosphatase activity was lower at altitude, but

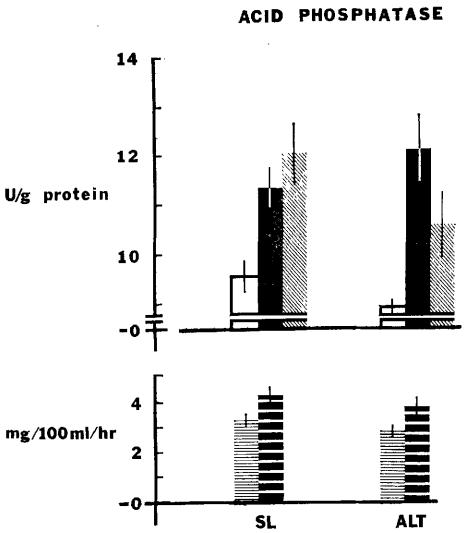

FIG. 4. Acid phosphatase changes (means ± S.E.) in liver, serum, and Morris hepatoma 7793 from rats exposed for 8 weeks to sea level or 4500 m simulated altitude. Same symbols are used as in Fig. 3.

not different at sea level. But in relation to normal livers, this activity was augmented at both altitudes.

The responses at 450 m. The stress of the hypobaric chamber, but without the high altitude, produced no results of significance for the present study excepting the following (as compared to the corresponding sea level controls): (i) the rates of weight gain of the rats bearing hepatoma 5123C were retarded by this treatment; (ii) hepatoma 5123C began to grow at 450 m later than at sea level, but the final mass of this tumor (6.93 ± 1.05 g) was not significantly different from control; (iii) the hepatic activity of cathepsin D was significantly increased under these conditions in the nontumorous rats and decreased in the rats bearing 5123C.

Discussion. The present experiments were undertaken in the belief that a clearer indication of the mechanisms involved in hypoxia might be provided by comparing the development of homologous tumors of known and reproducible characteristics, i.e., Morris transplantable hepatomas, under rigorously standardized ambient conditions. Thus, the present data indicate that the growth of Morris hepatomas 5123C and

7793 was significantly impaired by exposure of the hosts for 8 weeks to 4500 m simulated altitude. The results, therefore, extend to these 2 hepatoma lines the findings of different workers in other tumors (1–17), and substantiate that the Morris hepatomas are suitable models in which to examine the hypoxic effects on tumor growth.

Since autolysis in other physiological and pathological tissue regressions is often mediated by autophagy and lysosomal hydrolysis (21, 34, 40, 41), the possibility was considered that a similar process might be occurring in hypoxic tumorous tissue, i.e., tumor growth might be proceeding at a normal rate while concomitant autolytic destruction might modulate its apparent development. This conjecture seemed strengthened by the presence of abundant lysosomes in tumors (22–31), and the ability of hypoxia to induce membrane permeability changes in lysosomes (20, 21). However, the present results would seem to suggest that, to the contrary, lysosomally-mediated, progressive autolytic destruction of the growing tumors is probably not a primary factor in their smaller mass at altitude. For, instead of increasing, hepatoma hydrolase activities were unchanged at 4500 m as compared to sea level. Moreover, if, owing to hypoxia-enhanced labilization of lysosomal membranes, an increased release of these hydrolases from the hepatomas had occurred, then the diffusion of lysosomal enzymes into the circulation should have raised the altitude serum enzyme activity above its sea level counterpart, when in fact the former tended to be decreased. It is, of course, possible that the released lysosomal enzymes were rapidly incorporated into the liver, but then a positive correlation between the activity of lysosomal enzymes in tumor and liver would be expected, and none was demonstrated in this study. It is also conceivable that lysosomal labilization caused diffusion of enzymes intracellularly without concomitant elevation of serum hydrolase activity, but the autolytic breakdown that should have resulted was not evident in these hepatomas. Nevertheless, measurements of hepatoma both free and total hydrolase activities during the course of altitude exposure are needed before the possibility of an autolytic mechanism of tumor growth depression at altitude can be definitely denied.

The present findings of large increases in the liver hydrolase activities of the hepatoma-bearing rats at sea level corroborate clinical data on similar hepatic lysosomal alterations during the growth of various tumors (26, 27, 40). Two explanations for this effect have been proposed: the liver may be exposed to (i) an increased load of foreign matter which is liberated into the circulation by the growing tumor, or (ii) a specific agent released by the tumor which stimulates enhanced hepatic lysosomal activity. The observation in the present report that at altitude the retardation of hepatoma growth was associated, by contrast, with generally a diminished hepatic lysosomal hydrolase response could be consistent with either speculation, i.e., either (i) consequent to its smaller growth, the hepatoma released fewer metabolites or autolytic debris into the blood stream and, thus, promoted a weaker endocytic drive to the liver lysosomal system, or (ii) the slowly growing tumor liberated less of the specific lysosome-activating factor.

Except for the striking elevation of hepatic cathepsin D, the activities of the 4 representative hepatic and the 2 serum lysosomal hydrolases were, under the conditions of these experiments, only slightly affected in the nontumorous rats by eight weeks' exposure to 4500 m simulated altitude. These findings are in contrast to the marked rat liver lysosomal alterations noted by Nelson (20). However, the altitude levels used in that study were considerably higher than in the present, and the exposures were all acute (maximum duration: 4 hr). Since it is established that acute hypoxia is a potent stimulus to lysosomes (46), the present responses may indicate that, during prolonged tissue hypoxia, compensatory adjustments become manifest in regard to the cellular control processes of lysosomal stabilization and cellular integrity.

The tendency of lysosomal hydrolase activities to generally be augmented in the hepatomas as compared to host livers conforms to previous findings (24, 25, 29–31). Therefore, the absence of such elevations in beta-glucuronidase and cathepsin D activities in the hepatomas from the altitude rats may be of interest. Since cathepsins have been implicated in tumor growth and spread (43), the correlation of low growth with low cathepsins may indicate that these reflect the regulation of tumor mass in a regressing situation. However, since the regulation of intracellular acid hydrolases is sensitive to so many diverse, local environmental influences, and the internal milieu of tumors so labile in this regard, the meaning of these observations with respect to tumor growth depression at altitude remains uncertain.

Summary. The present results indicate, therefore, that the growth of Morris hepatomas 5123C and 7793 was impaired by exposure of the hosts to 4500 m altitude, and that this effect involved alterations in the activities of lysosomal enzymes in both hepatomas and host livers.

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1. Warburg, O., Wind, F., and Negelein, E., Klin. Wochenschr. **5**, 829 (1926).

2. Campbell, J. A., and Cramer, W., Lancet **214**, 828 (1928).

3. Karczag, L., Arch. f. Exper. Zellforsch. **6**, 178 (1928).

4. Campbell, J. A., J. Pathol. Bact. **35**, 379 (1932).

5. Pollack, M. A., Taylor, A., and Sortomme, C. L., Cancer Res. **2**, 828 (1942).

6. Sundstroem, E. S., and Giragossintz, G. A., Proc. Soc. Exp. Biol. Med. **27**, 511 (1930).

7. Sundstroem, E. S. and Michaels, G., *in* "Memoirs of the University of California" (C. B. Lipman & E. R. Hedrick, eds.), pp. 1–409. University of California Press, Berkeley and Los Angeles (1942).

8. Barach, A. L., and Bickerman, H. A., Cancer Res. **14**, 672 (1954).

9. DiPaolo, J. A., Cancer Res. **22**, 299 (1962).

10. Heston, W. E., and Pratt, A. W., J. Nat. Cancer Inst. **22**, 707 (1959).

11. Mori-Chavez, P., J. Nat. Cancer Inst. **21**, 985 (1958).

12. Mori-Chavez, P., J. Nat. Cancer Inst. **28**, 55 (1962).

13. Mori-Chavez, P., J. Nat. Cancer Inst. **29**, 945 (1962).

14. Mori-Chavez, P., and Salazar, M., Proc. Amer. Ass. Cancer Res. **3**, 253 (1961).

15. Mori-Chavez, P., and Salazar, M., J. Nat. Cancer Inst. **35**, 193 (1965).

16. Mori-Chavez, P., Upton, A. C., Salazar, M. J., and Conklin, J. W., Cancer Res. **30**, 913 (1970).

17. Tannock, I. F., and Steel, G. G., J. Nat. Cancer Inst. **45**, 123 (1970).

18. Mori-Chavez, P., *in* "Mechanisms of Invasion in Cancer" (Pierre Denoix, ed.), pp. 73–86. Springer-Verlag, Berlin (1966).

19. Mori-Chavez, P., and Salazar, M., Nat. Cancer Inst. Monograph **14**, 309 (1964).

20. Nelson, B. D., Amer. J. Physiol. **211**, 651 (1966).

21. Abraham, R., Goldberg, L., and Grasso, P., Nature, **215**, 194 (1967).

22. Essner, E., and Novikoff, A. B., J. Cell Biol. **15**, 289 (1962).

23. Kampschmidt, R. F., and Wells, D., Cancer Res. **28**, 1938 (1968).

24. Kampschmidt, R. F., and Wells, D., Cancer Res. **29**, 1028 (1969).

25. Roth, J. S., Hilton, S., and Morris, H. P., Cancer Res. **24**, 294 (1964).

26. Scherstén, T., Wahlqvist, L., and Jilderos, B., Cancer **27**, 278 (1971).

27. Scherstén, T., Wahlqvist, L., and Johansson, L-G., Cancer **23**, 608 (1969).

28. Shamberger, R. J., Biochem. J. **111**, 375 (1969).

29. Shamberger, R. J., Hozumi, M., and Morris, H. P., Cancer Res. **31**, 1632 (1971).

30. Wagner, R. L., and Roth, J. S., Cancer Res. **27**, 2053 (1967).

31. Wattiaux, R., and Wattiaux-De Conick, S., Eur. J. Cancer **4**, 193 (1968).

32. Blatteis, C. M., and Tucker, E. F., J. Appl. Physiol. **15**, 1146 (1960).

33. Morris, H. P., and Wagner, B. P., Methods in Cancer Res. **4**, 125 (1968).

34. Filkins, J. P., Amer. J. Physiol. **219**, 923 (1970).

35. Wattiaux, R., and deDuve, C., Biochem. J. **63**, 606 (1956).

36. Gianetto, R., and deDuve, C., Biochem. J. **59**, 433 (1955).

37. Fiske, C. H., and Subbarow, Y., J. Biol. Chem. **66**, 375 (1925).

38. deDuve, C., Pressman, B. C., Ginanetto, R., Wattiaux, R., and Appelmans, F., Biochem. J. **60**, 604 (1955).

39. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **143**, 265 (1951).

40. Butterworth, S. T. G., J. Path. **101**, 227 (1970).

41. Weissmann, G., New Engl. J. Med. **273**, 1143 (1965).

42. Ghadially, F. N., and Perry, E. W., Cancer **18**, 485 (1965).

43. Allison, A. C., *in* "Lysosomes in Biology and Pathology," pp. 178–204. American Elsevier, New York (1969).