STUDIES ON A SUBSTANCE THAT PROMOTES TUMOR HOMOGRAFT SURVIVAL (THE "ENHANCING SUBSTANCE")

ITS DISTRIBUTION AND SOME PROPERTIES*, ‡

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Like homografts of normal tissues, tumors grafted between unrelated strains of mice may survive for a short period of time but are, in most cases, ultimately rejected. However, it is now well established that in certain tumor-host combinations, tumor homografts can be induced to survive and grow until the death of the mouse, by pretreating the host with killed tissue preparations (see references 2 and 3 for extensive bibliography). This phenomenon has been called the "enhancing effect." Possibly similar are the accelerant effects of certain tumor preparations on tumor isografts (4–7), and the prolongation of the life of normal skin homografts in pretreated mice (8). The observation that the γ-globulin fraction from rabbits or mice immunized with killed tissues from the donor strain can also induce homograft survival (9), indicates that the mechanism has an immunologic basis and that the active substance is an isoantigen. The possible modes of action by which antibody to donor tissues may increase the survival of homografts has been discussed in recent publications (8, 10).

The enhancing factor has been demonstrated in tumor, spleen, kidney, and liver (11), but not in muscle, brain, or in lyophilized whole blood (12). Its presence in untreated whole blood has been demonstrated (13). However somewhat special test conditions were needed. Fractionation studies have shown enhancing activity to be present in mitochondrial and microsomal preparations (14), and in high speed supernate fractions (15). Accelerant activity has been found in mitochondrial and microsomal preparations (5), in a nucleoprotein prepared from the isolated nuclei of ascites tumor cells (6), and in an acetone-soluble tumor fraction (7). With the exception of the acetone-soluble factor, in all cases in which these factors were investigated the

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‡ Part of these data have been presented elsewhere (1).

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active factors were non-dialyzable, labile to heat, and to extreme alkaline or acid pH, and resistant to the action of enzymes.

The present study deals with quantitative measurements of enhancing activity in organs and in certain fractions prepared from tumor tissue and with some chemical properties of the active substance.

Materials and Methods

The Assay.—Sarcoma I, which arose in strain A mice and which was carried in this line was used as the homograft (3). Mice of strain B10.D2 (a C57BL/10 subline), 7 to 12 weeks old when treatment was begun, were used as hosts. Tissues to be fractionated or tested for activity were obtained from strain A mice, washed in saline, and stored in a deep-freeze at −15°C. until used. Before injection, all tissues or tissue fractions were lyophilized, ground in a mortar, and suspended in saline by homogenizing in a glass homogenizer. Dosage was based on Kjeldahl nitrogen and the total dose was distributed over 3 intraperitoneal injections 4 days apart. Two to four dose levels were given and 10 mice (5 female, 5 male) were used at each level. Approximately 10 days after the last injection, live bits of tumor were transplanted subcutaneously into the dorsal region of the host mice with a trocar. A positive test for the active substance was indicated by the progressive growth of the tumor until the death of the mouse.

Although the tumor was transplanted into an untreated group of host mice in each experiment, these control groups are not included in the following tables since the tumor grew in only two untreated mice out of the several hundred used in all experiments.

Chemical Determinations.—Nitrogen was determined by a semi-micro Kjeldahl method. Nucleic acids were extracted by the procedure described by Ceriotti (16). DNA was determined by the method of Dische (17) corrected for interference as described by Dische (18). RNA was determined by the orcinol method as described by Ceriotti (16). Hexosamine was determined by the Elson-Morgan method as modified by Boas (19).

EXPERIMENTAL

The Bioassay.—

The response to the injection of graded doses of killed tissue was influenced to a considerable extent by changes in the growth characteristics of the tumor used as the graft. Hence, the apparent activity of a single tumor preparation varied considerably from experiment to experiment and it was necessary that a wide dose range be covered to insure at least one level in the sensitive range. With this procedure and with the small numbers of mice that it was practical to use at each dose level, a graded response to dose was not always evident. Since this was true, the results obtained with the different dose levels were, in most cases, combined and are presented in the tables as a single value. That the response to dose is quantitative when large numbers of animals are used is shown by Fig. 1 in which 14 individual dose-response curves obtained with similar tumor preparations in 10 different experiments are averaged. It is also apparent from Fig. 1 that the tumor homograft can be induced to survive by relatively small doses of killed tissue and grow until it kills the mouse, 50 per cent deaths being obtained with a dose of approximately 0.15 mg. N or approximately 1.5 mg. dry weight of lyophilized tumor in an average experiment. Neither sex nor age appeared to be important variables in the bioassay. However, the response to a preparation was

much reduced when the tumor was transplanted as a cell suspension rather than by trocar as described above.

Distribution in Organs and Tissues.—Tumor, liver, spleen, and kidneys from A strain mice were collected, washed, and homogenized. Stomachs were re-

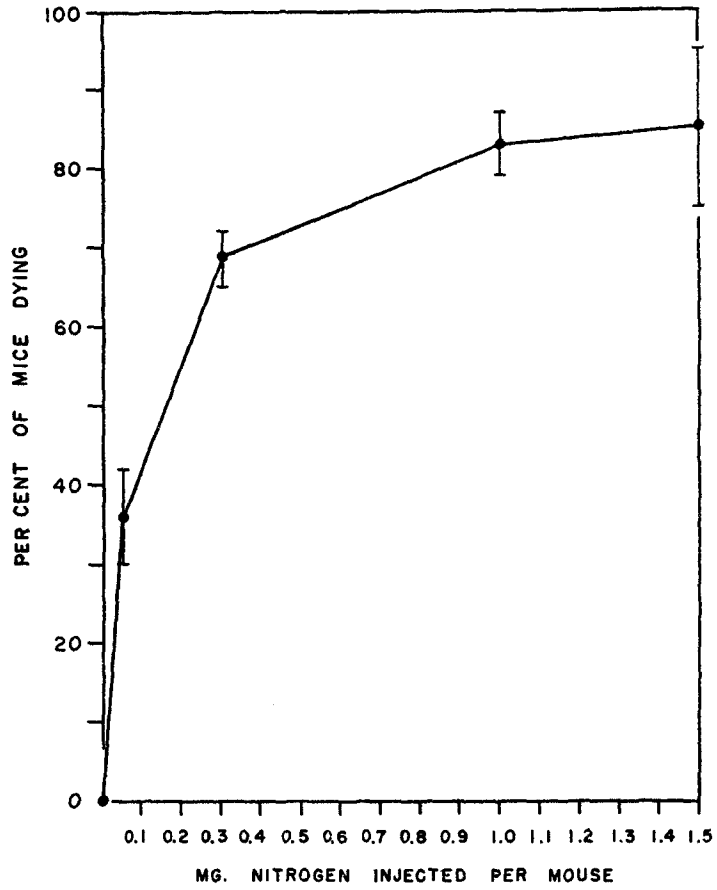

FIG. 1. Dose response curve. Points on the curve were obtained by averaging 14 individual dose response curves when 10 mice were used for each point. Only 5 of the 14 curves extended to 1.5 mg. N/mouse. Vertical lines represent the standard error of the mean.

moved, washed, and the glandular mucosal lining was scraped from the pyloric region with a spatula. All preparations were freeze-dried and nitrogen analyses were made on the dried materials. Stomach mucosa was of interest because of the high concentrations of blood group substances in such preparations from other animals and analyses on the lyophilized mucosa preparation from mouse stomach showed the presence of 1.2 per cent hexosamine as compared to 0.55

per cent for the part of the stomach remaining after the glandular mucosa had been removed. In agreement with Kaliss and Snell (11), tumor and spleen were of approximately equal activity while kidney, liver, and the stomach preparations possessed relatively small amounts of activity (Table I).

Activities of Tumor Fractions.—In a typical fractionation, tumor tissue was divided into 4 different fractions by the following procedures:—

Approximately 260 gm. wet weight of Sarcoma I was homogenized in a Waring blendor with 200 ml. of 0.14 M saline. To the homogenate were added 450 ml. of saline and the suspension was centrifuged in a refrigerated centrifuge at 3°C. for 30 minutes at 4500 *g*. The supernate was separated, and a portion was adjusted to pH 5.4 with 0.1 N HCl, allowed to stand 15 minutes in the refrigerator then centrifuged at 4500 *g* for 30 minutes. The resulting supernate was lyophilized (*fraction 1*) and the fraction precipitated by the acid was washed with 0.14 M saline at pH 5.4 (*fraction 2*). This fraction contained most of the ribonucleoprotein (RNAP) of the tissue; in terms of lipide-free dry weight, 17 per cent of this fraction was RNA.

TABLE I

Distribution of Enhancing Activity in Organs and Tissues

Tissue	Per cent of mice dying out of 30*
Tumor (sarcoma I)	47
Spleen	40
Kidney	00
Liver	3.3
Stomach glandular mucosa	3.3
Stomach after removing glandular mucosa	13.3

* Total dose levels of 1.0, 0.3, and 0.05 mg N per mouse were administered to groups of 10 mice.

The sediment obtained by centrifuging the initial homogenate was homogenized with 0.14 M saline and centrifuged at 2000 *g*. This step was repeated three times, the supernate being discarded each time. The resulting product is referred to in following experiments as low speed sediment (*LSS*). A desoxyribonucleoprotein (DNAP) fraction (*fraction 3*) was prepared from the LSS by extracting with 1 M NaCl essentially as described by Mirsky and Pollister (20). The nucleoprotein was purified by precipitating it four times from 0.14 M saline. In some experiments, the DNAP fraction was extracted with distilled water rather than with 1 M NaCl. DNAP fractions formed highly viscous solutions in distilled water or 1 M NaCl and from 27 to 37 per cent of the lipide-free dry weight was DNA. The material remaining after extraction with molar NaCl was washed two more times with molar NaCl and three times with distilled water to give *fraction 4*. All fractions were freeze-dried and stored in a deep-freeze until used. Nitrogen was determined on the lyophilized materials and nitrogen, DNA, RNA, and hexosamine were determined after extracting weighed amounts of the fractions in a Soxhlet apparatus for 4 hours with alcohol and 4 hours with ether and drying in a desiccator. Lipide was estimated by evaporating an aliquot of the extracts at 110°C. and weighing. The presence of hexosamine was considered to indicate the presence of a mucopolysaccharide or mucoprotein. However, it is probable that at least some of the hexosamine contained in the supernate at pH 5.4 was a component of smaller molecules. Hexosamine, lipide, and RNA were present to some extent in all fractions (Table II).

The supernate at pH 5.4 (fraction 1, Table II) appeared to be less active than the other fractions while the insoluble residue fraction (fraction 4, Table II) appeared to be the most active. The presence of high levels of activity in the fraction precipitated at pH 5.4 and in the DNAP fraction was in agreement with earlier reports that microsomal fractions and a nucleoprotein prepared from isolated nuclei possessed accelerant or enhancing activity. However, the active factor did not appear to be a desoxyribonucleoprotein since fractions which possessed no detectable DNA (fraction 1) or only trace amounts (fraction 2) possessed considerable activity. On the other hand, even the purest DNAP obtained was contaminated by a detectable amount of hexosamine (fraction 3 a). Neither was there any apparent relationship between RNA concentration and activity, residue and DNAP fractions being fully as active as the RNA-rich fraction precipitated at pH 5.4.

TABLE II

Analysis and Activity of Tumor Fractions

Fraction No.	Description	Mg. DNA / Mg. N*	Mg. RNA / Mg. N*	Mg. hexosamine / Mg. N*	Mg. lipide / Mg. N*	Per cent of Mice dying out of 30‡
1.	Supernate pH 5.4	0.0	0.204	0.047	2.10	57
2.	Ppt. pH 5.4 (RNAP)	0.013	1.023	0.022	3.44	67
3.	Sol. 1 M NaCl (DNAP)	1.89	0.297	0.018	10.45	70
3 a. §	"	2.76	0.060	0.007	2.33	
4.	Low speed sediment insoluble 1 M NaCl (residue)	0.143	0.102	0.034	2.11	83

* Kjeldahl nitrogen determined on lipide-free fractions.

‡ Total dose levels of 1.0, 0.3, and 0.05 mg. N/mouse were administered to groups of 10 mice.

§ This relatively pure DNAP fraction was prepared and tested in an earlier experiment. At levels of 2.0 and 0.75 mg. N per mouse it resulted in the death of 10 and 8 out of 10 mice respectively.

In further studies on the ribonucleoprotein fraction the acid precipitate obtained by adjusting the pH to 5.4 was resuspended in and reprecipitated twice from a 0.5 M $NaHCO_3$ solution according to a procedure for purifying a ribonucleoprotein (20). The material insoluble in 0.5 M $NaHCO_3$ was discarded each time. The reprecipitated fraction was dialyzed overnight against distilled water and lyophilized. After this treatment the fraction no longer possessed enhancing activity. To determine at which step activity was lost from the acid-precipitated materials, portions of an active pH 5.4 precipitate were resuspended in saline and adjusted to pH 7.2 with $NaHCO_3$ or in 0.5 M $NaHCO_3$ (final pH 8.0). After centrifuging these suspensions for 30 minutes at 4500 *g*, the sediment was separated and the material in the supernate was again precipitated by adjusting the pH to 5.4. Both fractions were lyophilized.

As shown in Table III, the activity appeared to be mainly associated with the materials that were centrifuged from the alkaline suspension. In these fractions

the concentration of RNA was reduced while the concentration of hexosamine appeared to be somewhat increased.

The fact that materials sedimented at low centrifugal speeds, such as the LSS and the insoluble residue fraction (fraction 4) were highly active, made them particularly useful for studies on the stability characteristics of the enhancing substance, since added reagents could be removed by sedimenting the tumor fraction in a centrifuge.

Extraction with Organic Solvents.—It has been suggested that the antigens involved in transplantation may be lipides (21) likewise, the preparation of an acetone-soluble accelerant fraction from a mouse mammary tumor has been reported (7). On the other hand, after extraction with alcohol, ether, or dioxane for short periods of time (14) or with cold acetone (15), tumor preparations were still capable of promoting homograft survival. However, all lipides may

TABLE III

Activities of Reprecipitated RNAP Fractions

Fraction	Mg. DNA / Mg. N	Mg. RNA / Mg. N	Mg. hexosamine / Mg. N	Per cent of mice dying out of 30*
Ppt. pH 5.4	0.013	1.023	0.022	67
Ppt. pH 5.4 insoluble in 0.5 M $NaHCO_3$	0.0	0.691	0.032	60
Ppt. pH 5.4 soluble in 0.5 M $NaHCO_3$	0.0	1.182	0.017	23
Ppt. pH 5.4 insoluble saline pH 7.2	0.03	0.708	0.024	77
Ppt. pH 5.4 soluble in saline pH 7.2	0.0	1.276	0.020	37

* Total dose levels of 1.0, 0.3 and 0.05 administered to groups of 10 mice.

not have been completely extracted under the relatively mild conditions used in the last two cases and the lipide fractions were not tested for activity.

To determine whether or not the lipide fraction possessed enhancing activity, the following procedures were carried out:—

One or two gm. samples of lyophilized whole tumor were extracted overnight with 50 ml. of organic solvents or a mixture of solvents at temperatures ranging from −20 to room temperature. In some cases, the mixture of organic solvent and lyophilized tumor was heated to 50 or 60°C. in a water bath for 2 hours then allowed to stand at room temperature overnight. The portion of the tumor insoluble in the solvent was filtered out, washed with 100 ml. of the same solvent, and dried at room temperature. The extracts were evaporated *in vacuo* at room temperatures or lower and weighed. Lipide extracts were suspended in a saline solution containing 1 per cent tween 80 and injected into 10 mice.

When injected at a single dose level of 40 mg. dry weight, the extracted residues appeared to be fully active except when the solvent was 80 per cent alcohol or 3:1 alcohol:ether at 60°C. (Table IV). However, when tested at lower dosage levels, extraction with alcohol:ether at 5°C. caused a marked decrease in activity while extraction in a Soxhlet apparatus for 4 hours with

alcohol and 4 hours with ether, appeared to destroy most of the activity. None of the mice receiving injections of the lipides were killed by growth of the subsequently inoculated tumor; however, the lipide injections sometimes caused the tumors to survive for a longer time and to grow on a much larger size before regressing. Attempts to obtain the survival of tumor homografts

TABLE IV

Activity of Lyophilized Tumor after Extraction with Organic Solvents

Solvent	Temperature*	Per cent dying		
		Exp. 1‡	Exp. 2	Exp. 3§
None	Room temperature	100	100	
	50°C.	90	80	
80 per cent alcohol	Room temperature	50		
	50°C.		00	
Acetone	Room temperature	70		
	50°C.		80	
3:1 alcohol:ether	Room temperature	60	80	
	50°C.	70	70	
	60°C.		40	
None				70
3:1 alcohol:ether	Overnight in refrigerator			22.5
" " "	Overnight in refrigerator followed by Soxhlet extraction			7.5

* In Experiments 1 and 2 the tumor was allowed to stand in the solvent overnight at room temperature or was heated to 50 or 60° for 2 hours, then allowed to stand overnight at room temperature.

‡ A total dose of 40 mg lipide-free dry weight per mouse administered to 10 mice.

§ Total dose levels of 2.0, 1.0, 0.2, and 0.05 mg. N per mouse administered to groups of 10 mice.

until the death of the mouse, by increasing the total dose of lipide extract to 300 mg. per mouse, by distributing the dose over 6 injections at 3 day intervals or by suspending the lipides in a 2 per cent solution of lyophilized porcine serum (Mann Research Laboratories, Inc.) (22) were unsuccessful.

pH Stability.—The accelerant factor studied by Shear *et al.* (5) was destroyed by 2 N NaOH while the active factor studied by Day *et al.* (15) was destroyed after exposure to pH 2 or lower or to alkaline solution at a pH of 11. Since, in neither of these studies was the stable pH range clearly defined, the following experiment was carried out.—

LSS was homogenized in saline at a concentration of 1.74 mg. N/ml. The homogenate was divided into 10 ml. portions and the pH was slowly adjusted to the desired level with 0.1 N HCl or 0.1 N NaOH using a pH meter while the homogenate was rapidly stirred with a magnetic stirrer. After standing for 1 hour at room temperature, the suspensions were carefully neutralized, diluted to the required concentrations, and tested at the dose levels of 1.0 and 0.33 mg. N/mouse. Since the pH was readjusted to neutrality after exposure of the LSS to alkaline or acid pH, only irreversible losses in activity were measured in this experiment.

While the procedure employed may have resulted in some loss of activity due to high local concentrations of acid or alkali, it is apparent that the active

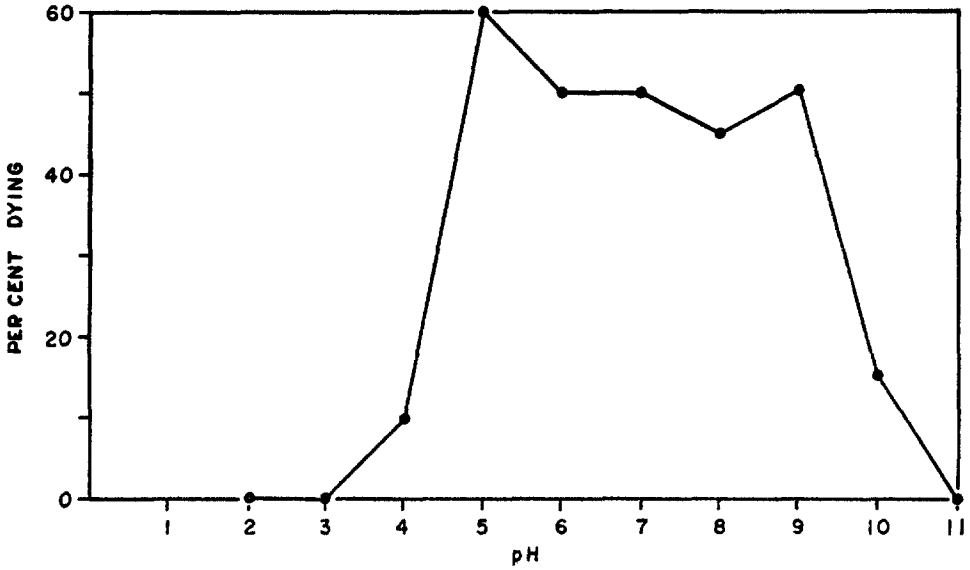

FIG. 2. pH stability curve. The ordinate represents the per cent of mice dying out of 20 when 2 groups of 10 mice received LSS at levels of 1.0 or 0.33 mg. N per mouse. The pH was adjusted back to neutrality after 1 hour at room temperature.

substance was quite labile to dilute acid or alkali, activity being rapidly lost when the pH dropped below 5 or rose above 9 (Fig. 2)

Effects of Certain Enzymes.—In early experiments it was found that treatment of a suspension of LSS with trypsin caused it to become very viscous, probably owing to the release of DNA, so that particulate materials could no longer be sedimented by centrifugation. For this reason, in those experiments in which it was desirable to remove trypsin after the incubation period, the washed insoluble residue (fraction 4) was used. This fraction did not contain a great deal of DNA and did not become viscous when treated with trypsin.

Samples of insoluble residue weighing 400 mg. were homogenized with 15 ml. of 0.14 M saline. To the homogenates were added 10 ml. of 0.1 M phosphate buffer (pH 7) containing 50 mg. of crystalline trypsin, 50 mg. of crystalline hyaluronidase or buffer only. A sample of

insoluble residue weighing 235 mg. was homogenized with 20 ml. of unbuffered saline containing 5 mg. of a lyophilized cell-free extract of *Vibrio cholerae* containing the receptor-destroying enzyme (RDE)[1] (final pH 6.8). The mixtures were incubated for 4 hours with frequent shaking in a 37° water bath. At the end of the incubation period, an aliquot containing 75 mg. dry weight of the insoluble residue fraction was removed from each mixture and centrifuged at 16000 *g* for 15 minutes. The supernate was carefully poured off and the residue was washed once with a small amount of saline, centrifuging as before. The first supernate and the wash were combined and the supernates and the residues were lyophilized. Hexosamine was determined in both the residue and supernatant fractions. Analysis of the crystalline hyaluronidase showed the presence of considerable hexosamine and the values obtained for this supernatant fraction were corrected accordingly. No hexosamine was found in the trypsin and because of the small amount of RDE available no analysis was made on this preparation.

The remainders of the incubation mixtures, with the exception of the RDE mixture, were divided into two equal portions. One portion was centrifuged and washed as described above, discarding the wash supernate. The portion of the incubation mixture that was not centrifuged was diluted to the proper concentration with saline and injected at levels of 1.0, 0.2, and 0.05 mg. N/mouse. The residue from the centrifuged portion was reconstituted in saline to the initial volume and diluted in the same way as the uncentrifuged portion. The supernate of the centrifuged portion was injected in amounts equivalent to 1.0 or 0.2 mg. N of the uncentrifuged suspension. The entire RDE incubation mixture not used for hexosamine analysis, was centrifuged and separated into residue and supernate as already described.

Since the results obtained with enzymes were sometimes equivocal, parts of the experiment described above were repeated several times and desoxyribonuclease was also tested using 5 mg. of desoxyribonuclease and 0.025 molar $MnCl_2$ in the final incubation mixture. The results of these experiments are added together and are included in Table V.

Incubation with trypsin caused a significant increase in the per cent of the total hexosamine found in the supernate, 78.2 per cent of the total being solubilized. Incubation with hyaluronidase did not increase the amount of hexosamine in the supernate and the slight increase in hexosamine in the supernate after incubation with the RDE preparation may have been at least partially due to hexosamine present in the RDE preparation.

When injected with the tumor fraction both trypsin and hyaluronidase caused a significant reduction in activity (groups 2, 3, 6, 7; Table V). However, when the enzyme was removed after the incubation period, the material that had been incubated with trypsin appeared to be as active as the uncentrifuged control even though most of the hexosamine had been removed. The material incubated with hyaluronidase also appeared to regain activity when the enzyme was removed. However, both the washed control and the washed hyaluronidase-treated materials appeared to be less active than the uncentrifuged control. Little significance was attached to this apparently decreased activity since in other experiments, there was no loss in control preparations under similar

[1] We are indebted to Dr. Karl Meyer, College of Physicians and Surgeons, Columbia University, for this preparation.

conditions. Neither DNAase nor RDE had an appreciable effect on activity. The effects of incubation with lysozyme and ribonuclease were also tested in preliminary experiments. Neither of these enzymes appeared to alter activity.

Effects of Urea and 90 per cent Phenol.—

LSS was homogenized with saline or 50 per cent urea previously adjusted to pH 9 with HCl. As the final pH of the urea homogenate was 9, an aliquot of the saline homogenate was adjusted to pH 9 with 0.1 N NaOH to control the effects of the alkaline pH. The suspensions were allowed to stand in the refrigerator for 7 hours. The neutral saline suspension and part of the urea homogenate were centrifuged for 30 minutes at 30,000 *g*. The supernates were

TABLE V

*Effects of Enzymatic Digestions on the Activity of a Sarcoma I Fraction**

Group	Enzyme	Per cent of mice dying‡			Per cent of total hexosamine in supernate
		Total mixture§	Washed residue§	Supernate‖	
1	None	47 (30)	20 (30)	10 (10)	12.5
2	Hyaluronidase	13.3 (30)	26.7 (30)	0 (10)	13.5
3	Trypsin	10 (30)	43 (30)	0 (10)	78.2
4	RDE		43 (30)	0 (10)	20.4
5	None	45.6 (120)			
6	Hyaluronidase	19.1 (120)			
7	Trypsin	13.1 (120)			
8	None	45 (60)			
9	DNAase	45 (60)			

* Insoluble residue (fraction 4, Table II) used for groups 1 to 4, LSS for groups 5 to 9.

‡ Numbers in parenthesis are the total numbers of mice injected.

§ Groups 1 to 4; total doses of 1.0, 0.3, and 0.05 mg. N per mouse administered to groups of 10 mice. Groups 5 to 9 total doses of 1.5, 0.5, and 0.05 mg. N per mouse administered to groups of 10 mice.

‖ Total dose equivalent to the volume of uncentrifuged mixture containing 1.0 mg. of N administered to 10 mice.

separated and, along with the residues and the uncentrifuged urea and saline homogenates, were dialyzed overnight in running water at 5°C. The dialyzed preparations were freeze-dried and tested for enhancing activity.

Sarcoma I was also fractionated by extraction with 90 per cent phenol with no prior treatment of the tumor tissue essentially as described by Howe and Kabat (23), except that the phenol-soluble fraction was precipitated by the addition of 2 volumes of alcohol.

As shown in Table VI, treatment with urea or phenol resulted in complete loss of activity.

Effects of Oxidizing Agents.—

Samples of LSS weighing 364 or 728 mg. were homogenized in 20 ml. of phosphate buffered saline (pH 7) or in buffered saline containing $NaIO_4$, $KMnO_4$, $K_2Cr_2O_7$ or H_2O_2 at a concentration of 0.01, 0.001, or 0.0001 molar. After standing for a period of time, the suspension was

TABLE VI

Effects of Urea and Phenol on the Activity of a Sa I LSS Fraction

Treatment	Mg. N/mouse*	Per cent of mice dying‡
Saline	1, 0.2, 0.05	60 (30)
Soluble in 50 per cent urea	1, 0.2	0 (20)
Insoluble in 50 per cent urea	1, 0.2, 0.05	0 (30)
50 per cent urea (not fractionated)	1.0	0 (10)
Saline, pH 9	1.0	60 (10)
Soluble 90 per cent phenol	3.0	0 (20)
Insoluble 90 per cent phenol	3.0	0 (20)

* Each dose was administered to 10 mice.

‡ Numbers in parenthesis are the total numbers of mice injected.

TABLE VII

The Effects of Oxidizing Agents on the Enhancing Activity of a Sarcoma I LSS Fraction

Oxidizing agent	Molarity of oxidizing agent	Time exposed	Washed*	No. of mice dying out of 10	
				1.5 Mg. N‡	3.0 mg. N‡
None§	—	—	Yes	9	8
$NaIO_4$	0.01	1 hr.	Yes	0	1
"	0.01	20 min.	"	0	0
"	0.001	1 hr.	"	3	8
"	0.0001	1 "	"	10	10
$NaIO_4$ inactivated with glucose	0.01	1 hr.	Yes	10	10
$NaIO_4$‖	0.01	1 hr.	No	0	
None‖	—	—	No	10	
$KMnO_4$	0.01	1 hr.	Yes	2	
"	0.001	1 "	"	6	
H_2O_2	0.01	1 hr.	Yes	10	
"	0.001	1 "	"	10	
$K_2Cr_2O_7$	0.01	1 hr.	Yes	8	
"	0.001	1 "	"	9	

* Centrifuged for 15 minutes of the indicated time at 3°C., sediment washed with glucose solution.

‡ Dose per mouse.

§ At dose levels of 0.5 and 0.05 mg. N per mouse, numbers of mice dying were 9 and 0 out of 10 respectively.

‖ At the end of the indicated period of time glucose was added to inactive excessive periodate.

centrifuged at 4500 *g* for 20 minutes, the supernate was discarded and the residue was washed once with 10 ml. of 2.5 per cent glucose, and centrifuged again. The glucose wash was poured off and the sediment homogenized in saline. Dosage was calculated with the assumption that essentially all of the LSS was recovered in the sediment at the centrifugal speed used. In some cases, the mixtures were not centrifuged but glucose was added to inactivate excess periodate ion. To determine the effects of the products resulting from the action of periodate ion on carbohydrates present in the LSS, 200 mg. of glucose were added to 20 ml. of 0.01 M $NaIO_4$ in buffered saline and allowed to stand 1 hour at room temperature after which the mixture was used to treat a sample of the LSS as described above.

The results shown in Table VII demonstrate that the activity of the LSS was rapidly lost after exposure to dilute sodium periodate. The loss occurred in the uncentrifuged mixture as well as in the cases in which the periodate ion was washed away indicating that the active substance was not converted to a soluble form and removed. Treatment with $KMnO_4$ also, caused a reduction in activity although $KMnO_4$ was less effective than $NaIO_4$ under the same conditions. Hydrogen peroxide, potassium dichromate, and sodium periodate that had reacted with glucose had no effect on activity.

DISCUSSION

The donor and host mice used in the tests for the enhancing substance in the present experiments are genetically quite different. Thus the possibility that several chemically distinct substances may act together or separately to produce the enhancing effect must be considered. The failure to identify activity with any one cellular fraction may indicate that this is true. However genetic factors concerned with homograft acceptance or rejection are relatively few in number (24), and by analogy with other isoantigen systems (*i.e.* the blood groups) the several substances involved may all fall into the same chemical class. According to the latter view, the presence of activity in different cellular fractions would be due to the presence of a common particle. In this connection it is noted that membranous structures of the cell have been demonstrated in mitochondrial, microsomal, and nuclear preparations (25–27).

A significant finding in the present experiments was the presence of high concentrations of the activity in the insoluble residue fraction. In this fraction, the active substance appeared to be almost completely insoluble in water or salt solutions. Although the composition of the insoluble residue fraction, outside of the presence of large amounts of connective tissues, was unknown, it is unlikely that much cytoplasmic material was present.

Important clues to the chemical nature of the active substance(s) are its lability to periodate ion and to protein-denaturing agents. Although not conclusive, the evidence available suggests that the periodate may be acting on a carbohydrate component in its relatively specific capacity to split carbon-carbon bonds where there are adjacent hydroxyl or hydroxyl and amine groups. In favor of this hypothesis are the relative effectiveness of $NaIO_4$ in destroying

activity in comparison to that of other strong oxidizing agents, the lack of an appreciable effect of $NaIO_4$ on the enhancing activity of a globulin fraction obtained from mouse antisera (9), the relatively prolonged exposure to periodate ion needed to obtain extensive inactivation of certain biologically active proteins (28–30) and the similar inactivating effect of periodate ion on mucoprotein or mucopolysaccharide antigens (31, 32). An alternative, but in the view of the authors, less likely explanation of the effect of periodate is that the activity of the active substance is dependent on the hydroxylysine residues or terminal hydroxyamino acids of a protein.

That a protein does constitute a part of the active substance is evident from the inactivating effect of protein-denaturing agents. Indeed, the enhancing factor appeared to be more labile to protein-denaturing agents than are the antigenic properties of at least some proteins, which may retain their specificities even after extensive denaturation (33, see reference 34 for extensive bibliography). If on the basis of the inactivating effect of $NaIO_4$ the active substance is postulated to contain a carbohydrate moiety, the lability of the enhancing substance to protein-denaturing agents can be accounted for in two ways: (*a*) denaturation of a specific protein which is conjugated to a specific carbohydrate or (*b*) the dissociation of a protein which may or may not be specific from a specific carbohydrate. The latter process might eliminate the capacity of the enhancing factor to evoke antibody without altering the antigenic specificities of either the protein or carbohydrate. Whether one of these ways is correct cannot be decided on the basis of the present experiments; but, it may be pointed out that, when their structure is known, all isoantigens studied thus far consist of a specific non-protein heptene conjugated with a relatively nonspecific protein.

More complete characterization of the enhancing factor is largely dependent on obtaining the substance in a relatively pure state. Obvious difficulties are involved in isolating a substance that is apparently insoluble and at the same time labile to such agents as acid, alkali, urea, and phenol. The development of a serological test might overcome most of these difficulties, since serological activity might be more stable than the capacity to evoke antibody. It is also possible that, like some mucopolysaccharide haptens (35) a mucopolysaccharide fraction from the tumor can be reactivated by combination with a foreign protein.

SUMMARY

Using the death of mice due to the growth of tumor homografts as an end point, the distribution and properties of a tumor homograft–promoting substance(s), the "enhancing substance," were studied.

Activity was most largely present in tumor and spleen tissues while that in the liver, kidney, and stomach was relatively slight.

Activity was widely distributed in fractions prepared from tumor. Although DNAP and crude RNAP fractions were active, activity could not be associated with either RNA or DNA. The fraction possessing the highest concentration of activity was a residue fraction which was insoluble in water or in dilute or strong saline solutions.

Extraction of lyophilized tumor with acetone or 3:1 alcohol:ether reduced, but did not eliminate, activity in the insoluble fraction. However, activity was completely lost after extraction with 80 per cent alcohol at room temperature.

Enhancing activity of a tumor fraction was irreversibly lost after exposure for 1 hour to a solution more acid than pH 5 or more alkaline than pH 9. Activity was completely lost after exposure to 50 per cent urea or 90 per cent phenol.

Activity was not altered by incubation with trypsin, hyaluronidase, DNAase or the receptor-destroying enzyme of *V. cholerae*, but it was decreased when trypsin or hyaluronidase was injected with the incubated tumor fraction.

Activity was rapidly destroyed by treatment with dilute solutions of sodium periodate at pH 7. Under similar conditions, treatment with $KMnO_4$ resulted in less extensive destruction of activity while H_2O_2, $K_2Cr_2O_7$, and $NaIO_4$ previously inactivated by reaction with glucose had no apparent effect on activity.

These results are interpreted as indicating the presence of both carbohydrate and protein in the structure of the activity substance.

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