effect to a change in pH, it is conceivable that the tissue culture and the intact embryo results are related. Further studies in both systems may yield new information concerning the mechanism of action of the ammonia inhibition.

Summary. The antiviral effect of ammonium ions, previously shown in dog kidney cells against influenza virus, has been reexamined with 10 viruses in 10 tissue culture cell lines. Only influenza virus appeared to be affected by the inhibitor, which functioned in a number of cell systems. The effect was shown to be independent of virus inoculum size and therefore apparently on the host cell. The inhibition was demonstrated in suspended allantoic membrane cultures, and a marginal effect was also observed in intact chick embryos.

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Growth Characteristics of Ascitic Tumor Cells in the Heparinized Peritoneal Cavity of the Mouse.* (26771)

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Six years ago, at a conference on ascites tumors(1), we mentioned the occurrence of fluctuations in the amount of serous fluid and in its cellular content in mice bearing ascites tumor and given heparin intraperitoneally. Recently, we attempted to study the mechanism of this phenomenon by varying the method of administration, specifically by injecting heparin to some groups of animals at various intervals before tumor inoculation and to other groups after inoculation. In each experiment, the amount of ascitic fluid, its concentration of tumor cells, frequency of mitoses in these cells and extent of their implantation into the peritoneum were estimated, at requisite intervals (3 or 7 days) after inoculation. The results are reported and tentatively interpreted below.

Materials and methods. a) *Mouse and tumor strains.* Swiss Albino mice (Albino Farms, Red Bank, N. J.) of 24-27 g weight, mostly females, were used. As female mice with ascites tumors show a uniform and distinctive pattern of periuterine implant growth(1), they were preferred for our purposes. Krebs-2 ascites tumor was carried in our laboratory in serial intraperitoneal transfers. b) *Experimental technics.* Technics of tumor inoculation with requisite numbers of ascitic cells, of tumor cell counts, etc., have been described(2,3,4,5). Doses of approximately 10^5 tumor cells from ascitic fluid diluted (with .85% solution of NaCl) to a volume of .5 ml were injected intraperitoneally into lower *left* quadrant of the abdomen; the fluid subsequently accumulated in these mice was withdrawn by puncturing the *right* lower abdominal quadrant, thus avoiding trauma to granulating tissue and abundant bleeding (in heparinized animals) at sites of inoculation and of heparin injections (upper left quadrant). c) *Injections of heparin.* A standardized and commercially available solution of heparin (Panheparine Abbott) was used in our investigation. This solution, containing 1000 units (1 mg) per ml was diluted 1:20 with saline solution immediately before injections. Doses of .5 ml or 1.0 ml (25 to 50 units) were given before inoculation, but

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only .5 ml was given to inoculated animals which showed a tendency to bleed spontaneously during the first hours after injection. d) *Quantitative estimate of ascites tumor growth.* aa) *Amount of ascitic fluid.* The amount of exudate was estimated as proportionate to the volume of fluid obtained by insertion of a standard glass capillary in peritoneal wall: +++ = .5 ml or more; ++ = less than .5 but more than .1 ml; + = less than .1 ml; O = no fluid. The reliability of this estimate was shown by comparison with the amount of exudate measured at autopsy. bb) *Scale of peritoneal implantation*: no implant = 0; circular nodules only, 1 to 2 mm in diameter = +; 3 to 5 mm in larger diameter = ++; larger = +++. e) *Mitotic index counts.* Fluid smears were stained with Acetoorcein (2) for the purpose of counting mitoses. f) *Pattern of experiments.* aa) *Heparinization after tumor inoculation.* Three or 4 injections were given daily, starting on first day after inoculation. bb) *Heparinization before inoculation.* Three or 4 injections were given daily, the last 6 hours before inoculation. cc) *Combined method.* Heparin injections 48, 24, and 6 hours before inoculation and the 4th injection 24 hours after inoculation. dd) *Recording the results.* In one-half of the mice in each experimental and control group (inoculated but not heparinized), ascitic fluid was withdrawn for volume estimate and tumor cell counts on the 4th day after inoculation, and the autopsies for recording implantation were performed on the 5th day. In the other half of the animals the fluid was withdrawn and the mice were sacrificed on the 8th day.

Results. Mice were divided in 3 series according to 3 patterns of heparinization outlined in Materials and methods. Each series included 3 groups, each of 20 mice: 2 groups were inoculated on the same date and heparinized by the same plan, but examined for records at different time intervals, on the 4th or the 8th day. The third group, inoculated on the same date but not heparinized served as control. The data obtained in all groups of mice were tabulated in Table I and illustrated in Fig. 1-6.

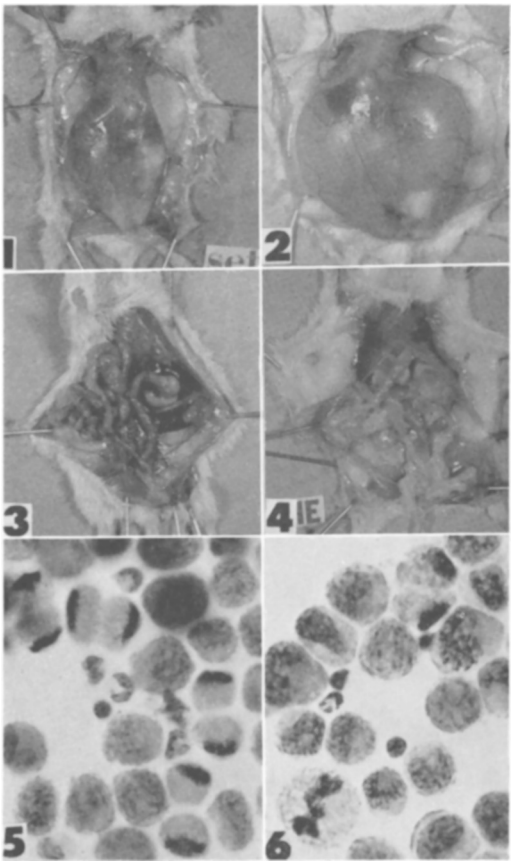

FIG. 1, 3, and 5. Mice given 50 units of heparin intraper., 48, 24, and 6 hr before inoculation of Krebs-2 ascites tumor.

FIG. 2, 4, and 6. Controls inoculated simultaneously with exp. mice but not heparinized.

FIG. 1-4. Mice examined on 8th day after inoculation.

FIG. 5-6. On 5th day.

Note difference in amount of fluid (Fig. 1-2) and in extent of implantation (Fig. 3-4); implant at site of inoculation in Fig. 1; frequency of mitoses in anaphase shape in Fig. 5 (×400, Aceto-orcein).

The data in the various series and groups suggested the following: 1. The combined method of heparinization of mice before and after ascites tumor cell inoculation (Series III) inhibited consistently and significantly all 3 phenomena of growing ascites tumor, *i.e.*, fluid accumulation (Fig. 1 as compared with Fig. 2), increase in concentration of free tumor cells and their implantation in the peritoneum (Fig. 3 as compared with Fig. 4). Heparinization only before inoculation (Series II) produced similar effects but mostly at a lower scale, and the same treatment applied

TABLE I. Free Growth and Implantation of Ascitic Tumor Cells in Heparinized Swiss Albino Mice Inoculated with Krebs-2 Ascites Tumor (20 Mice in Each Group).

Series	Group	Timing of heparinization*	Interval between inoc. and recording of results (days)	Results (avg and variation extremes in each group)			
				Amt of ascitic fluid†	Conc. of tumor cells in fluid (thousands/mm³)	Mitotic index of tumor cells	Extent of implant growth†
I	1	Heparinization starting 24 hr after inoc.	3	++ + - +++	42.3 36.1-51.4	7.7 5.2-6.8	+ + - ++
	2	Controls (nonheparinized)	"	++ ++ - +++	48.2 40.8-56.4	7.1 5.6-9.1	++ + - +++
	3	As Group 1	7	++ + - +++	48.5 42.2-54.8	7.8 4.9-9.4	+ + - ++
	4	Controls	"	+++ ++ - +++	52.7 43.2-59.8	6.5 4.4-8.8	++ ++ - ++
II	1	Heparinization ending 6 hr before inoc.	3	+ + - ++	46.9 39.2-55.5 56% in anaphase	10.2 7.8-12.5	+ 0 - +
	2	Controls	"	++ ++ - +++	57.2 41.4-67.5 28% in anaphase	7.1 4.9-9.4	++ ++ - +++
	3	As Group 1	7	+ + - ++	49.4 43.2-54.2	6.9 5.6-9.2	+ 0 - +
	4	Controls	"	++ ++ - +++	56.6 44.9-65.1	7.4 5.1-8.8	+++ ++ - +++
III	1	As Series II and one inj. 24 hr after inoc.	3	+ 0 - ++	47.7 37.2-52.3 48% in anaphase	12.1 8.9-15.2	0 0 - +
	2	Controls	"	++ + - +++	59.7 40.2-67.4 2% in anaphase	7.2 6.2-9.4	++ + - ++
	3	As Group 1	7	+ + - ++	41.2 29.5-54.2	7.4 6.2-8.8	+ 0 - +
	4	Controls	"	++ ++ - +++	59.1 49.1-69.5	7.2 5.5-9.1	+++ ++ - +++

* 3 or 4 daily inj. of 25 or 50 units of heparin.
† For estimate of fluid volume and scale of implants, see *Materials and methods.*

entirely after inoculation (Series I) resulted only in irregular fluctuations in the level of these phenomena.

2. Mitotic index of tumor cells in ascitic fluid was higher in heparinized mice of Series II and III on the 4th day after inoculation than in controls, but was lower on the 8th day, suggesting that the majority of mitotic cells had not completed their division; the high proportion of anaphase figures suggested arrest of the mitotic cycle in this stage (Fig. 5 as compared with Fig. 6).

3. Concentration of ascitic tumor cells was considerably decreased in Series II and III, in spite of a substantial decrease in the volume of their diluent, *i.e.*, ascitic fluid as compared with controls. Thus, there was greater reduction in number of ascitic cells than in volume of fluid, *i.e.*, the process of cell proliferation was inhibited more strongly than the process of fluid exudation.

4. Some effects of heparin as recorded on the 4th day were apparently reversed by the 8th day; amount of fluid increased through slow accumulation of scanty but continuously exudated fluid, which may be in part responsible, for the further drop in tumor cell concentration. Some implants appeared, mainly at the site of abdominal puncture (Fig. 3), suggesting that some inoculated tumor cells invading the puncture wound in this location escaped the effect of heparinization. However, the surface of serosa remained free from implants. Thus, it may be emphasized that of all principal features of growing ascites tumors, only implantation

was inhibited completely in several heparinized animals (Series II and III), and for an indefinite period.

Discussion. There are varying opinions as to the nature and mechanism of ascitic fluid exudation from vessels of the peritoneum which are invaded by inoculated tumor cells (1,6,7). However, it is a consensus that this invasion is a prerequisite for appearance of intracavitary fluid and a starting point for free growth of tumor cells. In our experiment, heparinization of the peritoneal cavity inhibited implantation of cells into serosa more consistently and more completely than it inhibited other features of ascites tumor growth. Accordingly, prevention of tumor cell implantation may be considered as the primary effect of heparinization, which was responsible for other effects of this procedure.

Implantation of tumor cells invading the peritoneal serosa occurs through their penetration into connective tissue of subserosa made available by trauma to the epithelial surface(8). It has been repeatedly reported that both in human transcoelomic metastases (9) and in analogous phenomena labeled as ascites tumors in rats and mice(10,11,12) the first step in the process of implantation is the embedding of tumor cells by fibrin clotted at the site of slight injury of peritoneal serosa by tumor cells. "Next to the cancer cells themselves, fibrin is the most important factor in the life history of peritoneal implantation. It is fibrin which holds the cancer cells in place by splinting them against the denuded peritoneum. Fibrin is both the temporary framework and the scaffolding of the organized implants"(9). Consideration of these phenomena may provide interpretation of our findings reported above.

It is known(12) that heparin is not only anti-prothrombin but also anti-thrombin, preventing thrombin from converting fibrinogen to fibrin. Its effect *in vivo* is more lasting than *in vitro* and our daily injections induced a continuous heparinization (as was shown by the prolonged fluid state of the shed blood). Clinical experience has shown that continuous heparinization may prevent accretion of fresh fibrin clot and thus allow time for its resorption. It may be presumed that in our experiments a similar mechanism released tumor cells embedded in the fibrin clot attached to peritoneum, and that damaged serosa healed after elimination of fibrin clot. If this is correct, the currently applied postoperative heparinization for prevention of thromboses may be of additional help in cancerous patients by preventing implantation of locally spilled or systemically circulating tumor cells.

It should also be noted that all of the mast cells which are an abundant source of heparin disappear from the peritoneal fluid of mice within 2 or 3 days after inoculation of tumor cells(2) and reappear only in cases of complete regression (by chemical or radioactive agents) of tumor growth. This phenomenon, suggesting antagonism between mast cell migration from connective tissue to peritoneal cavity and invasion of serosa by tumor cells, is being investigated in the light of our experiments.

Summary and conclusions. 1. In a large proportion of Swiss Albino mice which had received 3 daily intraperitoneal injections of heparin (25 or 50 units) immediately before inoculation of Krebs-2 ascites tumor, the amount of ascitic fluid and its concentration of tumor cells were consistently and significantly lower than in controls. 2. Macroscopically visible implantation of ascitic cells from the fluid into the peritoneum was considerably reduced and sometimes completely absent in overwhelming majority of heparinized mice (Series II). 3. An additional injection of heparin (twenty-four hours after inoculation) increased these effects (Series III) while heparinization given entirely after inoculation (Series I) induced only wide fluctuations in the extent of ascites tumor growth. 4. Accordingly, inhibition of implantation was considered to be the primary and direct effect of heparinization, which may account for reduction of the amount of ascitic fluid and of its concentration of tumor cells as secondary effects. 5. It was accepted, in the light of research by other investigators and by ourselves, that embedding of free tumor cells within a fibrin clot from the cavitary fluid initiated their implantation into

the serosa and that preventive heparinization arrested implantation in this early stage.

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A Method for Determination of Parathyroid Secretion Rate in the Rat.* (26772)

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While secretion rates of some endocrine glands, notably thyroid, have been measured by a variety of technics, virtually no attempt has been made to estimate parathyroid hormone secretion rate (PSR) in individual small laboratory animals. This is partly due to the difficulty in obtaining adequate serial samples of blood in the same animal and, in part, to a failure to develop technics comparable to those utilized in determining the thyroxine secretion rate. The availability of an ultramicro method for determination of serum calcium(1) has led to an attempt to determine PSR in individual rats, based on a principle similar to that used for measuring thyroxine secretion rate(2). This report presents the feasibility of such a technic.

Experimental. One hundred and thirty-three female rats of a local strain, each weighing about 200 g, were housed in cages maintained at uniform temperature (78 ± 2°F) and illuminated only during daylight hours. They were fed Purina Laboratory chow *ad libitum* and water was available at all times. The rats were thyroparathyroidectomized (TPEx) surgically, under ether anesthesia. Completeness of TPEx was checked by administering I^{131}, and when radioactivity over the neck region was less than background this indicated an absence of thyroid I^{131} pick-up. One day prior to TPEx the laboratory chow was replaced by a diet in which calcium content was less than .016%, in order to prevent calcium in the feed from affecting serum calcium levels following TPEx. Serum calcium was determined by a chelating procedure utilizing ethyelene diamine tetra-acetate (EDTA) as the complexing agent. As little as 20 lambda (20 μl) of serum was found to be sufficient for each calcium determination. Blood samples were collected from the tail vein directly into polyethyelene microcentrifuge tubes and centrifuged in a Beckman microfuge. Twenty lambda of serum were titrated against EDTA in a microtitrator in a highly alkaline medium utilizing calcein as a indicator. The endpoint of titration was represented by the disappearance of the blue-green color. With little practice it was possible to distinguish this endpoint clearly. An ultraviolet light source may also be used to aid in distinguishing the endpoint sharply. Serum calcium determinations by this technic checked against classical flame photometry methods were found to be well within limits of expected experimental error. Re-

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