

Role of Host and Tumor Calcium in Metastases¹

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SUMMARY

To define more clearly the role of tumor and host calcium in the development of metastases, a series of *in vivo* investigations were conducted with Walker tumor which demonstrated the following:

(a) Tumor calcium was modified by the level of serum calcium in the host. A reduction of tumor calcium occurred when tumors were grown in animals whose serum calcium was <5.0 mg % and a marked increase of calcium was observed in tumors from animals with serum calcium >11.0 mg %. Transfer of successive generations of tumor in hypocalcemic animals (serum calcium 5.0-8.9 mg %) failed to significantly alter tumor calcium, whereas those transferred in hypercalcemic animals (>11.0 mg %) demonstrated a progressive elevation of calcium.

(b) Host serum calcium levels failed to influence the development of metastases. The incidence of liver and lung metastases was similar following the transfer of tumor cells into normo-, hypo-, and hypercalcemic animals.

(c) As determined by the disappearance of labeled tumor cells from their injection sites, there was no difference in the invasive characteristics of cells obtained from low or high calcium-containing tumors.

(d) Plugs removed from low and high calcium-containing tumors were inserted into the legs of hypo- and hypercalcemic animals which were either untouched until sacrifice or were subjected to hind-quarter amputation 7 or 12 days after implantation. In no circumstances was a difference in the incidence of lung metastases observed.

These studies and recent observations by others suggest that the role of calcium in the phenomenon of tumor metastasis requires further clarification.

INTRODUCTION

In considering mechanisms of metastases formation it is generally accepted that local invasiveness and distant spread is the result of decreased adhesiveness of cancer cells which is related to a lowered calcium content of these cells. Evidence for this arises primarily from the investigations by Coman (7, 8) and his associates (14, 20) and from studies (3-6, 9, 10) which have compared the calcium content of tumor with that of normal tissue. In 1944 Coman (7) determined *in vitro* the

force required by micromanipulation to separate normal cells and tumor cells and concluded that cells from carcinoma of the lip and the cervix showed adhesiveness far less than that of normal cells. His suggestion that the decreased mutual adhesiveness of such cells was related to cell calcium gained support from the subsequent observations of Zeidman (20) who, employing similar *in vitro* technics, noted that adhesiveness of normal cells was reduced when placed in a calcium-free medium or when exposed to methylcholanthrene, a substance which lowers the calcium content of squamous epithelium.

The first observation that tumors possess less calcium than do normal tissues was made by Beebe in 1904 (3) and was confirmed by Clowes and Frisbie (6), who noted that calcium was lowest in rapidly growing tumors. Subsequently, Carruthers and Suntzeff (5) demonstrated a considerable decrease of calcium in both mouse and human squamous-cell carcinomas, and Shear (16), in a comprehensive review of the role of calcium in cancer, found reliable evidence of lowered calcium in the Jensen sarcoma, Rous sarcoma, and several other tumors. Brunschwig *et al.* (4) and Dunham *et al.* (10) found that human gastric and colonic tumors contained less calcium than did normal adjacent mucosa. Similarly, deLong *et al.* (9) reported that the calcium content of human intestinal cancers was 44% less than that in adjacent normal mucosa.

As a result of these studies, the conclusion that decreased adhesiveness and invasion of cancer cells were dependent upon their low calcium content seemed warranted. However, to our knowledge no investigations have been carried out *in vivo* to determine the significance of such findings in so far as metastatic incidence is concerned. It might be anticipated that the latter could be influenced by the content of the tumor calcium and by host calcium levels should they affect that of the tumor. This paper records the results of studies carried out to evaluate the effect of tumor and host calcium on metastases.

MATERIALS AND METHODS

Female Sprague-Dawley (Holtzman, Madison, Wisconsin) rats weighing 150-220 gm were employed in these studies. All animals were housed in individual cages and fed a standard laboratory diet (Purina Rat Chow) and tap water *ad libitum* until assigned to experimental groups. The Walker carcinoma was employed. Tumor was either injected intraportally (5000 cells in 0.5 ml of saline) or was inoculated as a suspension or as a solid plug subcutaneously (500,000 cells) into the left hind leg between the ankle and the knee.

The technic utilized to prepare tumor cell suspensions and to inject cells intraportally has been previously described (12).

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Tumor plugs were prepared by cutting a cylinder of firm tumor with a 13-gauge needle and sectioning it into 3-mm segments which were inserted subcutaneously.

Animals inoculated intraportally were sacrificed 14 days after tumor cell injection and livers were examined for metastases. Those implanted with tumor plugs or injected with tumor cells subcutaneously were sacrificed 21 and 30 days respectively after tumor transfer and examined for lung metastases.

To produce hypocalcemia, animals were parathyroidectomized and maintained on a calcium-free, vitamin D-free synthetic diet with distilled water for 2 weeks prior to tumor inoculation and subsequently until sacrifice. Parathyroidectomies were performed with the aid of the Zeiss operating microscope. The two glands, one on the lateral aspect of each thyroid lobe, were easily visualized and removed with a minimum of trauma to the thyroid. The diet consisted of 24% casein, 68% sucrose, and 5% fat (corn oil) with complete mineral and vitamin supplement exclusive of calcium and vitamin D.

Hypercalcemia was produced by the daily feeding of ground chow (Purina) with the addition of 8000 units of crystalline vitamin D₂ (Calciferol-Nutritional Biochem. Corp.). All animals were maintained on this diet for 2 weeks prior to tumor inoculation and subsequently until sacrifice.

Animals were sacrificed by decapitation and were exsanguinated. Tumor and normal tissues were removed, frozen on dry ice, and stored at -20°C until analyzed. All analyses were carried out within 2 weeks. Preliminary observations demonstrated that calcium determinations remained constant for at least 6 weeks. Only small, firm, non-necrotic tumors trimmed of surrounding tissue were employed. Serum calcium was determined by ethylenediaminetetraacetic acid titration using the Oxford Titrator and is expressed as mg/100 ml of serum. Tumor and normal tissue calcium was determined employing a modification of the method described by Baron and Bell (2). Tissues were treated by sonication (Branson Sonifier) to disrupt the cells completely before precipitation of protein with trichloroacetic acid. The calcium was precipitated as the oxalate, converted to carbonate, dissolved in HCl, and titrated with ethylenediaminetetraacetic acid. This method gave excellent replicate results on normal tissues and tumors. Recovery of added calcium was consistently complete. All determinations were run in duplicate and agreed within ±1.5%. Extensive trials of ashing techniques failed to provide consistent results in our laboratory. Results are reported as mg % of wet weight of tissues.

For a decade Walker tumor has been propagated in this laboratory by the subcutaneous inoculation of 1 ml of tumor brei in normal rats. Such animals develop a firm 1-1.5 cm tumor 7 to 10 days later. To determine the effect of altered host serum calcium on tumor calcium, tumors were grown in hyper- and hypocalcemic rats. The growth pattern of such tumors did not differ from that in normal animals. Approximately 10 days after inoculation, animals were sacrificed for determination of serum and tumor calcium. To determine the effect of propagation of successive generations of tumors in low calcium animals, a normal tumor was injected into a number of hypocalcemic rats. At each transfer, tumor from the rat with the lowest blood calcium was inoculated into additional hypocalcemic

animals. Tumor propagation in animals with high serum calcium was similarly carried out.

Animals implanted with tumor plugs were subjected to left hind leg amputations 7 or 12 days later, care being taken not to manipulate the tumor site.

⁵¹Cr-labeled tumor cell suspensions were prepared from 9 tumors. Calcium analysis was carried out on a portion of each tumor. The technic of labeling and sample counting has been described (11). Five million labeled cells in a volume of 1 ml were injected in the lower left hind leg, which was amputated at intervals from 15 min to 24 hr after inoculation. Radioactivity of legs was determined and results are expressed as % of injected activity and/or number of tumor cells remaining in the leg.

RESULTS

Relation of Host Serum Calcium to Tumor Calcium. To evaluate the effect of altered host serum calcium on tumor calcium, tumors grown in normo-, hypo-, and hypercalcemic animals were analyzed for their calcium content (Chart 1). Those obtained from 43 normal rats having a serum calcium between 9.0 and 10.0 mg % contained 5.2 ± 1.7 mg % of calcium. Tumors from hypocalcemic animals had a significant reduction in calcium only when serum calcium of such animals was below 5 mg %. Under such circumstances tumor calcium averaged 4.0 ± 1.7 mg %. Hypercalcemic rats grew tumors with significantly higher calcium than did normal animals. In those rats tumor calcium values were greater than 8 mg %. Just as in the tumors, liver and muscle calcium of hypocalcemic animals differed little from normal, whereas in hypercalcemic animals an elevation of calcium was observed in those tissues. Liver of normal rats (12 rats) contained 2.3 ± 0.6 mg % of calcium. Those (10 rats) with a serum calcium of 5.0-5.9 mg % had a liver calcium of 2.0 ± 0.4 mg %, and those whose serum calcium was >12.0 mg % (16 rats) demonstrated 4.9 ± 1.2 mg % of calcium in liver. Changes in muscle calcium (normal 4.1 ± 0.7 mg %) were of similar magnitude.

In an attempt to further alter tumor calcium, successive generations of tumor were transferred through hypo- and hypercalcemic animals during a period of approximately 2

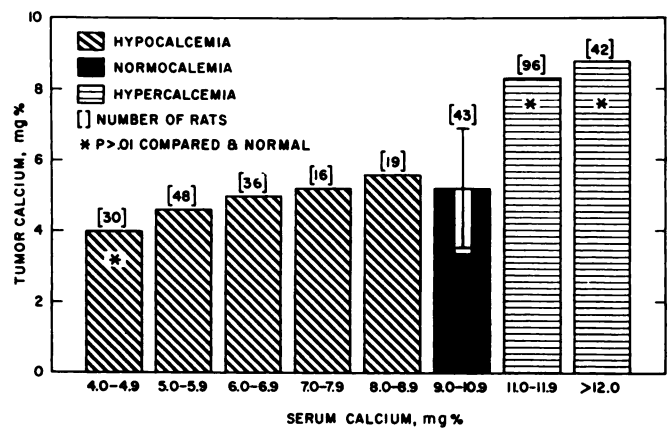


Chart 1. Relation of serum calcium to tumor calcium.

Table 1

Generations	Low calcium		High calcium	
	Serum calcium (mg %)	Tumor calcium (mg %)	Serum calcium (mg %)	Tumor calcium (mg %)
Initial		4.8		5.0
1-10	5.3 ± 0.7	6.5 ± 2.7	11.3 ± 0.8	6.6 ± 1.7
11-20	6.2 ± 1.0	5.1 ± 1.5	12.1 ± 0.6	7.8 ± 4.9
21-30	5.6 ± 1.1	4.3 ± 1.5	11.6 ± 0.6	12.1 ± 3.1
31-40	5.0 ± 1.1	4.4 ± 2.0	11.5 ± 0.7	11.7 ± 4.4

Effect of successive transfer of tumor in hypo- and hypercalcemic rats.

years (Table 1). Transfer of tumors through 40 generations in low calcium rats failed to significantly alter the tumor calcium, whereas tumors passed through hypercalcemic animals progressively increased in calcium content through approximately 30 such transfers and then failed to rise further during the next 10 generations.

Effect of Host Calcium on Development of Metastases. To evaluate the effect of host calcium on the development of hepatic metastases, 5000 tumor cells obtained from a tumor grown in a normocalcemic rat and having a calcium of 6.2 mg % were inoculated intraportally into normo-, hypo-, and hypercalcemic rats (Chart 2). Animals maintained on a chow diet and sacrificed 14 days following inoculation of cells failed to demonstrate a significant difference in incidence of liver metastases (47, 45, and 41% respectively). Since hypocalcemic animals on a synthetic diet had a further reduction in serum calcium, the incidence of metastases in such animals was compared with similarly fed control rats and again no significant difference was observed (38% in the former and 30% in the latter).

Further evaluation of the effect of host calcium on metastases was carried out by the subcutaneous injection of 500,000 tumor cells similarly into animals maintained normo-, hypo-,

and hypercalcemic. At sacrifice 30 days after inoculation, no significant difference in incidence of lung metastases was recorded. Fifty-five of 93 (59%) normocalcemic rats demonstrated lung tumors, and 17 of 30 (57%) hypocalcemic and 13 of 21 (62%) hypercalcemic animals had metastases.

Effect of Tumor Calcium on Disappearance of Labeled Tumor Cells from Injection Site. To obtain information relative to the effect of tumor calcium on the invasive properties of tumor cells, suspensions of labeled cells were prepared from 15 tumors grown in normo-, hypo-, and hypercalcemic animals. Calcium in the tumors varied between 2.1 and 9.0 mg %. Cell suspensions were inoculated subcutaneously into the legs of normal animals and the legs were amputated at intervals from 15 min to 24 hr after injection. There was no difference in disappearance rate of tumor cells from low or high calcium-containing tumors (Chart 3).

Lung Metastases following Implantation of Hypo- and Hypercalcemic Tumor Plugs (Table 2). Tumor plugs prepared from tumors of varying calcium content grown in hypo- and hypercalcemic animals were implanted subcutaneously into the left hind legs of animals with varying serum calcium. Animals in each group were sacrificed 14 or 21 days following implantation. Those with low calcium tumors failed to demon-

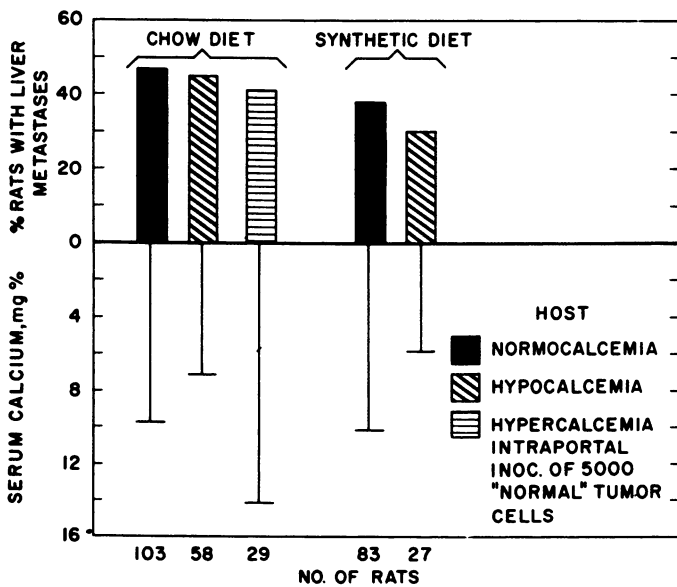


Chart 2. Host calcium and liver metastases.

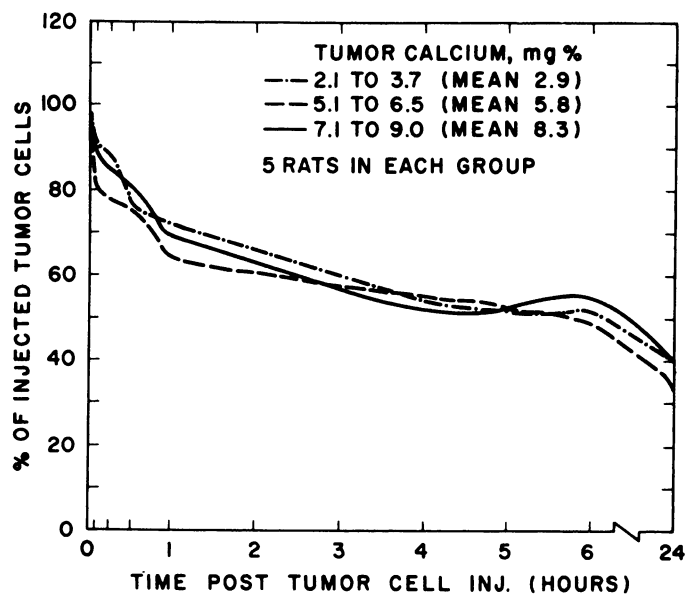


Chart 3. Effect of tumor calcium on disappearance of labeled tumor cells from injection site.

Table 2

	Tumor calcium (mg %)	Serum ^a calcium (mg %)	Sacrifice time: days post implant	Metastases	
				No. of rats	% positive
No amputation					
A	Low	Low	14	24	17
			21	24	71
B	Low	Normal	14	23	9
			21	22	45
C	"Normal"	Normal	14	24	12
			21	54	76
D	High	High	14	24	17
			21	24	87
E	High	Normal	14	24	29
			21	22	77
Amputation					
7 days post implant					
A	Low	Low	21	46	26
B	High	High	21	23	26
Amputation					
12 days post implant					
A	Low	Low	21	45	60
B	Low	Normal	21	17	65
C	High	High	21	22	64

Lung metastases following implantation of hypo- and hypercalcemic tumor plugs.

^a At sacrifice 14 and 21 days post implantation.

strate an increased incidence of lung metastases; those with high calcium tumors had no decrease in such lesions. At each sacrifice the incidence of lung metastasis was similar whether tumors of low, "normal," or high calcium content were implanted into animals with low, normal, or high serum calcium. The only exception, which is unexplained, is the low incidence of metastases in animals sacrificed 21 days after implantation of tumors with low calcium into animals with normal serum calcium. Likewise, no difference in incidence or degree of metastases was observed in 2 groups of similarly managed animals subjected to hind limb amputation 7 or 12 days following insertion of plugs.

DISCUSSION

The precise role of calcium in various facets of tumor biology was a favorite subject for investigation during the first half of this century (16). Only the studies of Coman (7, 8) and his associates (14, 20) regarding tumor cell adhesiveness, however, were concerned with its relation to any aspect of the phenomenon of metastasis. These investigators suggested that decreased adhesiveness of cancer cells was related to their decreased calcium content and that, indeed, much of the phenomenon of metastasis could be ascribed to the low calcium content of tumors. Recently, Weiss (17, 18), in several outstanding critical reviews relative to the tumor cell periphery and metastasis, has called for reappraisal with modern methodology of the older observations relative to tumor and normal tissue calcium content. In his opinion "there are not enough data to make sweeping generalizations about the calcium contents of tumours and their normal counterparts." In this regard, Hickie and Kalant (13), employing atomic absorption

spectrophotometry, have observed that the Morris hepatoma 5123tc contained 100% more calcium than did normal rat liver. Our findings relative to the calcium content of Walker tumor and normal rat tissue are in keeping with those of the latter investigators.

The possibility that tumor calcium values were influenced by blood content of samples has been considered. Sacrifice by decapitation and exsanguination was carried out to minimize this possibility. Moreover, it has been demonstrated by us (19), employing angiography, that the blood supply of the tumor employed is sparse, suggesting that the vascular space of the tumor is considerably less than that in muscle and liver, thus arguing against the possibility that the higher content of calcium in the tumor is a result of contamination by blood.

As a result of Coman's hypothesis, it might be anticipated that cells from low calcium-containing tumors should be less adherent to each other than those cells from plugs with a higher calcium content and, consequently, more cells should become available for dissemination from the former than the latter. Since it has been demonstrated (12) that metastatic incidence is related to numbers of tumor cells, it could be anticipated that more metastases would occur in animals implanted with low calcium tumors than in those bearing tumors with a high calcium content. It was considered that perhaps our failure to observe a difference in the incidence of lung metastases in hypo- and hypercalcemic animals implanted with low and high calcium-containing tumors might be a consequence of a difference in the invasive properties of cells from the 2 types of tumor subsequent to their separation. Since, however, their egress from an inoculation site was found to be similar, this explanation seems unlikely. Such disappearance

of label from an area of injection has been found² (11) to be related to a departure of living cells rather than to loss of tag. Since levels of host serum calcium (and parathyroidectomy) were of no pertinence in the development of metastases, it is unlikely that more cells were disseminated from tumors with lowered calcium but failed to become overt metastases because of the effects of parathyroidectomy and hypocalcemia in the host.

Recently Mizumoto and Honjo (15) reported that metastasis formation following implantation of ascites hepatoma and Yoshida sarcoma was markedly enhanced by the administration of parathyroid hormone. Since such an enhancement was not observed in animals receiving calcium chloride solution, they concluded that the effect was not the result of hypercalcemia caused by this hormone. Our failure to note enhanced metastases in hypercalcemic hosts is in keeping with their observations. Failure by us to find a decrease in metastases in parathyroidectomized animals tends, however, to minimize the importance of their findings relative to parathyroid hormone and suggests that in physiologic quantities this hormone has little significance in the formation of metastases.

Thus, results in this model system suggest, albeit indirectly, that cell adhesiveness was not sufficiently altered, despite the wide range of tumor calcium, to influence cell separation from implants and, consequently, the incidence of metastases. Such findings are not necessarily in conflict with Coman's hypothesis relating decreased mutual adhesiveness of neoplastic cells to their decreased calcium content or, more precisely, to a decrease in binding by the cell membrane. For, until methodology becomes available which can quantitate calcium in the cell periphery or within the cell, proper evaluation of this concept remains impossible.

How much of the increased or decreased tumor calcium observed by us was due to bound or diffusible (intracellular) calcium is impossible to say. Since it has been presumed that there is an abundance of calcium available in the serum and extracellular fluid of cancer patients, the low calcium of tumor cells has been attributed to a lack of binding sites for calcium. If intracellular calcium is independent of bound calcium, it may be considered why, in the presence of an adequate calcium milieu, total tumor cell calcium should be low.

Weiss has recently noted (17) that "the often-made statement that the tumour cell periphery does not bind calcium cannot be accepted uncritically." He has pointed out that in one of the very few systematic studies made in this regard, it was shown (1) that the calcium-binding capacity of the surfaces of Ehrlich ascites tumor cells and isolated liver cells were equal. Moreover, lymphocytes and erythrocytes require even higher concentration of calcium to reverse their charge, indicating a lower calcium-binding capacity than in tumor cells.

Our observation that the content of tumor calcium could be increased when tumors were grown in hypercalcemic hosts and reduced only when in markedly hypocalcemic animals suggests that the ability of tumors to take up and retain calcium

may not be impaired, but that an adequate concentration of this ion must be available to tumor tissue. The paucity of blood supply in certain experimental tumors may limit the availability of calcium to tumor cells. Whatever the explanation, the findings denote that tumor calcium levels are not entirely independent of those in the host. While the level of calcium in tumors may be manipulated over a wide range by alteration of host calcium, this does not seem to affect the incidence of metastases resulting from such tumors. From these studies and recent observations of others, it would seem that the role of calcium in the process of tumor metastasis requires further clarification.

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REFERENCES

1. Bangham, A. D., and Pethica, B. A. The Adhesiveness of Cells and the Nature of the Chemical Groups at Their Surfaces. *Proc. Roy. Soc. Edinburgh, A.*, 28: 43-50, 1960.
2. Baron, D. N., and Bell, J. L. Compleximetric Determination in Pathological and Physiological Specimens. *J. Clin. Pathol.*, 12: 143-148, 1959.
3. Beebe, S. P. The Chemistry of Malignant Growths. II. The Inorganic Constituents of Tumors. *Am. J. Physiol.*, 12: 167-172, 1904-1905.
4. Brunschwig, A., Dunham, L. J., and Nichols, S. Potassium and Calcium Content of Gastric Carcinoma. *Cancer Res.*, 6: 230-232, 1946.
5. Carruthers, C., and Suntzeff, V. Calcium, Copper and Zinc in the Epidermal Carcinogenesis of Mouse and Man. *Cancer Res.*, 6: 296-297, 1946.
6. Clowes, G. H. A., and Frisbie, W. S. On the Relationship Between the Rate of Growth, Age, and Potassium and Calcium Content of Mouse Tumors (Adenocarcinoma, Jensen). *Am. J. Physiol.*, 14: 173-192, 1905.
7. Coman, D. R. Decreased Mutual Adhesiveness, a Property of Cells From Squamous Cell Carcinomas. *Cancer Res.*, 4: 625-629, 1944.
8. Coman, D. R. Mechanism of the Invasiveness of Cancer. *Science*, 105: 347-348, 1947.
9. deLong, R. P., Coman, D. R., and Zeidman, I. The Significance of Low Calcium and High Potassium Content in Neoplastic Tissue. *Cancer*, 3: 718-721, 1950.
10. Dunham, L. J., Nichols, S., and Brunschwig, A. Potassium and Calcium Content of Carcinomas and Papillomas of the Colon. *Cancer Res.*, 6: 233-234, 1946.
11. Fisher, B., and Fisher, E. R. Metastases of Cancer Cells. In: Harris Busch (ed.), *Methods in Cancer Research*, Vol. 1, pp. 243-288. New York: Academic Press, 1967.
12. Fisher, E. R., and Fisher, B. Experimental Studies of Factors Influencing Hepatic Metastases: I. The Effect of Numbers of Tumor Cells Injected and Time of Growth. *Cancer*, 12: 926-928, 1959.
13. Hickie, R. A., and Kalant, H. Calcium and Magnesium Content of Rat Liver and Morris Hepatoma 5123tc. *Cancer Res.*, 27: 1053-1057, 1967.
14. McCutcheon, M., Coman, D. R., and Moore, F. B. Studies on the Invasiveness of Cancer. Adhesiveness of Malignant Cells in Various Human Adenocarcinomas. *Cancer*, 1: 460-467, 1948.

²B. Fisher, E. R. Fisher, C. Guzman, C. Copeland, and E. Caceres, The Dissemination of Subcutaneously Inoculated Tumor Cell Suspensions, submitted for publication.

15. Mizumoto, R., and Honjo, I. Parathyroid Hormone and Metastasis of Experimental Tumor. I. Effect of Parathyroid Hormone on the Metastasis Formation of Experimental Tumor. *Gann*, 56: 421-424, 1965.
16. Shear, M. J. The Role of Sodium, Potassium, Calcium and Magnesium in Cancer: A Review. *Am. J. Cancer*, 18: 924-1024, 1933.
17. Weiss, L. The Cell Periphery and Metastasis. *In: Proceedings Seventh Canadian Cancer Research Conference*, pp. 292-315. Toronto: Pergamon Press, 1967.
18. Weiss, L., and Mayhew, F. The Cell Periphery. *N. Engl. J. Med.*, 276: 1354-1361, 1967.
19. Yates, A., and Fisher, B. Experimental Studies in Regional Perfusion. *Arch. Surg.*, 85: 827-836, 1962.
20. Zeidman, I. Chemical Factors in the Mutual Adhesiveness of Epithelial Cells. *Cancer Res.*, 7: 386-389, 1947.