EVIDENCE THAT THE L-ASPARAGINASE OF GUINEA PIG SERUM IS RESPONSIBLE FOR ITS ANTILYMPHOMA EFFECTS*,‡

I. Properties of the L-Asparaginase of Guinea Pig Serum in Relation to Those of the Antilymphoma Substance

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The action of guinea pig serum in bringing about *in vivo*, the regression of 3 transplanted mouse and rat lymphomas was first described by Kidd in papers from this laboratory in 1953 (1, 2). Several highly specific features were shown: first, that inhibition was produced only by guinea pig serum, sera from other animal species being completely devoid of effect. Secondly, susceptibility to the effects of guinea pig serum was found to be sharply confined to certain tumor cell lines. Thus, although 3 lymphomas were found initially to be sensitive to guinea pig serum, 14 other tumor cell lines of similar morphology were not inhibited in subsequent tests. Since this time, however, a number of additional cell lines of widely differing histological types, notably the Walker carcinosarcoma, the rat fibrosarcoma ACMCA2, and recently 10 different newly induced mouse lymphomas, have been shown to be guinea pig serum–sensitive (3–5). Thirdly, although guinea pig serum proved highly effective *in vivo* against tumor cells of sensitive lines, particularly lymphomas 6C3HED and A2, it produced no sign of toxicity in the hosts and furthermore proved innocuous for Lymphoma 6C3HED cells when held in contact with them *in vitro* during several hours at 37°C.

The experiments performed at this time, however, did not make it possible to determine which of the constituents of guinea pig serum were responsible for the antilymphoma effects. In this paper the question has been examined further, using cells of Lymphoma 6C3HED, and in a companion paper observations are reported which throw light on a particular character of these neoplastic cells that may determine their susceptibility to the effects of guinea pig serum *in vivo*.

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Since guinea pig serum has long been known to be a particularly rich source of the different fractions of complement, it was initially considered that these or similarly acting substances might be responsible for inhibition of tumor growth. A number of immunological experiments made by the author were deemed incompatible with such an explanation, however, and a search was made for an entirely different possibility. One such possibility was suggested by an observation made more than 40 years ago by Clementi, namely that the serum of guinea pigs, but not of other mammals, is a rich source of the enzyme L-asparaginase (6). The activities and properties of this enzyme in guinea pig serum were therefore compared with those of the substance responsible for the antilymphoma effects, with the results now to be described.

Methods

The hypothesis that L-asparaginase might be the antilymphoma substance in guinea pig serum was tested by obtaining preparations and fractions of the serum and determining whether their antilymphoma effect could be predicted by measurements of L-asparaginase activity. Thus, using whole guinea pig serum the effects of heat and alterations in pH on these activities were examined. Similar comparisons were made in specimens of serum obtained from newborn guinea pigs and in protein fractions of normal serum obtained by means of salt precipitation, starch block electrophoresis, and chromatography on DEAE cellulose.

Mice.—ZBC mice (C3H backcross), obtained from the late Dr. J. J. Bittner of the University of Minnesota, Minneapolis, were used in all the present experiments. They were maintained on Purina chow and water *ad lib.*

Tumor Cells.—Cells of the mouse lymphoma 6C3HED were used in all experiments to be described. A cell line of the lymphoma has been maintained by serial subcutaneous transfer in C3H or ZBC mice in this laboratory since 1946.

Implantation and Measurement of Tumor Growth.—The techniques used were modified from those described by Kidd (1). Solid subcutaneous tumors were removed under sterile conditions and gently squeezed through a stainless steel mesh into 10 to 15 ml of cooled Ringer's solution or buffered glucose-Ringer's solution. The lymphoma suspension was then transferred to a 25 ml measuring cylinder which was placed in the refrigerator at 4°C. The larger particles were allowed to settle, leaving a suspension of single cells, which was suitably diluted to contain 100,000 living cells per ml, when counted by the method of Schreck (7). Portions of the tumor cell suspension were examined on each occasion for bacteriological contamination by inoculation of nutrient broth and nutrient agar slopes. They proved consistently to be sterile.

The procedure in experiments to assay antilymphoma activity of guinea pig serum preparations was as follows: ZBC mice, weighing 23 to 26 gm, separated from the main stock and placed in groups of three at least 1 week previously, were implanted on each side with 50,000 Lymphoma 6C3HED cells. Cell suspensions were injected through shaved areas in the groins to form subcutaneous blebs inferolaterally, just beneath the costal margins. In each experiment up to 70 groups were used. 1 to 2 hours after tumor cell implantation, mice in a number of these groups were injected intraperitoneally with guinea pig serum in quantities of 2.0, 1.0, 0.5, and 0.25 ml, at least 2 groups receiving each amount. Substances under test for antilymphoma activity were injected into further groups of animals, each substance being injected into all the animals of at least 1 group. A number of groups interspersed amongst the rest were used as untreated controls.

In every experiment control animals were examined daily from the 7th day after implanta-

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tion. When tumors first became palpable in these, daily examination of all the experimental animals was begun and the presence and size of the tumors as judged by palpation was recorded.

After using the implantation technique described, tumors first appeared as papular lesions 0.2 to 0.3 cm in diameter in the skin of the implantation site, where they were readily detected both visually and by palpation. In untreated animals, tumors appeared in different experiments after an average of 9 to 12 days, but within any one experiment there was considerable consistency. Table I illustrates this with the results from a number of groups of untreated

TABLE I

*The Times of First Appearance of Tumors in ZBC Mice Implanted with Cells of Lymphoma 6C3HED**

Experiment	Mice from group No.	Outcome of implantation Number of tumors in each group of mice with the following latent periods					
		8 days	9 days	10 days	11 days	12 days	Average
1	1	—	5	5	—	—	9 days
	6	—	6	—	—	—	
	11	—	5	1	—	—	
	54	—	6	—	—	—	
	59	—	5	1	—	—	
2	1	—	5	1	—	—	9 days
	6	—	4	2	—	—	
	9	—	4	2	—	—	
	34	—	6	—	—	—	
	59	—	1	4	1	—	
	60	—	5	1	—	—	
3	1	—	—	—	4	2	11 days
	8	—	—	2	2	2	
	19	—	—	—	6	—	

* Each test group consisted of 3 ZBC mice implanted with 50,000 6C3HED cells in each side, as described in the text, and afterwards untreated.

controls which were part of experiments to be described later. In the control groups of each experiment, tumors appeared in a similar manner, the majority on the same day and only rarely with an interval of more than 1 day from this. From such a base line in the controls, the inhibitory activity of small quantities of guinea pig serum or derivatives of it, could be measured from the delay in the appearance of tumors in treated animals.

Delay occurred in a stepwise fashion, being just detectable (an average of 3 days) in animals given 0.25 ml of guinea pig serum; it was greater in animals given 0.5 ml (an average of 6 days) and increased to complete tumor suppression when 1.0 to 2.0 ml of guinea pig serum was given. Mice implanted with lymphoma cells in the way described were considered "cured" if no tumor appeared and they remained healthy during an observation period of 60 days. In practice 30 days was the longest observed latent period of any tumor.

Placing these findings on a numerical basis involves a number of fundamental difficulties,

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TABLE II

Relationship of the Dosage of Guinea Pig Serum Used in Treatment to the Degree of Inhibition of Cells of Lymphoma 6C3HED

Experiment*	Outcome of implantation‡								
	Controls: day of tumor appearance	Days of tumor inhibition in groups of mice treated with the following quantities of guinea pig serum§, ‖							
		2.0 ml		1.0 ml		0.5 ml		0.25 ml	
		Total	Average	Total	Average	Total	Average	Total	Average
1	9	72	72	72	72	36	39	16	16
		72		72		32		19	
		—		72		47		15	
		—		72		34		16	
		—		72		46		18	
		—		—		—		11	
2	9	70	70	70	67	39	36	15	14
		—		63		32		12	
3	11	71	71	71	68	44	48	14	13
		—		64		51		12	

* Other parts of the experiments designated are shown in Table I.

‡ Groups of 3 ZBC mice were implanted with 50,000 lymphoma cells in each side. Details of the methods of lymphoma cell implantation and of treatment with guinea pig serum are described in the text.

§ To explain the manner of calculating the number of days of tumor inhibition the example of Experiment 2 will be quoted. In this and all other cases, calculations were based on groups of 3 mice, each implanted with 2 tumors. It was seen in Table I that most tumors in Experiment 2 were first observed on the 9th day after implantation. If all had been observed on this day, then in the test period, which here would be the 9th day and the 11 successive days there would have been $12 \times 6 = 72$ "tumor days." In fact there was a scatter in the time of appearance of tumors in control animals, so that there was an average number of 70 tumor days in each group. Complete suppression of tumor growth in a group of animals treated with 2 ml of guinea pig serum, as shown in the table, therefore caused 70 days of tumor inhibition.

‖ L-Asparaginase assays of the guinea pig serum samples used in treatment: Experiment 1, 69.2 units/ml; Experiment 2, 87.1 units/ml; Experiment 3, 69.3 units/ml.

but it has been found useful in practice to measure in each group of 3 treated animals the total number of days in which tumor appearance was delayed beyond the average of that found in control groups.[1] A limit was made at the 11th day after the appearance of tumors in untreated controls; from this time there was little change in the pattern of the "chartings," and, of tumors suppressed to this time, less than 10 per cent appeared subsequently. In Table II it will

[1] For instance, if in all the animals of control groups, tumors appeared on the 10th day, and in a group of treated animals 3 tumors appeared on the 14th and 3 on the 16th days, there would be $3 \times 4 + 3 \times 6 = 30$ "days of tumor inhibition."

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be seen that measurements of the number of "days of tumor inhibition" made in the way described show a progressive rise in groups of animals treated with increasing quantities of guinea pig serum, providing a suitable set of standards in each experiment to assay antilymphoma activity.

After their first appearance tumors grew progressively to bring about the death of the host animal 24 to 32 days after implantation. The subcutaneous tumors were by this time ulcerated and hemorrhagic and there was extensive visceral metastasis.

Guinea Pig Serum was obtained from market-bought adult animals of random breed and either sex. 15 to 20 ml of blood was obtained from each under aseptic conditions by cardiac puncture, without anesthesia. Most animals were bled on several occasions but at intervals of at least 6 weeks between bleedings. Serum from groups of 15 to 30 animals was pooled, and kept at −20°C until required. Samples from each batch of pooled serum were inoculated into nutrient broth and nutrient agar; they consistently showed no evidence of bacterial contamination.

Newborn animals (within 48 hours of birth) were rendered unconscious by placing them above a quantity of solid CO_2. Under aseptic conditions the thorax was then opened and the animals exsanguinated by cardiac puncture. Approximately 2 ml of blood was obtained from each animal. The serum from each animal was separated and kept frozen as already described.

Assays of L-Asparaginase Activity were performed by a modification of the method described by Meister, Levintow, Greenfield, and Abendschein (8) measuring the rate of ammonia production from a standard L-asparaginase solution. In this 0.2 ml samples of enzyme containing solutions were added to incubation mixtures at 37°C consisting of 1 ml 0.1 M sodium borate buffer pH 8.5, 1 ml 0.04 M L-asparagine, and 0.8 ml of 0.15 M NaCl. Immediately after adding the enzyme and 60 minutes later, 1 ml samples were withdrawn, and protein precipitated with tungstic acid solution. Ammonia in the supernatant was estimated by nesslerization, using with each batch of assays, standards containing 0.1 and 0.05 mg ammonia nitrogen/ml (as ammonium sulfate), and measuring ammonia concentrations in test solutions from a standard curve.

The unit of L-asparaginase activity employed was that which, under the conditions described, liberated 1 micromole of ammonia from L-asparagine in 1 hour.

Protein Concentration was measured by the Folin-Ciocalteu method as modified by Kunkel and Tiselius (9).

RESULTS

Inactivation of L-Asparaginase and the Antilymphoma Substance of Guinea Pig Serum at Different Temperatures.—Earlier experiments in this laboratory (2) showed that the antilymphoma activity of guinea pig serum was relatively easily destroyed by heating; in guinea pig serum kept at 56°C slight inactivation occurred, and at 66°C this became complete. Table III shows the result of an experiment in which a number of samples of guinea pig serum were incubated at varying temperatures for 30 minutes. At 56°C 15.2 to 20.7 per cent of L-asparaginase activity was lost, but at 66°C the enzyme was almost entirely (82.9 to 87.8 per cent) destroyed.

In the experiment shown in Fig. 1 a comparison was made between the L-asparaginase and antilymphoma activities of 3 samples of a pooled batch of guinea pig serum which were heated at 46°, 56°, and 66°C respectively, for 30 minutes.

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A further sample left at room temperature for the same period, that is under conditions in which both activities were stable, served as a standard. At 46°C the antilymphoma activity was perfectly stable, 1 ml of heated guinea pig serum completely inhibited 5 of the 6 tumors implanted; similarly, at this temperature L-asparaginase activity was unaltered. After incubation at 56°C, the guinea pig serum was still clearly inhibitory to tumor growth, but there was an obvious diminution in its effectiveness, for although the time of tumor appearance was delayed from 11 days in untreated controls to 15 to 27 days in this group, only 2 out of 6 implanted tumors were completely suppressed. The decline in antilymphoma activity was accompanied by a fall in L-asparaginase

TABLE III

The Inactivation of L-Asparaginase in Guinea Pig Serum by Heat

Sample No.	Initial L-asparaginase activity	L-Asparaginase activity (per cent of initial values) after incubation for 30 min. at the following temperatures			
		46°C	56°C	60°C	66°C
	units/ml*				
1	55.7	99.5	80.1	—	15.3
2	72.9	—	79.3	63.3	17.1
3	70.2	—	84.8	68.7	12.2
4	62.1	—	83.2	70.1	14.8

* Micromoles of ammonia liberated in 1 hour, under the standardized conditions previously described.

assays to 80.1 per cent of the value of unheated samples. At 66°C the antilymphoma activity of guinea pig serum was almost completely destroyed. In 2 of the 3 treated animals a slight delay of 2 to 3 days in tumor appearance occurred, compared with the controls, in the other animal there was no detectable inhibition. This corresponded closely with the destruction of L-asparaginase; only 15.3 per cent of enzyme activity remained.

Within the limits of the experimental method, then, these results showed a close similarity between the heat stability of L-asparaginase and the antilymphoma agent. This relationship was also found when the stability of these two activities to change in pH was examined.

Inactivation of L-Asparaginase and the Antilymphoma Substance of Guinea Pig Serum at Different pH Values.—L-Asparaginase in guinea pig serum was found to be notably unstable to changes in pH, and in a particular pattern; the enzyme was considerably less stable at acid than at alkaline values. The similar pattern of instability of the antilymphoma substance is shown in Fig. 2.

At pH 7.0 L-asparaginase was stable, but when incubated for 2 hours at 4.3 pH units above this value, 37.9 per cent of enzyme activity was lost and the

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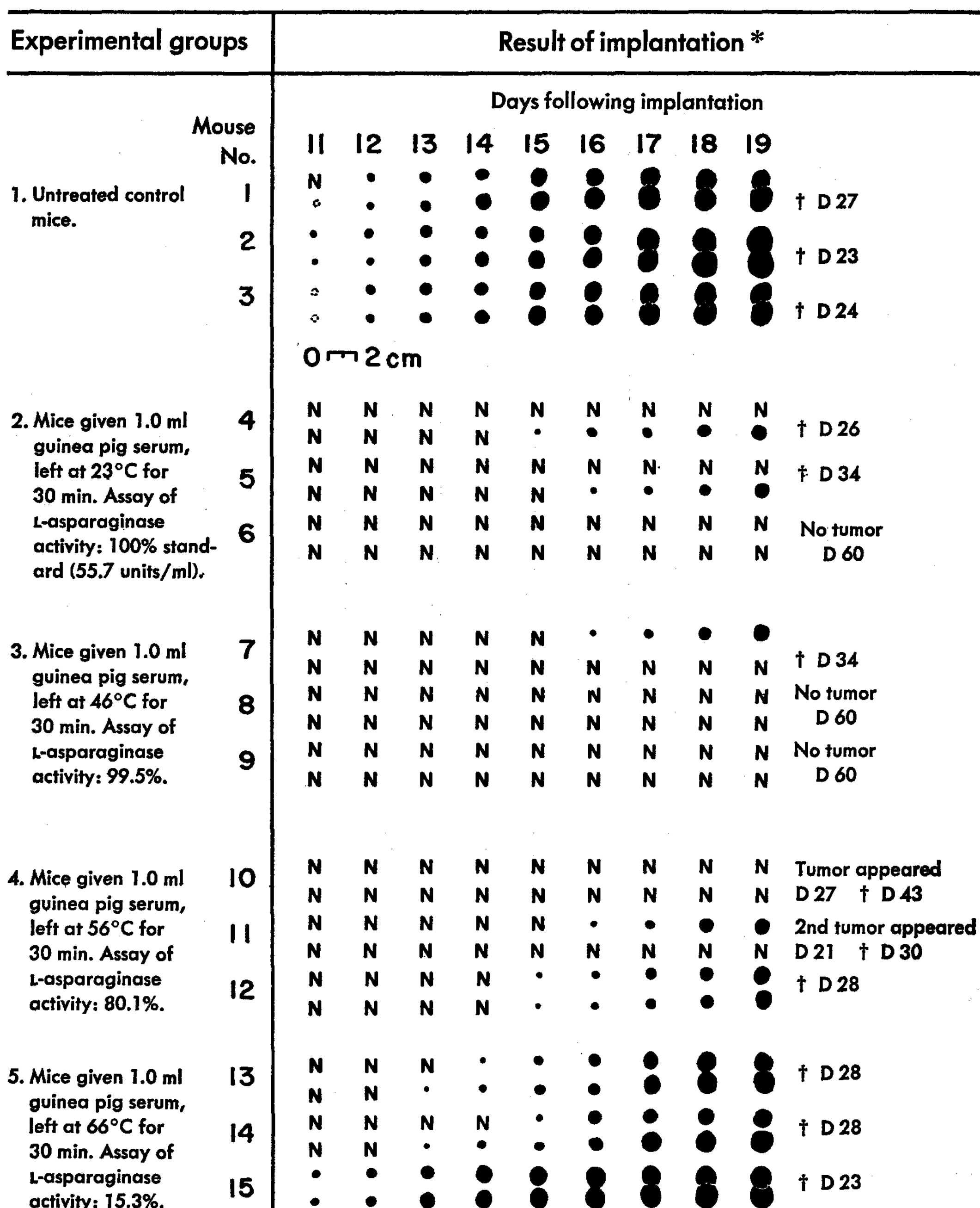

* 3 ZBC mice in each experimental and control group were implanted with 50,000 6C3HED cells subcutaneously in each flank.

FIG. 1. A comparison of the heat stability of L-asparaginase and antilymphoma activities in guinea pig serum.

Portions of a pooled quantity of guinea pig serum were incubated for 30 minutes in water baths at 23°, 46°, 56°, and 66°C. At the end of this time all were transferred to water at 0°C, and samples were immediately taken for assay of L-asparaginase activity. The sera were injected into experimental animals to measure their antilymphoma activity within 30 minutes of the end of incubation.

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Experimental groups	Mouse No.	Result of implantation (Days following implantation 10 11 12 13 14 15 16 17 18 19 20 21 22; scale 0–2.0 cm)
1. Untreated control mice.*	1	† D 27
	2	† D 27
	3	† D 25
2. Mice given 1.0 ml guinea pig serum,‡ left at pH 7.00 for 2 hours. Assay of L-asparaginase activity: 100% standard (62.9 units/ml).	4	† D 29
	5	No tumor D 60
	6	† D 31
3. Mice given 1.0 ml guinea pig serum, left at pH 11.23 for 2 hours. Residual L-asparaginase activity: 62.1%.	7	† D 28
	8	† D 30
	9	† D 29
4. Mice given 1.0 ml guinea pig serum, left at pH 11.96 for 2 hours. Residual L-asparaginase activity: 2.3%.	10	† D 27
	11	† D 26
	12	† D 26
5. Mice given 1.0 ml guinea pig serum, left at pH 5.30 for 2 hours. Residual L-asparaginase activity: 69.6%.	13	† D 28
	14	† D 30
	15	† D 29
6. Mice given 1.0 ml guinea pig serum, left at pH 4.40 for 2 hours. Residual L-asparaginase activity: 8.3%.	16	† D 25
	17	† D 27
	18	† D 25

* In 2 further groups of untreated controls, all tumors appeared between the 10th and 13th day, the average being the 12th day.

‡ In a group of mice given 0.5 ml guinea pig serum, all tumors appeared from the 13th to the 21st day, the average being the 16th day.

FIG. 2. A comparison of the stability at different pH values of L-asparaginase and antilymphoma activities in guinea pig serum. Method of tumor cell implantation as described in Fig. 1.

Varying quantities of N HCl or N NaOH were added from a microburet to 5 ml samples of a pooled quantity of guinea pig serum and these were incubated at 23°C for 2 hours. At the end of this time the acid added to the serum samples was neutralized with an equal quantity of N NaOH or correspondingly the alkali added was neutralized with N HCl. The final volume of each sample was made up to 10 ml by the addition of Ringer's solution. After samples were removed for assay of L-asparaginase, and within 30 minutes of the end of incubation, the sera were injected into experimental animals to measure their antilymphoma activity.

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serum was notably less effective in inhibiting tumor growth than before. Thus, treatment with 1 ml of the preparation of guinea pig serum maintained at pH 7.0 completely suppressed 3 of 6 implanted tumors. The same quantity of guinea pig serum after incubation at pH 11.3 suppressed only 1 of 6 tumors. At pH 11.96 enzyme activity was almost completely destroyed, and the serum produced no significant degree of tumor inhibition. At acid pH values a decrease from neutrality of as little as 1.7 pH units, to pH 5.3, caused a loss of 30.4 per cent of L-asparaginase activity, again accompanied by a significant loss of inhibitory effectiveness on lymphoma growth, all 6 implanted tumors grew out and delayed only 6 to 7 days more than the controls. Further acidifica-

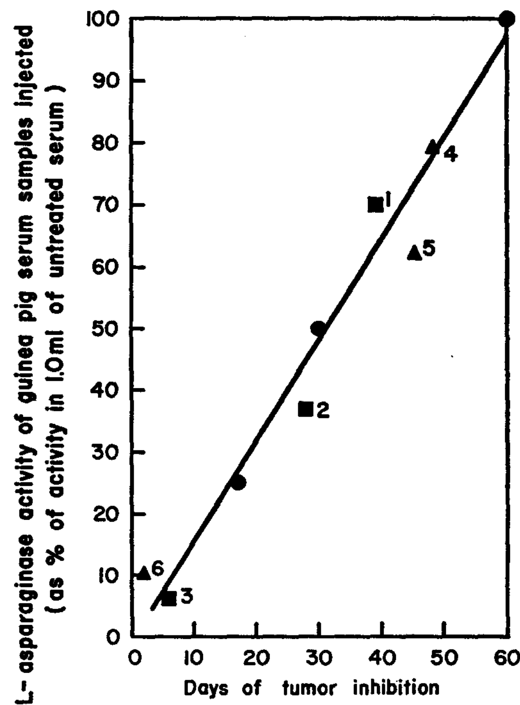

FIG. 3. The growth inhibition of Lymphoma 6C3HED by preparations of guinea pig serum incubated at different pH values, and its relationship to their L-asparaginase activities.

The experimental method used was that described under Fig. 2. The way in which the number of days of tumor inhibition was derived has been described in the section entitled Methods. ● represents the tumor inhibition produced by 1.0, 0.5, and 0.25 ml quantities of guinea pig serum left at 23°C for 2 hours at pH 7.0. ■ represents L-asparaginase activity and tumor inhibition produced by 1.0 ml of guinea pig serum after incubation at the following acid pH values; 1, pH 5.30; 2, 4.70; 3, 4.40. ▲ represents similar findings after incubation at the following alkaline pH values; 4, pH 10.0; 5, 11.23; 6, 11.96. Each point shows the average number of days of tumor inhibition in groups of 3 treated animals.

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tion to pH 4.4 destroyed all but 8.3 per cent of enzyme activity, and the serum then had no demonstrable effect on the tumors. The expression of these results, together with results from further groups of mice treated with guinea pig serum incubated at other pH values, is shown graphically in Fig. 3. Through a wide range of pH variation, antilymphoma activity, measured by "days of tumor inhibition" closely paralleled the enzyme assays of the sera.

These results were confirmed in another experiment performed in a similar way to that shown in Fig. 2, but employing an implantation dose of 500,000 lymphoma cells on each side. Here, although tumors in the controls appeared with a shorter latency than usual, 7 to 8 days, and normal guinea pig serum was less effective in inhibiting tumor growth, the same correspondence was maintained between L-asparaginase activity of guinea pig serum samples incubated at different pH values and their effect on the tumor.

Having thus compared the destruction of antilymphoma and L-asparaginase activities by artificial treatment of whole guinea pig serum, a natural source of guinea pig serum was sought in which one or both activities might be reduced.

Failure of Serum from Newborn Guinea Pigs to Inhibit Tumor Growth.—The serum of the newborn animal of many mammalian species differs considerably in protein composition from that of the adult (10). Now, Ainis *et al.* (11) found that serum from newborn guinea pigs had no inhibitory activity against the Murphy-Sturm sarcoma, a tumor cell line inhibited strongly by serum from adult guinea pigs. On testing lymphoma 6C3HED a closely similar result was obtained as is shown in Fig. 4. 2 ml of adult guinea pig serum completely suppressed all tumor growth in 3 treated animals, but 2 ml of neonatal serum failed to produce any significant degree of tumor inhibition. Assays of L-asparaginase activity of 10 neonatal sera which were pooled to form the material used in the test contained from 0 to 5.1 per cent of the enzyme activity of the adult serum.

The results of the experiments which have been described thus far were therefore consistent with the supposition that in guinea pig serum, L-asparaginase and the antilymphoma substance were identical. Clearly, it was important to attempt to characterize L-asparaginase as a protein, to purify it from guinea pig serum, and to determine whether it did in fact possess antilymphoma activity.

Partial Purification of L-Asparaginase from Guinea Pig Serum.—The experiments to be described form a succession of steps by which L-asparaginase was separated in increasing degree from other proteins in guinea pig serum.

At each of these steps all the fractions separated were tested for antilymphoma activity, to determine not only whether those containing L-asparaginase produced tumor inhibition, but also to determine whether any other protein fraction could produce the same effect.

1. Precipitation of L-Asparaginase by high salt concentration: L-Asparaginase was first concentrated from guinea pig serum by precipitation with other

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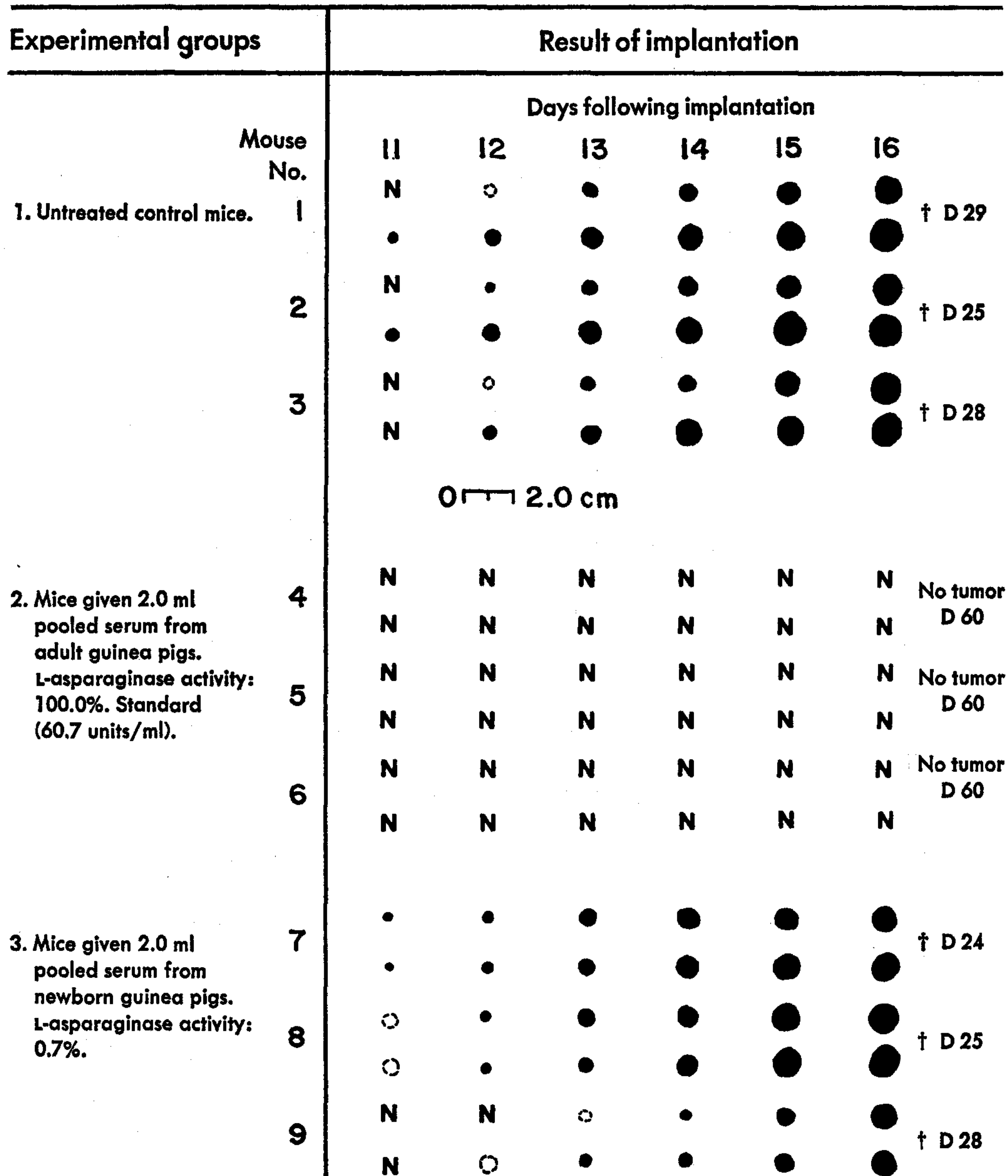

FIG. 4. The failure of serum from newborn guinea pigs to inhibit the growth of Lymphoma 6C3HED. Method of lymphoma cell implantation as in Fig. 1.

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proteins insoluble in 15 gm per cent sodium sulfate, as in the experiment shown in Fig. 5. Precipitate and supernatant were separately dialyzed against buffered 1.0 gm per cent sodium chloride, following which solutions of each were made up in a volume equal to that of the whole guinea pig serum from which they were derived. 68.5 per cent of the recovered L-asparaginase activity was in the precipitated fraction, which contained only 25.5 per cent of the total recovered protein. When the antilymphoma activities of the supernatant and precipitate were assayed they were found to correlate very closely with measurements of the L-asparaginase activities. Thus, in the experiment shown in Fig. 5, 2 ml of the sodium sulfate precipitated fraction with 103.9 units of L-asparaginase activity caused complete suppression of tumor growth in all 3 treated animals; 1 ml of solution from this fraction completely suppressed 4 of 6 implanted tumors. Whole guinea pig serum possessing similar amounts of enzyme activity, produced similar inhibitory effects, 1 ml with 81.0 units suppressed 5 of 6 implanted tumors and 0.5 ml suppressed 4 of 6. The supernatant produced considerably less powerful inhibition but again closely corresponding to the assay of its enzyme activity.

2. Electrophoresis: In a previous publication (13), it was shown that when guinea pig serum was electrophoresed on a starch block in 0.1 M veronal buffer at pH 8.5 by the method of Kunkel and Slater (14), L-asparaginase activity formed a single peak in the α_2-globulin fractions. It was also shown, and has been confirmed using different buffer systems, that inhibition of lymphoma growth was produced only by fractions from the electrophoretic separation which contained L-asparaginase activity and that the degree of inhibition was directly related to this.

To obtain a more highly purified enzyme preparation from electrophoresis than was possible using whole guinea pig serum, a fraction twice precipitated from 15 gm per cent sodium sulfate, as described previously, was used as starting material. It will be seen in Fig. 6 that this consisted mostly of γ-globulin, migrating to the cathode, in a direction opposite to that of L-asparaginase. The enzyme appeared as a single peak, and moving faster than most other components, in a region of relatively low protein concentration. In the most active L-asparaginase–containing fraction (No. 32) the ratio of enzyme activity to protein concentration was increased by a factor of 88 in comparison with that of the original serum. Nevertheless, considerable enzyme activity was lost in the purification process, from the activity possessed by the 100 ml of whole guinea pig serum which formed the starting material, only 23 per cent was recovered from the starch block.

When each of the eluted fractions from the starch block was tested *in vivo* only the fractions containing L-asparaginase produced a detectable degree of tumor inhibition. The quantitative relationship between L-asparaginase and antilymphoma activities of the protein fractions is clearly shown in Fig. 7. The

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Experimental groups	Mouse No.	10	11	12	13	14	15	16	17	18	19	20	21	22	23	
		Days following implantation														
1. Untreated control mice.*	1	○	○	•	•	•	•	•	•	•	•	•	•	•	•	† D 27
		N	•	•	•	•	•	•	•	•	•	•	•	•	•	
	2	•	•	•	•	•	•	•	•	•	•	•	•	•	•	† D 29
		•	•	•	•	•	•	•	•	•	•	•	•	•	•	
	3	•	•	•	•	•	•	•	•	•	•	•	•	•	•	† D 29
		•	•	•	•	•	•	•	•	•	•	•	•	•	•	
		0 ⌐ 2.0 cm														
2. Given 1.0 ml guinea pig serum,‡ L-asparaginase activity: 81.0 units/ml.	4	N	N	N	N	N	N	N	N	N	N	N	N	N	N	No tumor
		N	N	N	N	N	N	N	N	N	N	N	N	N	N	D 60
	5	N	N	N	N	N	N	N	N	N	N	N	N	N	N	No tumor
		N	N	N	N	N	N	N	N	N	N	N	N	N	N	D 60
	6	N	N	N	N	N	N	N	N	N	N	N	•	•	•	† D 43
		N	N	N	N	N	N	N	N	N	N	N	N	N	N	
3. Given 0.5 ml guinea pig serum.§	7	N	N	N	N	N	N	N	N	N	N	N	N	N	N	† D 33
		N	N	N	N	N	N	N	N	N	N	N	N	•	•	
	8	N	N	N	N	N	N	N	•	•	•	•	•	•	•	† D 31
		N	N	N	N	N	N	N	N	•	•	•	•	•	•	
	9	N	N	N	N	N	N	N	N	N	N	N	N	N	N	No tumor
		N	N	N	N	N	N	N	N	N	N	N	N	N	N	D 60
4. Given 2.0 ml fraction 1 (precipitate). (L-asparaginase activity: 63.4% of that in whole guinea pig serum.)	10	N	N	N	N	N	N	N	N	N	N	N	N	N	N	No tumor
		N	N	N	N	N	N	N	N	N	N	N	N	N	N	D 60
	11	N	N	N	N	N	N	N	N	N	N	N	N	N	N	No tumor
		N	N	N	N	N	N	N	N	N	N	N	N	N	N	D 60
	12	N	N	N	N	N	N	N	N	N	N	N	N	N	N	No tumor
		N	N	N	N	N	N	N	N	N	N	N	N	N	N	D 60
5. Given 1.0 ml fraction 1.	13	N	N	N	N	N	N	N	N	N	N	N	N	N	N	† D 34
		N	N	N	N	N	N	N	N	N	N	•	•	•	•	
	14	N	N	N	N	N	N	N	N	N	N	N	N	N	N	No tumor
		N	N	N	N	N	N	N	N	N	N	N	N	N	N	D 60
	15	N	N	N	N	N	N	N	N	N	N	N	N	N	N	† D 36
		N	N	N	N	N	N	N	•	•	•	•	•	•	•	
6. Given 2.0 ml fraction 2 (supernatant). (L-asparaginase activity: 29.2% of that in whole guinea pig serum.)	16	N	N	N	N	N	N	N	•	•	•	•	•	•	•	† D 32
		N	N	N	N	N	N	N	N	N	N	N	N	N	N	
	17	N	N	N	N	N	N	N	N	N	N	N	N	N	N	† D 34
		N	N	N	N	N	N	N	•	•	•	•	•	•	•	
	18	N	N	N	N	N	N	N	N	N	N	N	N	N	N	† D 42
		N	N	N	N	N	N	N	N	N	N	N	N	N	•	
7. Given 1.0 ml fraction 2.	19	N	N	N	N	○	•	•	•	•	•	•	•	•	•	† D 33
		N	N	N	N	•	•	•	•	•	•	•	•	•	•	
	20	N	N	N	N	N	N	N	N	N	•	•	•	•	•	† D 34
		N	N	N	N	•	•	•	•	•	•	•	•	•	•	
	21	N	N	N	•	•	•	•	•	•	•	•	•	•	•	† D 31
		N	N	N	○	•	•	•	•	•	•	•	•	•	•	

(Column heading: Result of implantation)

* In 4 additional groups of untreated controls tumors appeared on the 9th to 11th day.
‡ In a further group of animals given 1 ml of guinea pig serum all tumors were suppressed.
§ In a further group of animals given 0.5 ml of guinea pig serum, 2 mice developed tumors on the 17th to 24th day.

Fig. 5. The effect of preparations of L-asparaginase obtained by sodium sulfate precipitation of guinea pig serum on the growth of Lymphoma 6C3HED *in vivo*.

L-Asparaginase was precipitated from guinea pig serum by the addition of an equal volume of 30 gm per cent sodium sulfate as described by Meister (12), leaving the mixture at 20–25°C for 2 hours. After separation from the supernatant the precipitate was dissolved in distilled water and reprecipitated as before. Protein preparations were dialyzed for a period of 24 hours at 4°C against 0.15 M sodium chloride with 0.01 M phosphate buffer at pH 7.0, before assay for enzyme activity and before injection into experimental animals.

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eluate with the maximum L-asparaginase activity (No. 32, 56.7 units per ml) caused complete tumor suppression in all treated animals. Eluates on either side of this were proportionately less effective.

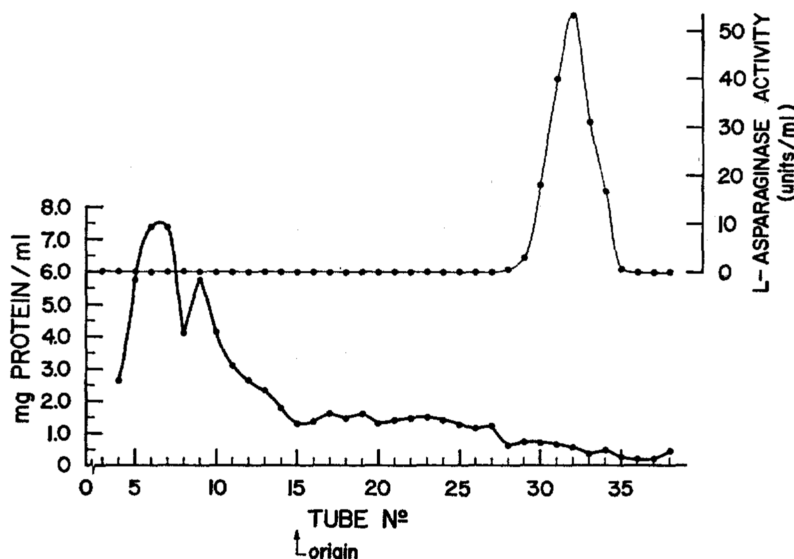

FIG. 6. Purification by electrophoresis of L-asparaginase from other guinea pig serum proteins insoluble in 15 gm per cent sodium sulfate.

100 ml of pooled guinea pig serum was mixed with an equal volume of 30 gm per cent $NaSO_4$ and allowed to stand at 20° to 25°C for 1 hour. The precipitate was recovered by centrifuging, dissolved in 100 ml of distilled water, and reprecipitated. After dissolving this second precipitate in distilled water it was dialyzed against 0.1 M veronal buffer at pH 8.4 and concentrated to a volume of 20 ml in collodion bags (obtained from the Schleicher and Schüll Company, Keene, New Hampshire) to the outside of which suction was applied. The dialyzed protein was electrophoresed by the technique of Kunkel and Slater (16), in a block of potato starch 45 cm in length, 35 cm in breadth, and 1.3 cm depth formed from a slurry in the veronal buffer described. A current of 700 v at approximately 100 ma for 40 hours was used, and the block kept at 2°C. After electrophoresis the block was cut into 1 cm strips from each of which protein was eluted using 7 ml of 0.15 M NaCl. Eluates were dialyzed against 0.15 M NaCl and 0.01 M phosphate buffer at pH 7.3, to remove veronal, following which samples were removed to measure protein concentration and L-asparaginase activity. The eluates were then injected without delay into test animals to assay their tumor inhibitory activity.

From the "chartings" of this and other experiments in which L-asparaginase was purified by electrophoresis, however, it is obvious that such enzyme preparations were consistently less effective in inhibiting tumor growth in relation to their L-asparaginase assays than was whole guinea pig serum. In the present experiment, each of 12 mice, placed in 4 separate groups of 3 at intervals between the rest were treated with 1 ml of whole guinea pig serum containing

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Experimental groups	Mouse No.	9	10	11	12	13	14	15	16	17	18	19	20	21	22	
		Result of implantation — Days following implantation														
1. Untreated controls.*	1	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	‡		† D 24
	2	N / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •			† D 23
	3	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •			† D 26
				0—2.0 cm												
2. Mice given 2.0 ml eluate No. 29 L-asparaginase 6.2 units.	123	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •			† D 25
	124	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •			† D 26
	125	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •			† D 23
3. Mice given 2.0 ml eluate No. 30 L-asparaginase 38.6 units.	126	N / N	N / N	N / o	N / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	† D 24
	127	N / N	N / N	N / N	N / N	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	† D 32
	128	N / N	N / N	N / N	N / •	N / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	† D 33
4. Mice given 2.0 ml eluate No. 31 L-asparaginase 85.5 units.	129	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / •	N / •	N / •	N / •	• / •	• / •	• / •	† D 34
	130	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	No tumor D 60
	131	N / N	N / N	N / N	N / N	N / N	N / N	N / N	• / N	• / N	• / N	• / N	• / N	• / N	• / N	† D 37
5. Mice given 2.0 ml eluate No. 32 L-asparaginase 113.4 units.	132	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	No tumor D 60
	133	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	No tumor D 60
	134	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / N	No tumor D 60
6. Mice given 2.0 ml eluate No. 33 L-asparaginase 66.1 units.	135	N / N	N / N	N / N	N / N	N / N	N / N	N / N	• / •	• / •	• / •	• / •	• / •	• / •	• / •	† D 34
	136	N / N	N / N	N / N	N / N	N / N	N / N	N / •	N / •	N / •	N / •	N / •	N / •	N / •	N / •	† D 35
	137	N / N	N / N	N / N	N / N	N / N	N / N	N / N	N / •	N / •	N / •	N / •	N / •	N / •	N / •	† D 36
7. Mice given 2.0 ml eluate No. 34 L-asparaginase 35.6 units.	138	N / N	N / N	N / N	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	† D 27
	139	N / N	N / N	N / N	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	† D 29
	140	N / N	N / N	N / •	N / •	N / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	† D 28
8. Mice given 2.0 ml eluate No. 35 L-asparaginase 1.5 units.	141	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •				† D 25
	142	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •				† D 26
	143	o / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •	• / •				† D 24

* For the growth pattern of tumors in other controls and in animals treated with varying quantities of whole guinea pig serum, see Experiment 1 of Tables I and II.

‡ Tumors were not examined on the days left blank.

FIG. 7. Inhibition of the growth of 6C3HED tumors by electrophoretically separated fractions of guinea pig serum which possessed L-asparaginase activity. Method of lymphoma cell implantation and treatment of implanted animals was that shown in Fig. 1.

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69.2 L-asparaginase units. No tumor appeared in any of these treated animals. Now, the only fraction from electrophoresis which produced complete suppression of tumor growth in a group of 3 animals contained 113.4 units, 64 per cent more enzyme activity than in 1 ml of whole guinea pig serum. A similarly decreased effectiveness of purified L-asparaginase preparations in inhibiting tumor growth, in relation to their enzyme activity, was found in extracts containing smaller amounts of the enzyme.

A further experiment performed in a similar way but using for electrophoresis veronal buffer at pH 8.6, provided results which confirmed all the significant findings in the previous experiment. 2 ml injections of the eluate having maximum L-asparaginase activity (65.7 units/ml) suppressed 5 of 6 implanted tumors. But 1 ml of whole guinea pig serum, containing 82.9 units/ml, suppressed 16 of 18 implanted tumors in 9 animals. The degree of tumor inhibition brought about in these 2 cases may be considered to be approximately equal. It was necessary therefore to treat mice with purified preparations containing 59 per cent more L-asparaginase activity than was present in amounts of whole guinea pig serum which brought about a similar result. The possible significance of this finding will be discussed later.

3. Chromatography on DEAE cellulose: To bring about further purification of L-asparaginase a three stage process was used. In this, enzyme was precipitated from guinea pig serum with proteins insoluble in 15 gm per cent sodium sulfate; as in the previous section these were subjected to electrophoresis on the starch block, and eluates containing the maximum L-asparaginase activity were now pooled and absorbed on a column of DEAE cellulose. Enzyme was eluted from the column by the schedule shown in the Fig. 8. Of numerous elution schedules which were tried, this was the most satisfactory for the specific requirements of the experiment, namely to produce the highest degree of purification consistent with obtaining the eluted materials in sufficient concentration to allow testing for their antilymphoma activity.

In the present experiment the total yield of L-asparaginase activity in the eluates represented only 12 per cent of that in the 200 ml guinea pig serum used as starting material. This result was somewhat disappointing for the maximum ratio of L-asparaginase activity to protein concentration was thus raised very little compared with that of the material obtained simply by electrophoresis; it now reached a factor of 118 compared with the original whole guinea pig serum. Attempts to further purify L-asparaginase were unsuccessful owing to the considerable instability of the partially purified enzyme. L-asparagine, glutathione, L-cysteine, or versene when added to enzyme solutions brought about no notable stabilizing effect.

L-Asparaginase appeared in 2 peaks, which in 2 other experiments have been separated considerably more widely. Most of the enzyme was eluted before the peak in protein concentration.

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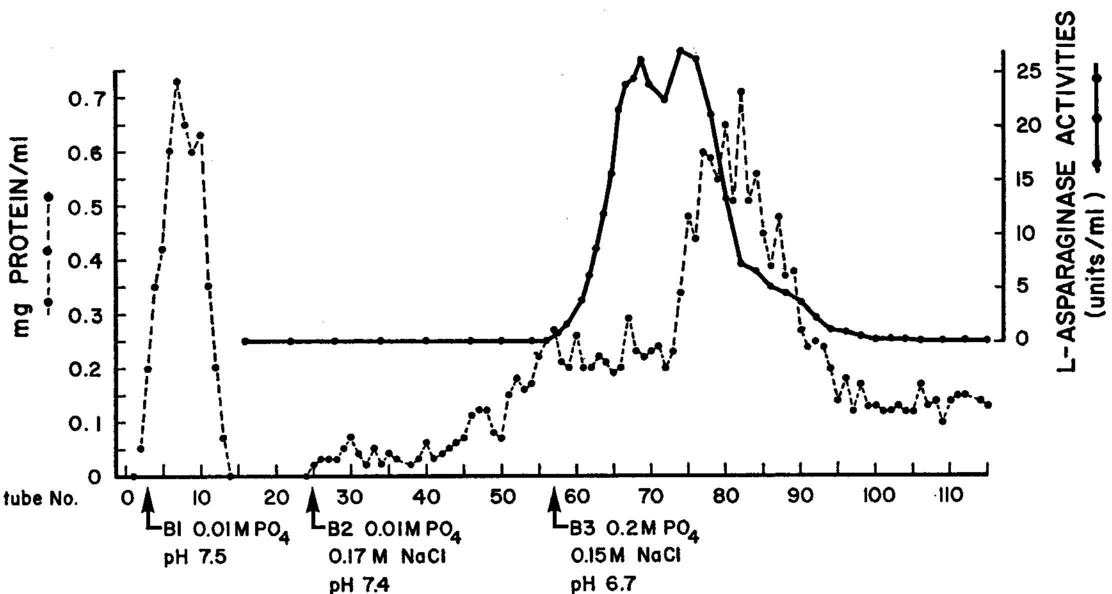

Fig. 8. Further purification of L-asparaginase by chromatography on DEAE cellulose.

200 ml of guinea pig serum was used as starting material for the 3 stage purification of L-asparaginase. Double precipitation of protein in 15 per cent sodium sulfate and starch block electrophoresis of the concentrate in 0.1 M veronal buffer at pH 8.4 were carried out as in the previous experiment. Strips of the starch block were eluted in distilled water and 4 eluates containing the maximum L-asparaginase activity were pooled, making a volume of 55 ml which was slowly run onto a column of DEAE cellulose (0.9 $\pm$ 0.1 mEq/gm, Mann Research Labs, New York) 12 cm in length and 2.5 cm in diameter. Approximately 350 ml of starting buffer (0.01 M sodiumphosphate at pH 7.5) was run through the column. Gradient elution was then begun. The starting buffer was contained in a 150 ml flask into which 0.01 M sodium phosphate with 0.17 M sodium chloride (pH 7.4) was mixed. After 200 ml of the second buffer had been added and as preliminary experiments showed the elution of L-asparaginase was beginning, it was replaced by 0.2 M sodium phosphate and 0.15 M sodium chloride (pH 6.7) and gradient elution was continued. All procedures were carried out in a cold room at 2–4°C. 7 ml fractions of fluid emerging from the chromatographic column were collected and samples were removed from these for the measurement of protein concentration and L-asparaginase activities. The fractions were then injected into mice for assay of their tumor inhibitory activity.

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Eluates were tested for their antilymphoma activity as before, but here to obtain an effective assay it was necessary to give each animal 2 injections of 2 ml at intervals 6 hours apart. Such is the extraordinary resilience of mice that

Mice "cured" *												1	1	1																
Tumors completely suppressed											1	3	4	4	3	1														
Average latent period of tumors (days)	9		9		9		9		12		17	16	18	18	14	15	16	12	12	10	9	9	9	9	9	9	9	9		9
Maximum and minimum latent periods of tumors (days)	9		9		9		9 \ 11		10 \ 15		16 \ 19	16	16 \ 19	17 \ 19	14	15 \ 16	15 \ 18	11 \ 12	9 \ 12	9 \ 11	9	9	9 \ 10	9	9 \ 11	9	9	9		9

* Animals healthy and without evidence of tumor growth 49 days after implantation.

FIG. 9. The antilymphoma activity of L-asparaginase fractions separated by chromatography on DEAE cellulose.

3 ZBC mice in each experimental group were implanted with cells of Lymphoma 6C3HED in the standard way described in previous experiments. For the outcome of implantations in the groups which served as untreated control mice and in those given 0.25 to 2.0 ml quantities of whole guinea pig serum immediately following implantation, see Experiment 3 of Tables I and II.

In the present experiment, to assay tumor inhibitory activity of the eluates from the chromatographic column, 2 injections each of 2 ml of eluates were given intraperitoneally with an interval of 6 hours between injections, as described in the text.

these large volumes of fluid produced no apparently harmful effects; treated mice remained sleek and active and 18 hours later their weights were not significantly different from those before the experiment. No change in the effectiveness of the graded quantities of guinea pig serum used as a standard for tumor inhibitory activity was observed when these were administered, diluted in Ringer's solution in the same volume as the eluates.

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The results of measurements of antilymphoma activity are shown in Fig. 9. As in previous experiments, only those fractions possessing L-asparaginase activity produced tumor inhibition, but, in contrast to the previous findings in which a single peak of enzyme activity occurred and in which tumor inhibition was proportional to the enzyme assays, here tumor inhibition was notably more powerful in fractions from the first enzyme peak than from the second.

A definite degree of tumor inhibition was produced by fractions 61 and 62 which provided 19.3 u. of L-asparaginase activity to each mouse, and tumor inhibition increased proportionately with enzyme assays to the height of the first peak; fractions 68 and 69, providing 101.9 units, produced 63 days of tumor inhibition, measured in the way already described, and "cured" 1 out of 3 mice. Similarly fractions 70 and 71, providing 93.6 L-asparaginase units to each mouse (and just past the enzyme peak), produced 65 days of tumor inhibition and cured 1 of 3 mice. With later eluates, although enzyme activity rose again in a second peak, there was a steady decline in the lymphoma inhibition they produced and even at the height of the second peak, fractions 74 and 75, providing 105.5 units of enzyme per mouse and more than any other fraction, produced only 42 days of tumor inhibition and cured none of the implanted animals. The difference between tumor inhibitory activity and enzyme activity became more marked with the decline from the second peak. These findings are subject to a number of possible interpretations which will be discussed later.

DISCUSSION

The observations of others that guinea pig serum is unique amongst numerous animal sera in producing inhibition of the growth of certain lymphomas *in vivo* (1, 2) and also in containing L-asparaginase (6) led to the experiments here described.[2] The results provide substantial indication that this enzyme is responsible for lymphoma inhibition. Within the limits of the experimental methods used, the stability of the antilymphoma substance at different temperatures is indistinguishable from that of L-asparaginase; so too, the stability at different pH values. Furthermore, guinea pig serum from newborn animals, which possessed no L-asparaginase activity, produced no inhibition of tumor growth. In purification of L-asparaginase both by salt precipitation and electrophoresis the degree of tumor inhibition brought about by different fractions was directly related to their enzyme activity.

The fact that antilymphoma activity of L-asparaginase preparations purified in 2 stages by salt precipitation and electrophoresis was persistently about ⅓

[2] Old, L. J., Boyse, E. A., Campbell, H. A., and Daria, G. M., (Nature, 1963, in press) have recently found that animals of 4 further species of the superfamily Cavioidea, to which the guinea pig belongs, have L-asparaginase activity in their sera, in this respect differing from animals of other rodent families. Furthermore, in all sera tested a close correlation was found between assays of L-asparaginase and antilymphoma activities.

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less than that produced by amounts of whole guinea pig serum of similar enzyme assay requires some consideration. A number of experiments showed this was not due to the greater volumes or lower total protein concentrations of the purified enzyme preparations. Other unreported experiments showed that it was not due to impurities introduced from the starch block or dialyzing apparatus which might have had an effect preventing tumor inhibition. It remains possible that L-asparaginase was split during purification from a cofactor enhancing its antilymphoma activity, or more particularly, responsible for maintaining its stability. The instability of L-asparaginase is very marked when it is purified to any extent, as has been abundantly shown in the present experiments, and it is therefore quite likely that inactivation of purified enzyme would continue in treated animals at a faster rate than when the enzyme was given in whole guinea pig serum.

The 2 peaks of L-asparaginase activity found in the present experiments after separating guinea pig serum proteins by chromatography on DEAE cellulose, and by Wriston (15) in other experiments using whole serum, may indicate the existence of 2 L-asparaginase isozymes. But this is not necessarily so; it is possible, particularly in view of the instability of the enzyme, that one or both forms were produced as artefacts during the purification procedure; such changes have been observed with a number of other purified proteins (see, for instance, Lindskog, reference 16, and Kaplan and Grumbach, reference 17).

The difference in the present experiments, between the antilymphoma activities of materials from the 2 peaks may have various causes. Thus, the 2 forms of L-asparaginase may have inherently different capacities for tumor inhibition. But alternatively the present observations may be due to a difference in stability *in vitro* of the 2 enzyme forms. The effect of such a difference would be accentuated by the experimental technique used, for all protein fractions were given to treated mice in 2 injections 6 hours apart.

That enzymes might have considerable potentiality as tumor inhibitory agents was recognized by Bergel and discussed by him in a recent monograph (18). Earlier, Haddow, Bergel, and their associates treated mice bearing spontaneous mammary tumors with xanthine oxidase, and produced a measurable inhibition of tumor growth (19, 20). Ledoux subsequently showed that preparations of ribonuclease when injected intraperitoneally brought about inhibition of a number of different lines of mouse ascites tumors (21). More significantly, detectable inhibition was also produced in the growth of solid tumors of several cell lines (22). de Lamirande found that desoxyribonuclease could produce *in vivo* some degree of inhibition of the growth of the Ehrlich ascites tumor (23). In the experiments mentioned it was necessary, however, to treat animals with large quantities of crystalline enzyme to produce any detectable degree of tumor inhibition. If L-asparaginase is in fact the tumor inhibitory agent of guinea pig serum, then its inhibitory activity on tumor cells of sensitive lines is

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very much more powerful than that produced by the other enzymes tested thus far. The considerable potency of L-asparaginase may be related to its ability to act on a specific abnormality in the metabolism of sensitive tumor cells. This will be considered in the succeeding paper.

SUMMARY

A number of the properties of the L-asparaginase present in guinea pig serum have been examined and shown to be indistinguishable from those of the agent responsible for inhibiting cells of lymphoma 6C3HED *in vivo*.

The patterns of instability of the enzyme to changes in temperature and pH were found to parallel closely those of the antilymphoma agent. L-Asparaginase activity was essentially absent from the serum of newborn guinea pigs and this failed to inhibit 6C3HED cells. On separating guinea pig serum proteins by salt precipitation, electrophoresis, and chromatography on DEAE cellulose, antilymphoma activity was found only in fractions which contained L-asparaginase.

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