is stable for at least 3 months at room temperature without loss of sensitivity or specificity.

The bentonite test can be applied to purified virus or sap from infected plants. The presence of sap components from bean, tobacco, and barley in high concentrations neither inhibited specific flocculation nor caused nonspecific flocculation. The sap extracts need not be clarified nor treated in any way except for dilution. Preliminary evidence indicates that undiluted sap may be used provided the virus antigen is not in sufficient excess to cause a prozone effect.

The presence of potato virus "X" in tubers from infected potato plants was also detected by the bentonite flocculation test. This has not hitherto been possible with other serological tests(12). There is reason to believe that the list of viruses similarly detectable in crude plant or seed extracts can be greatly extended.

Summary. A flocculation test using a highly stable antibody-sensitized bentonite was used to detect plant viruses in both crude and purified preparations. Conversely, antisera could be titrated with bentonite suspensions coated with purified virus. In both systems, the bentonite flocculation test proved rapid, sensitive and specific.

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Further Observations on Tumor Extracts Causing Hemolysis *in vitro*.* (28802)

M. D. REYNOLDS AND G. H. FRIEDELL (Introduced by S. Warren)
Cancer Research Institute, New England Deaconess Hospital and Pathology Department, Boston University Medical Center, Boston, Mass.

Many hypotheses have been proposed to explain the anemia of cancer. We were interested in extending the work of Sherman *et al*(1) who postulated at least two mechanisms responsible for the anemia of tumor-bearing hamsters: (1) a direct hemolytic effect occurring in or near the tumor itself, and caused by a factor extractable from the viable portions of the tumor(2), a more gradual effect caused indirectly by a factor extractable from necrotic portions of the tumor. Earlier publications by Sherman(2) and by Friedell *et al*(3) discuss this indirect effect. This paper presents additional observations on the factor causing the direct hemolytic effect.

Materials and methods. Tumors. The methylcholanthrene (MCP)-induced hamster sarcoma of Lutz(4) and a spontaneous hamster melanoma (MM2) originally obtained by us from the laboratory of Dr. W. B. Patterson were used. Golden hamsters,

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maintained on Purina Chow and water *ad lib*, were implanted with tumor in the flank when the animals weighed between 70 and 90 g.

After 4 to 6 weeks of growth these tumors weigh from 40 to 80 g. The peripheral part of the tumor is composed of firm, grayish white, glistening tissue with scattered minute foci of hemorrhage. The central portion of the tumor, comprising one-third to two-thirds of the tumor volume, is composed of somewhat softer, reddish brown tissue, fairly sharply demarcated from the peripheral portion. The central portion is termed the necrotic part, the peripheral portion is considered the viable.

Extracts. Separate saline extracts of the necrotic and the viable portions of the above 2 tumors and of various fresh normal tissues from nontumor-bearing hamsters were prepared according to the method used by Sherman *et al*(1).

Aqueous extracts were also prepared, using the following method. Tumors were separated into necrotic and viable portions, according to the gross appearance of the tumor tissue, and frozen. As needed, the frozen tumor tissue was thawed and cut into small pieces. For each gram of tissue (wet weight) 2 ml of cold distilled water was added. The fragments were then crushed, using a mortar and pestle or, when necessary, a Lourdes homogenizer. Care was taken to keep the tissue cold. The resulting homogenate was dialyzed at 7°C for 3 days against distilled water. The contents of the bag were then centrifuged in the cold at 25,000 $\times$ *g* for 25 minutes. An inert precipitate appeared and was discarded. The supernate, with pH about 6.7, was frozen until used. If necessary, the material was recentrifuged after thawing. In most cases, the thawed supernate was passed through a Seitz EK filter. The protein content of the resulting filtrate averaged 0.12% (biuret). If the thawed material was not filtered, a culture was taken to rule out the presence of hemolytic organisms. Finally, the extract was made isotonic by adding 1/10 part of 9.0% of NaCl solution.

Preparations containing ether-extractable material were made by adding 2-3 times as much diethyl ether to a given volume of the aqueous extract, at a point in the above procedure just before adjustment to isotonicity. The mixture was shaken thoroughly for about 5 minutes and set aside for the layers to separate. The ether layer was removed to a stoppered, graduated cylinder. The latter was placed in a water bath at 38°C, the stopper removed and the ether evaporated. The last traces of ether were removed by placing the cylinder in an air incubator at 38°C for 30 minutes. The residue in the graduate was resuspended in a convenient volume of saline. It will be referred to as the reconstituted ether extract. Ether remaining in the water layer in the separatory funnel was evaporated off at 40°C.

Assay. Heparinized blood was obtained by cardiac puncture from normal hamsters. The cells were washed 3 times with saline and a one percent cell suspension in saline was made. To 0.25 ml of extract in a small tube, 0.5 ml of cell suspension was added. This tube was incubated at 38°C for 3-5 hours, then spun in a Clay-Adams Serofuge for 60 seconds. The supernate was examined for hemolysis and the appearance of the red cell button was noted. Controls consisted of (1) extract plus saline and (2) red cells plus saline. All determinations were run in duplicate.

Autolysis of normal tissue. A homogenate of normal hamster lungs in saline (2 ml per gram wet weight of tissue) was made and potassium penicillin G (500 units/ml) added to the extract. The mixture was incubated at 37°C for 72 hours.

Effect of heating. The effect of boiling on extract activity was studied as follows. A hemolytic aqueous extract of viable tumor (MM2) was spun at 100,000 $\times$ *g* for 60 minutes (Spinco ultracentrifuge) to clear the solution as well as possible. One aliquot of the supernate was boiled for 3 minutes; another portion was not. The hemolytic effect was then tested.

Results. Saline extracts of 13 MCP tumors made according to Sherman *et al*(1) varied in ability to produce hemolysis *in vitro*. In 8 cases the viable extract was active and the necrotic extract inactive; in 2

TABLE I. Effect of Extraction by Diethyl Ether of Viable Tumor Extract.

Material assayed*	Degree of hemolysis
Original aqueous extract—before ether extraction	+++
Aqueous phase—after ether extraction	0
Reconstituted ether extract (volume equal to ⅓ of original)	++++

* All material was made isotonic before assay, and the 3 samples were assayed simultaneously.

cases the reverse was true; in 3, neither portion showed activity. However, in one instance, a tumor weighing 60 g and estimated to be 95% necrotic was used. The necrotic portion could have contained, at best, only traces of viable tissue, and could be considered almost "pure" necrotic tissue. This time the extract of the necrotic part caused no hemolysis. It was found that addition of fresh hamster serum (as a source of complement) to nonhemolytic extracts of viable tumor tissue failed to activate them.

Aqueous extracts were prepared (by the modified method) from the viable tissue of 4 MCP and 4 MM2 tumors. Five of these were active; 3 were inactive (Table I).

Two saline extracts of normal hamster tissue, consisting of pooled lungs, adrenals and kidneys from nontumor-bearing animals showed no hemolytic activity. The saline extract of autolyzed normal lung tissue was also negative. However, one aqueous extract prepared from pooled lungs, adrenals, kidneys and spleens from normal hamsters was hemolytic, and the hemolytic principle was apparently extractable into ether.

When an aliquot of one actively hemolytic aqueous extract was boiled for 3 minutes, a complete loss of activity resulted. The unheated portion promptly hemolyzed normal hamster red cells.

One aqueous extract was assayed for liver catalase-depressing activity in hamsters.† The results were negative.

Discussion. Several authors have investigated *in vitro* the hemolytic effect of tumor extracts or slices(5-13). Results vary, however. The mechanism or mechanisms of action are not clear, and the problem is complicated by the fact that so many factors can influence hemolysis. Additional information is badly needed on the physical and chemical properties of the direct hemolytic factor or factors.

The active material in the tumors we studied is present fairly consistently in viable tumor tissue and is usually absent from necrotic tumor tissue, as well as from extracts of several normal tissues and of autolyzed lung. Thus it is possible that the causative agent is present in increased amounts in viable tumor, but its presence does not signify neoplasia. This would be in accord with the familiar observation that malignant and normal tissue differ only quantitatively with respect to the presence of a given factor.

The evidence obtained so far suggests that the hemolytic agent is a protein or that it is bound to protein. It does not pass through a dialyzing membrane and it is destroyed by heat. Its solubility in ether further implies a lipid-protein complex, but this finding must be considered with caution. The complete removal of ether from a solution is a difficult procedure and it is possible, although unlikely, that the hemolytic activity of the reconstituted ether extract is due to traces of ether included in the fatty material. At the same time, the apparent loss of activity from the aqueous layer might possibly be due to denaturation caused by the efforts to remove traces of ether.

Summary. Further study has been made of the direct hemolytic factor present in hamster tumors. Hemolytic activity was generally present in viable tumor tissue and generally absent from necrotic tumor and normal tissue. The evidence so far implicates protein or protein-bound material as the causative agent.

† Assays kindly performed by Dr. M. Rechcigl, Jr., Nat. Cancer Inst.

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Effect of Hypochloremia on Cerebrospinal Fluid Chloride Concentration In a Patient with Anorexia Nervosa and in Dogs. (28803)

STANFORD B. FRIEDMAN,* W. GERALD AUSTEN,* RICHARD E. RIESELBACH,* JEROME B. BLOCK AND DAVID P. RALL
Medicine Branch, National Cancer Institute, National Institutes of Health, Dept. of HEW, Bethesda, Md.

There has been some question as to whether the cerebrospinal fluid (CSF) chloride concentration is the result of a passive transport process, or whether an active mechanism accounts for the normal CSF/plasma chloride ratio of approximately 1.15. In the dogfish (1) and the cat(2), an active mechanism has been postulated, though data reported by Held, Fencl and Pappenheimer(3) indicate that, normally, the gradient in dogs and goats can be accounted for by the existing potential difference between the CSF and blood.

Studies in a patient with chronic hypochloremia associated with anorexia nervosa demonstrated that the plasma chloride concentration was an important determinant of chloride concentration in CSF from the lumbar sac, but that CSF removed from higher in the neural axis was less influenced by the low plasma concentration of chloride. Preliminary work in dogs (Dogs 1-5) also indicated a tendency to maintain cisternal CSF chloride concentration over a wide range of plasma chloride concentrations.

Further studies with dogs (Dogs 6-8) demonstrated that changes in blood pH, which alter the potential difference between CSF and blood(3), could not account for the observed high CSF/plasma chloride ratio.

Materials and methods. Clinical studies. A 27-year-old woman† with anorexia nervosa (4) developed hypochloremia secondary to frequent self-induced vomiting. Extensive diagnostic studies failed to reveal evidence of an organic lesion involving the brain or meninges. On each of 2 occasions, after a blood specimen was obtained, a lumbar puncture was performed. The cerebrospinal fluid was obtained in the following manner. After collection of the first 2 ml, approximately 14 ml was removed over a 5-minute period. Then a third sample of 2 ml was obtained; the first and third specimens were analyzed by the clinical laboratory for chloride and potassium. Considering that the normal intraspinal (thoracic and lumbar) volume of cerebrospinal fluid is approximately 30 ml in adults of average size(5), the third specimen obtained from this 33 kg patient was assumed to be from the high thoracic area.

Dog studies. Hypochloremia of varying degrees was induced in 8 dogs of mixed breed, weighing from 12 to 15 kg, by surgically creating a gastric fistula. In Dogs 1-2,

* Present addresses: Depts. of Pediatrics and Psychiatry, Univ. of Rochester Med. Center, Rochester, N. Y. (S.B.F.); Dept. of Surgery, Massachusetts Gen. Hosp., Boston, (W.G.A.); Dept. of Medicine, Washington Univ. School of Med., St. Louis, Mo. (R.E.R.).

† Patient was under the care of Dr. Jacob Fishman, Adult Psychiatry Branch, Nat. Inst. Mental Health.