Disadvantages in the use of the hydroxamate derivatives include the manipulations involved in their preparation and the multiple spots obtained from the acids with multiple functional groups. The multiple spots are presumably due to failure to carry the preparative reactions to completion.

The colors in the developed chromatogram are relatively stable, although continued exposure to strong light causes a loss of contrast, principally by action on the ferric chloride background. Intense spots of the ferric hydroxamates tend to spread to other papers in contact with them, so that in filing it is well to insert blank sheets of paper between the chromatograms.

One dimensional chromatogram may be made on filter paper previously impregnated with ferric chloride, in which case the chromatogram may be examined visually at any stage of the development, but better results are usually obtained by use of non-impregnated paper.

Summary. A procedure for the application of filter paper partition chromatography to the separation of both volatile and non-volatile organic acids with chain lengths of about eight carbons or less is described. The potassium hydroxamate derivatives of the organic acids are prepared by reacting the methyl ester with about a two-fold excess of a mixture of potassium hydroxide and hydroxylamine in methyl alcohol. The hydroxamate derivatives obtained from about 10^{-6} mole of each of the esters are applied to the filter paper, the chromatogram developed with suitable solvents, and then sprayed with ferric chloride to make the derivatives visible as purple spots on a yellow background. Isobutyric acid and phenol gave the best results of the solvents tried for two-dimensional chromatograms of the dicarboxylic acids. R_F values for a number of hydroxamate derivatives are given for several solvents.

17025

Destructive Action of Human Cancer Extracts on Red Blood Cells *in vitro*.*

LUDWIK GROSS.

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It was recently observed in this laboratory that centrifugated or filtered extracts prepared from spontaneous mouse mammary carcinomas hemolyze mouse erythrocytes *in vitro*.[1]

Experiments reported in this study were designed with the purpose of determining whether extracts prepared from human cancer also exert a destructive action on red blood cells *in vitro*.

Materials and methods. Tumor extracts. Sterile specimens of tumors were obtained from the operating room.† The tumor tissue was weighed, cut with scissors into small pieces, then ground thoroughly for several minutes in a porcelain mortar, 0.85% solution of sodium chloride being added to obtain cell suspensions varying in concentration from 20 to 30%. The suspensions thus obtained were cleared from cells by 2 successive centrifugations at 5,000 t.p.m. for 10 minutes each; the final supernatant fluid was then used, and designated "tumor extract." In several cases, tumors of hard, fibrous consistency had to be ground into fine cell suspensions in a Latapie Grinding Apparatus (Thomas), and then centrifugated.

Cell suspensions prepared from human

* Published with the permission of the Chief Medical Director, Department of Medicine and Surgery, Veterans Administration, who assumes no responsibility for the opinions expressed or conclusions drawn by the author.

1 Gross, L., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 292.

† Most of the tumor specimens were obtained from the operating room of our hospital (Veterans Administration Hospital, Bronx, N. Y.); 10 of the 12 specimens of human breast carcinomas were obtained from the Memorial Hospital, New York City.

TABLE I.
Action of Cell-Free Human Tumor Extracts on Human RBC *in vitro*.

Tumor specimen				Results of the RBC test *in vitro*		
No. of test	Specimen diagnosis		Blood group of patient	RBC used for test	Hemolysis after 48 hr incub. 37°C	Agglutination after 24 hr incub. 37°C
1.	Carcinoma, breast	(F)	O pos.	O pos.	+	0
2.	" "	(F)	O pos.	O pos.	+	0
3.	" "	(F)	O pos.	O pos.	+	sl†
4.	" "	(F)	B pos.	B pos.	+	+
5.	" "	(M)	A pos.	A pos.	+	0
6.	" "	(F)	O pos.	O pos.	sl†	sl†
7.	" "	(F)	O pos.	O pos.	0‡	+
8.	" "	(F)	O neg.	O neg.	sl†	0
9.	" "	(F)	ND*	O pos.	+	sl†
10.	" "	(F)	ND*	O pos.	+	0
11.	" "	(F)	ND*	O pos.	+	+
12.	" "	(F)	ND*	A pos.	+	+
				B pos.	+	+
13.	Carcinoma, stomach	(M)	A pos.	A pos.	0	+
14.	" "	(M)	O neg.	O neg.	+	sl†
15.	" "	(M)	A pos.	A pos.	+	+
16.	" "	(M)	A pos.	A pos.	+	sl†
17.	" "	(M)	B pos.	B pos.	+	0
18.	" rectum	(M)	B pos.	B pos.	sl†	0
19.	" antrum	(M)	O neg.	O neg.	0‡	sl†
20.	" "	(M)	A neg.	A neg.	+	0
21.	" metastatic (primary undetermined)	(M)	A pos.	A pos.	sl†	sl†

* ND—Blood group not determined.
† sl Slight.
‡ Minimal hemolysis noticed.
(F) female.
(M) male.

breast cancer formed a fatty layer on the surface of the supernatant fluid following centrifugation; this fat had to be separated from the extract by passing the supernatant fluid through a sterile voile filter.

In a few instances, apparently infected tumors such as carcinoma recti, or that of the antrum, were used for the tests. To assure bacterial sterility, extracts prepared from such tumors were passed through a Seitz filter, and the filtrate was used for the tests. In addition, filtrates, as well as centrifugated extracts, were prepared also from several other bacteriologically sterile tumors.

The entire procedure followed in the preparation of the extracts was aseptic, and the extrates, or filtrates, used for the tests were found to be bacteriologically sterile, as evidenced by negative inoculations of ordinary culture media.

Normal Tissue Extracts. Control experiments were performed with cell-free extracts prepared from various freshly obtained and sterile human tissues: specimens of healthy muscle were obtained from patients in whom a limb was amputated; specimens of thyroid gland were obtained from patients in whom thyroidectomy was performed because of thyreotoxicosis; specimens of hypertrophic, but otherwise normal, breast gland were obtained from 5 males operated on for gynecomastia, and from one female in whom a plastic breast operation was performed; a specimen of placenta was obtained from a young woman in whom Caesarean Section was performed; finally, 2 small specimens of apparently normal liver were obtained from patients in whom a diagnostic liver biopsy was performed. From these various tissue specimens, centrifugated, cell-free, and bacteriologically sterile extracts were prepared in a manner identical with that described for the preparation of the tumor extracts.

Red Blood Cells (RBC). Freshly drawn, oxalated red blood cells from healthy human donors, as well as in some instances also from

guinea pigs, rabbits, chickens, and mice, were washed twice at 2,500 t.p.m. for 3 minutes each, and final one per cent suspensions of the red blood cells in 0.85% solution of sodium chloride were prepared. The washed red blood cell suspensions were freshly prepared for each test. In all instances, except when indicated otherwise in Table I, human erythrocytes of the same type were used as that of the patient from whom the specimen had been removed.

Technic of the Test. The test was performed in the following manner: one cc of the 1% RBC suspensions was mixed gently in a small (10 x 75 mm) tube with doses of the tumor, or normal tissue, extracts varying from 1 to 0.25 cc, and placed in an incubator at 37°C for 48 hours. The presence or absence of agglutination was determined after 24 hours of incubation at 37°C; agglutination, if any present, was graded "slight," "1+," and "2+." The presence or absence of hemolysis was determined by inspecting the tube, without previous centrifugation, after incubation at 37°C for 48 hours; hemolysis present only over the sediment was graded "slight"; if hemolysis was stronger so as to diffuse in the supernatant fluid to a level extending at least for 1 cm above the sediment, it was graded "1+."

Experimental. "Agglutinating" Action of the Human Tumor Extracts on RBC in Vitro. The curious clumping action of tumor extracts on red blood cells, observed in experiments with transplanted mouse carcinomas,[2] was reproduced in some of the tests performed with human cancer extracts mixed with human erythrocytes. The clumping potency of the tumor extracts was not a constant phenomenon (Table I). In some instances, it occurred promptly; in others, only very slightly, and with a considerable delay, or it was missing entirely. Even in the more pronounced cases, however, the examination of the sediment under the microscope failed to show a typical picture of agglutination (Fig. 1 to 3). The clumping of the cells was usually only slight, if at all present, and was far from being uniform; only some of the cells were affected;

FIG. 1.
Agglutinating action of human breast carcinoma extract on human erythrocytes *in vitro* after 24 hours of incubation at 37°C. Mag. ×67.

accumulation of either debris, or some unidentified precipitated masses could also be noticed. The interpretation of these findings was not facilitated by the observation that frequently a slight precipitation occurred also in control tubes containing tumor filtrates only, without erythrocytes, and incubated at 37°C for 24 hours.

In 8 experiments the tumor extracts were passed through a Seitz filter and the filtrates were mixed with the erythrocytes; in all these cases the agglutinating potency of the extracts was not diminished by filtration. In fact, the agglutinating potency of the filtrates appeared to be somewhat more distinct than that of the centrifugated extracts.

In 10 experiments the tumor extracts were heated to 56°C for 30 minutes in a water bath prior to mixing them with red blood cells. All such preheated extracts were found to have lost their ability to agglutinate erythrocytes *in vitro*.

A series of experiments was performed in which the human tumor extracts were mixed with erythrocytes of mice, rats, guinea pigs or chickens. In all these instances in which such extracts were found to agglutinate human red blood cells, they were also found to exert a similar agglutinating action on the erythrocytes of the various different species of the animals tested.

Hemolytic Action of Human Tumor Ex-

2 Gross, L., *J. Immunol.*, 1948, **59**, 173.

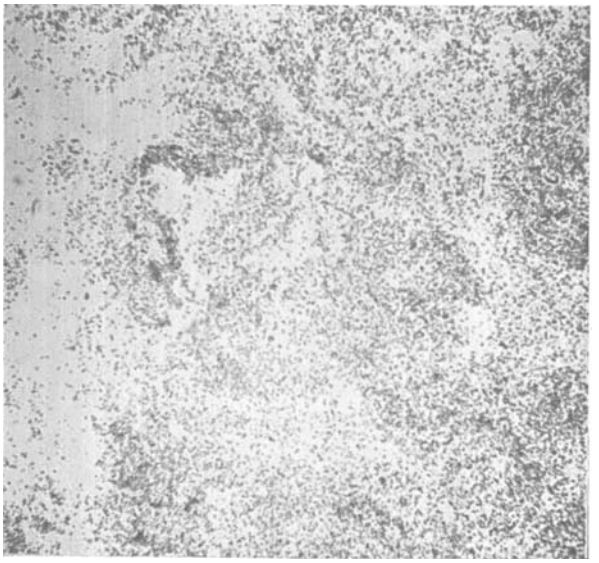


FIG. 2.
A slight clumping action of an extract from a human stomach carcinoma on human erythrocytes after 18 hours of incubation at 37°C. Mag. ×67.

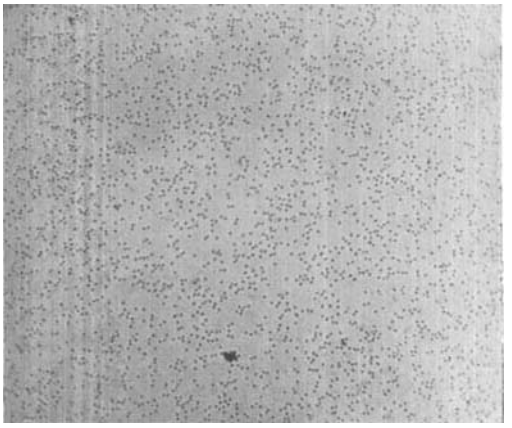


FIG. 3.
Human erythrocytes, 1% suspension, after 24 hours incubation at 37°C. Control slide. Mag. ×67.

tracts on Human RBC in Vitro. Most of the extracts prepared from human tumors were found to hemolyze (Fig. 4) human erythrocytes *in vitro* after an incubation for 48 hours at 37°C (Table I). Control tubes containing human red blood cell suspensions in physiological saline solution, and incubated simultaneously under otherwise identical conditions, remained unaltered.

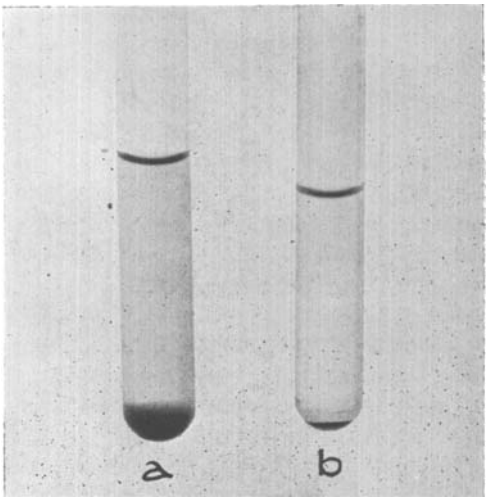


FIG. 4.
a. "Slight" hemolysis resulting from exposure (48 hours at 37°C) of human erythrocytes to a filtered extract prepared from human breast carcinoma. b. Control tube containing erythrocytes suspended in physiol. saline solution; no hemolysis.

In 8 experiments the tumor extracts were passed through Seitz filters, and the filtrates, as well as the corresponding centrifugated extracts, were tested for their hemolytic potency. In 3 instances the tumor extracts lost their

hemolytic potency after filtration; in 4 instances, the hemolytic potency of the extract was substantially diminished, but detectable, after filtration. Finally, in one instance, the hemolytic potency of the extract was not diminished after filtration.

In 10 successive experiments, human tumor extracts were heated to 56°C for 30 minutes in a water bath, prior to mixing them with the red blood cells. In all instances the hemolytic potency of these extracts was destroyed by heating.

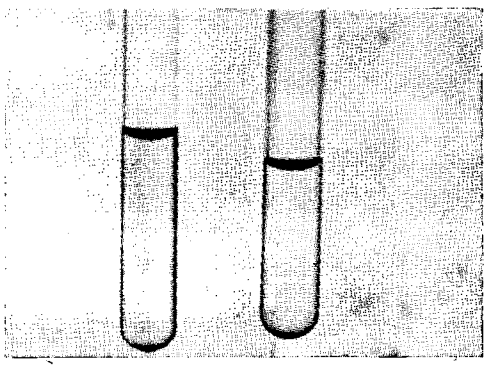
In 4 experiments, centrifugated tumor extracts, separated from the tumor cells, were incubated at 37°C for 4 hours, and then mixed with the erythrocytes. The potency of these pre-incubated extracts did not appear to be diminished, as compared with the corresponding control samples of fresh tumor extracts. This was in marked contrast to similar experiments showing that mouse tumor extracts lose their hemolytic potency when separated from the tumor cells, and incubated at 37°C for a few hours.[2]

An attempt was made to determine whether human tumor extracts would also hemolyze red blood cells of other species of animals. Experiments dealing with this question encountered, however, a rather unexpected difficulty; thus, it was found that washed mouse erythrocytes undergo spontaneous hemolysis when suspended in physiological saline solution, and incubated at 37°C for 12 to 24 hours; erythrocytes of rabbits, and to a lesser degree those of guinea pigs, were also found to be not suitable for a long incubation at 37°C.

In a few cases only, using potent human tumor extracts, was it possible to observe a slight hemolytic action of the human tumor extracts on the red blood cells of mice, rabbits, and guinea pigs, occurring comparatively promptly, and at a time when the control tubes with the respective erythrocytes suspended in physiological saline solution did not yet show any trace of hemolysis. The washed chicken erythrocytes appeared to be more resistant to spontaneous hemolysis when incubated at 37°C, and for that reason were found to be better suitable for these tests than the red blood cells of guinea pigs, rabbits or mice. In a few instances it was found that human tumor extracts slightly hemolyzed chicken erythrocytes after incubation for 48 hours at 37°C.

Experiments with Extracts Prepared from Non-Malignant Tumors. Four experiments were made with extracts prepared from non-malignant human tumors, *i.e.* from 3 prostatic adenomas (one centrifugated and 2 filtered extracts), and from a uterine myoma. In all instances washed erythrocytes of the same type were used for the tests as the type of the patient from whom the specimen had been removed. The results were as follows: the 2 filtrates prepared from the prostatic adenomas agglutinated the red blood cells after 24 hours of incubation at 37°C; the other 2 extracts did not agglutinate the erythrocytes. All 4 tests were negative for hemolysis after 48 hours of incubation at 37°C; after additional 24 hours of incubation, however, the prostatic adenoma extract, one of the 2 prostatic filtrates and the uterine myoma extract, produced a slight hemolysis of the corresponding erythrocytes.

Action of Normal Human Tissue Extracts on Human RBC in Vitro. Sterile, cell-free, centrifugated extracts freshly prepared from various normal human tissues, were mixed with 1% suspensions of washed human erythrocytes of the same type as that of the patient from whom the respective specimen



a b

FIG. 5.

Exposure of human erythrocytes (48 hours at 37°C) to the action of an extract prepared from human breast gland (gynecomastia). a) Extract from breast gland (1 cc), and RBC. No hemolysis. b) Physiol. saline solution, and RBC. Control tube. No hemolysis.

TABLE II.
Action of Cell-Free Extracts from Normal Human Tissues on Human RBC *in vitro*.

Organs tested	Blood group of patient	No. of individual extr. tested	Blood group or RBC used	No. of extr. causing hemolysis	No. of extr. causing agglut.
Muscle	A pos.	4	A pos.	0	1*
	A neg.	1	A neg.	0	0
"	O pos.	2	O pos.	0	0
	O neg.	1	O neg.	0	0
Placenta	B pos.	1	B pos.	0†	0
Thyroid	B pos.	1	B pos.	0	0
"	O pos.	1	O pos.	0†	0
"	O pos.	1	O pos.	0	0
"	AB pos.	1	AB pos.	0	0
Breast (F)	A pos.	1	A pos.	0	0
" (M)	O pos.	4	O pos.	1*	0
Liver	A neg.	1	A neg.	0†	0
	B pos.	1	B pos.	0	0

Each extract was prepared from a different patient. The agglutination was read after 24 hours of incubation at 37°C. The hemolysis was read after 48 hours of incubation at 37°C.
* "Doubtful."
† Slight hemolysis after 72 hours of incubation at 37°C.

had been removed; these mixtures were then incubated, at 37°C, for 48 hours (Fig. 5). There was only one instance of a "questionable" agglutination (Table II.); all other sediments were negative for clumping when inspected after either 24 or 48 hours of incubation. Of the 21 individual extracts tested, only one produced a minimal hemolysis after 48 hours of incubation; in 3 additional instances, a slight hemolysis resulted after 72 hours of incubation at 37°C.

Discussion. In previous experiments,[1,2] mouse mammary carcinoma extracts were found to hemolyze mouse erythrocytes, *in vitro*, after 2 to 3 hours of incubation at 37°C. Agglutination of the red blood cells by mouse tumor extracts was found to be less frequent, unless transplanted tumors were used for the preparation of the extracts.[2] Both observations have been recently confirmed by Zimmerman[3] and Salaman.[4]

At first, similar experiments performed with human tumor extracts in this laboratory gave negative results, except for occasional agglutination[5] (Fig. 6); in these preliminary tests the readings were made after a brief incubation at 37°C, not exceeding 3 or 6 hours. In the case of experiments with mouse tumor extracts a longer incubation was not necessary, and for that reason a similar procedure was followed in these tests dealing with human cancer. Later on, however, the human tumor extracts were allowed to act on human erythrocytes for longer periods of time; this modification in the technic of the test lead to positive results: agglutination, and hemolysis were found to result frequently. Even after a prolonged incubation, however, the hemolytic action of the human tumor extracts on human erythrocytes was found to be somewhat less pronounced than the similar action of mouse carcinoma extracts on mouse red blood cells.

The hemolytic, and particularly the agglutinating actions of the extracts prepared from various human tumors were far from constant; in some instances the hemolysis and/or agglutination occurred promptly and to a pronounced degree. In other cases, the destructive action of the tumor extracts was slight, and delayed. Since, however, the various extracts were prepared from different tumors, it was reasonable to expect differences in their ability to destroy the erythrocytes. Similar differences in the potency of the individual tumors were also observed in experiments dealing with mouse carcinomas.[2]

The question immediately arose whether cell-free extracts prepared from normal human tissues would also exert a similar destructive

[3] Zimmerman, H. M., (Montefiore Hospital, Bronx, N. Y.), personal communication to the author.

[4] Salaman, M. H., *Brit. J. Cancer*, 1948, **2**, 253.

[5] Gross, L., unpublished experiments.

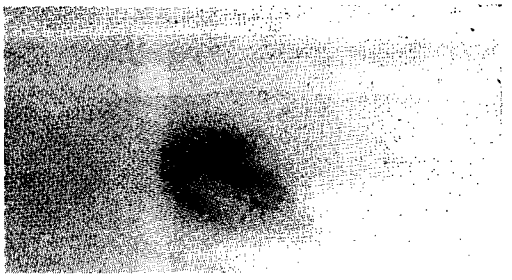


FIG. 6.
Agglutination of human erythrocytes by a human carcinoma (stomach) extract after an incubation, in test tube, at 37°C, for 6 hours. A drop of the sediment was then placed on a slide. The agglutination is visible macroscopically. Mag. ×1½.

action on human erythrocytes; this point was of particular interest in view of the fact that preincubated extracts from certain mouse tissues,[6] as well as *slices*[7,8] of normal animal or human tissues, had been found to hemolyze red blood cells *in vitro* after a prolonged incubation at 37°C. Accordingly, a series of experiments was performed with freshly prepared cell-free extracts from various human organs. With insignificant exceptions, the results of these tests were negative.

Anemia is a frequent associate of cancer. Some of the tumors, such as carcinomas of the intestinal tube, may ulcerate and bleed, giving apparently sufficient reason for the resulting anemia. In other instances, however, no bleeding occurs, and yet anemia also eventually develops. The exact cause of the progressive anemia so frequently encountered also in non-bleeding, and not ulcerated cases of cancer, has been a matter of controversy.

Experiments reported in this paper suggest that certain malignant tumors in man may exert a direct destructive action on the red blood cells *in vitro*. Should a similar phenomenon occur in the living host, a logical explanation of at least one of the causes of anemia in certain cases of cancer would be at hand. Such an explanation would be consistent with reports of recovery from anemia of patients relieved from tumors by surgical procedures.[9] On the other hand it appears difficult to assume that all cases of anemia in cancer are caused by a direct destruction of the red blood cells in the hosts; frank hemolytic anemia has been occasionally observed in neoplastic diseases,[10,11] but is far from being either frequent or usual. It is apparent, therefore, that some other mechanism would have to be also, at least partially, responsible for the development of anemia in certain cases of cancer. Thus, theoretically at least, one could assume that the destructive action of a diffusible substance, either liberated or produced by the tumor, may not be limited to erythrocytes, but may affect, in certain instances, among some other cells, also those of the bone marrow. This, however, is speculation only at the present time.

The fact remains unchanged, nevertheless, that human cancer cells either liberate or secrete a thermolabile substance which, *in vitro* at least, exerts a slow acting, but destructive influence on human erythrocytes.

Summary. Experiments reported in this paper suggest that extracts prepared from human cancer occasionally agglutinate, and frequently hemolyze red blood cells *in vitro*. These phenomena, but particularly the hemolysis, become evident only after a prolonged incubation of the tumor extracts with the red blood cells at 37°C. The agglutination is evident after 24 hours of incubation or earlier; the hemolysis requires 48 hours of incubation. Heating of the tumor extracts for 30 minutes to 56°C, prior to mixing them with the red blood cells, destroys their ability to either agglutinate or hemolyze the erythrocytes.

Mrs. Ruth G. Zahler rendered very ably technical assistance in this study. Dr. B. S. Gordon, Chief of the Clinical Laboratory was responsible for the pathological diagnosis of the slides. Mr. S. Shapiro, Chief of Medical Illustration, was responsible for the photographs.

[6] Gross, L., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 341.

[7] Maegraith, B. G., Martin, N. H., and Findlay, G. M., *Brit. J. Exp. Path.*, 1943, **24**, 58.

[8] Bruckmann, G., and Wertheimer, E., *Brit. J. Exp. Path.*, 1945, **26**, 217.

[9] Jones, E., and Tillman, C., *J.A.M.A.*, 1945, **128**, 1225.

[10] Wintrobe, M. M., *Clinical Hematology*, Philadelphia, Lea & Febiger, 1946.

[11] Stats, D., Rosenthal, N., Wasserman, L. R., *Amer. J. Clin. Path.*, 1947, **17**, 585.