CHEMICAL AND IMMUNOLOGICAL STUDIES ON THE AGENT PRODUCING LEUKOSIS AND SARCOMA OF FOWLS*

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Since the demonstration that fowl leukosis and sarcoma can be transmitted by cell-free filtrates (1, 2), many investigators have attempted to concentrate the causative agents and study their chemical properties. The earlier studies have been reviewed by Claude and Murphy (3). Ledingham and Gye (4), and Claude (5) have demonstrated that the tumor-producing substance present in filtrates from tumor extract can be sedimented by centrifugation at approximately 15,000 R.P.M. thus effecting a considerable purification of the active substance. Similar observations were made for the agent of fowl leukosis by Dr. J. H. Bauer who examined material from this laboratory in 1936, using leukotic plasma as the source of virus.

These studies are hindered by difficulties of obtaining large quantities of the agent, lability of the partially purified agent, and by the presence in normal tissues of large quantities of material sedimentable at high speed. Accordingly experiments were undertaken to increase the yield of agent, study its stability, and attempt to distinguish it by immunological means from normal material sedimentable at high speed.

There are three main sources of leukosis virus: (*a*) blood plasma or serum, (*b*) tissues with leukotic infiltration, and (*c*) sarcoma produced by an agent obtained from leukotic tissue. Plasma yields only small amounts of virus; leukotic tissue extracts contain large amounts of normal material sedimentable at high speed. As the crude extracts of sarcoma are usually very viscous, it is necessary to use smaller quantities of tumor or larger volumes. A polysaccharide has recently been shown to be responsible for the viscosity of these tumor extracts (6). It has been found to be similar if not identical with those isolated from human umbilical cord, vitreous humor, synovial fluid, and the mucoid hemolytic streptococcus (6). By the use of an enzyme

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(7) it has been possible to reduce the viscosity of the tumor extracts without affecting tumor-producing activity.

The activity of the agent in the various preparations studied was tested by inoculation into chicks in varying amounts. This was correlated with the N content of the crude preparation and with its fraction sedimentable at high speed.

Material and Methods

Strain 13 (8), which produces both sarcoma and erythroleukosis, and strain 1 (9), which produces erythroleukosis and myeloid leukemia, were used. The former is advantageous because one chicken can be injected at several sites thus enabling comparison of several different solutions in the same bird, though one occasionally succumbs to erythroleukosis without developing tumors at the site of injection. Experiments with leukosis strain 1 require several chicks for each dilution to be tested.

Barred Rock baby chicks from 2 to 20 days old were used except when noted. Intramuscular injections of the preparation were made in volume of 0.2 cc. and intravenous injections in volume of 0.25 cc. Tumors produced by strain 13 could be palpated from 15 to 25 days after injection and strain 1 produced leukemia after 3 to 6 weeks. Animals which had neither sarcoma nor leukosis were killed and autopsied 2 months after injection with sarcoma 13 and 3 months after injection with strain 1.

The successful inoculations are given as fractions of the total number of inoculations. When tumor extracts were tested, crude extracts and centrifuged materials were injected into the same bird. The values obtained are comparable, though it is possible that somewhat higher dilutions would have produced tumor.

Tumor-bearing fowls were killed and the tumor cut out under sterile conditions, wrapped in parchment or sealed in test tubes, and kept in a thermos flask containing solid carbon dioxide. Tumors kept under these conditions showed no loss of activity after several years. However, when all solid carbon dioxide was allowed to evaporate and the tumors were partially thawed for some time, the agent deteriorated.

A weighed amount of frozen tumor was ground with sand in a mortar that was chilled by immersion in a pan of ice water, and saline was added in small quantities until a uniform suspension of the desired volume was obtained. In most cases the saline was buffered at pH 7 by adding phosphate to a final concentration of 0.005 M PO_4. In several experiments approximately 0.5 mg. of enzyme preparation (6) was added to the extract which was warmed to 37°C. for 15 minutes. This caused an evident reduction in viscosity. To remove sand and large particles the material was chilled and centrifuged at 4000 R.P.M. for about one-half hour in the ice box in an angle centrifuge. The solution was then centrifuged for 15 minutes at 8000 R.P.M. in an air-driven vacuum ultracentifuge (10), the head of which was kept in the ice box when not in use. The clear supernatant was decanted and centrifuged at 27,000 R.P.M. for one hour; this was found to be sufficient to sediment almost all of the active material. The liquid in the tubes was then carefully decanted, and the tubes were allowed to drain and their mouths wiped with filter paper. A small gelatinous disc remained at the bottom. The adherent liquid was removed by washing the disc carefully twice with several milliliters of saline and decanting as before. The discs were then broken up with a stirring rod and small quantities of saline added until solution took place. It was seldom possible to redissolve

the material completely. The contents of the tubes were transferred to one tube, the insoluble matter was centrifuged off in the ice box, and washed with saline. The supernatant and washings were made up to a known volume and an aliquot portion was analyzed for nitrogen by the micro Kjeldahl method. The washed insoluble residue and samples of the extracts before centrifugation were analyzed for nitrogen. Solutions of

TABLE I

Activity of Material Sedimentable at High Speed from Tumor Extracts, Strain 13

Preparation No.	Amount of tumor	Extract		High speed sedimentable fraction				Activity expressed in high speed sedimentable N	
		Volume	Total N	Total N	Per cent in extract	Amount soluble in saline	Per cent soluble	Crude extract	High speed deposit (soluble fraction)
	gm.	*ml.*	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*mg.*
7422	15 *ca.*	112	60.5	0.80	1.3	—	—	—	6.5×10^{-5}
7516 I	12	104	40.2	1.76	4.4	1.06	60	1.4×10^{-5}	1.4×10^{-4}
7516 II	20	114	89.0	2.92	3.3	—	—	—	—
7423 I	12	100	72.8	0.73	1.0	—	—	6×10^{-6}	—
7560	12	95	61.0	4.22	6.9	3.90	—	—	3×10^{-4}
7423 II	12	1st 108	70.0	1.70	2.4	1.36	80	6.3×10^{-6}	3×10^{-5}
		2nd 140	25.8	1.75	3.6*	1.58	89	5×10^{-4}	2.4×10^{-5}
8072	11.3	1st† 52	49.0	1.35	2.8	0.90	67	2.2×10^{-5}	1.4×10^{-5}
	11.3	1st 52	49.0	1.45	2.9	1.00	69	2.2×10^{-5}	1.4×10^{-5}
8082	91	1st† 92	244.0	1.83	0.75	1.60	87	7.6×10^{-5}	7×10^{-5}
		2nd 97	78.5	5.52	2.3*	5.00	91	1×10^{-5}	—
8435	102	1st† 107	330.0	1.80	0.55	1.66	92	(1.7×10^{-4} neg.)	(6×10^{-4} neg.)
		2nd 42	35.0	2.16	—	2.02	93	2.0×10^{-6}	2×10^{-3} (8×10^{-5} neg.)
		2nd‡ 40	46.4	4.58	—	3.92	86	4×10^{-6}	1.6×10^{-4}
		3rd‡ 97	64.0	2.34	2.3*	—	—	9×10^{-6}	3.5×10^{-4}

* Total percentage of high speed sedimentable material after corresponding number of extractions.

† Enzyme-treated.

‡ These extracts were made with saline containing 5 per cent saturated Na_2SO_4 solution. The residue from the first extract was divided in half and the second extraction made in two parts. The residues from the second extractions were recombined for the third extraction.

the crude extracts and high speed deposits were injected into the breast and leg muscles of chicks. In this manner it was possible to determine the amount of nitrogen centrifugable at high speed, the amount of sediment which redissolved, and the comparative activity of the original extract.

The antigens used in precipitation and complement fixation tests were standardized on the basis of the amount of N they contained. In complement fixation tests 0.2 ml.

of serum and of antigen and about 2 units of complement were mixed. The complement was titrated against the antigen. The material was incubated for one hour at 37°C. and for an additional hour at room temperature and then 0.2 ml. of a 5 per cent suspension of sensitized sheep cells was added. In the precipitation test 0.15 ml. of serum was added to the same quantity of antigen dilution, incubated for 2 hours at 37°C., and placed in the ice box overnight. Direct readings are given. A second reading made after centrifugation at low speed yielded similar results.

Findings

Relation of Activity to Amount of High Speed Sedimentable Material.—The results obtained in nine experiments are shown in Table I. Activity of both crude extract and the partially purified material is expressed in terms of the smallest quantity of nitrogen centrifugable at high speed that produced a tumor. The table also shows the results of repeated extraction of tumors. From 1 to 7 per cent of the N present in crude extracts could be sedimented at 27,000 R.P.M. The first extract usually contained a smaller amount of material centrifugable at high speed than the second or third extracts, especially when large amounts of tumor were used. In the last experiment of Table I the first extract had little if any activity although the second extract was highly active. The second extract obtained using saline containing 5 per cent sodium sulfate had a higher activity and more material sedimentable at high speed than that extracted with saline alone.

Treatment of crude tumor extracts with approximately 0.5 mg. of enzyme at 37°C., resulting in a conspicuous reduction of the viscosity of these extracts, had no effect on the tumor-producing activity as the following data of four experiments indicate.

Dilution of extract	Enzyme-treated	Untreated
1:250	5/6	3/6
1:5	5/6	5/6
1:250	1/5	2/5
1:5	4/5	4/5
1:500	4/5	3/5
1:20	4/5	4/5
1:250	2/6	5/6
1:5	5/6	5/6

The successful inoculations are expressed as a fraction of the total number of injections.

Quantity of Heavy Material in Blood Plasma.—Data of centrifugation experiments with leukemic serum or plasma and normal sera are summarized in Table II. The total amount of material sedimentable at high speed was generally much less in normal extracts than in tumor extracts.

TABLE II

Amounts of Material Sedimentable at High Speed in Normal and in Leukotic Serum or Plasma

No. of serum (S) or plasma (P)	Volume centrifuged	Total N centrifuged	High speed deposit			Original activity of serum or plasma	Intensity of blood changes
			Total N	Per cent N in serum or plasma	N in 10 ml.		
	ml.	*mg.*	*mg.*	*per cent*	*mg.*	*mg. N*	
				Strain 1			
7537 P	19	90 *ca.*	0.42	0.5	0.22		±E
7677, 7797, 7703 S	41	250	2.9*	1.2	0.73	1.8×10^{-3}	+, ++, +E
8060 P	19	80	2.7	3.4	1.42		+++E
8062 P	13	89	1.4	1.6	1.08		++M, ±E
8146 P	7	29.5	0.37	1.2	0.53		±E
8144 P	6	15.6	0.20	1.3	0.33		+±E
8148 P	9	37	0.48	1.3	0.53	2.6×10^{-5}	+++E
8059 S	18	59	0.79	1.5	0.49		++±M, +E
8239 P	22	109.5	1.63	1.5	0.74		++±M, ++E
8320 P	14	73	0.36	0.5	0.26		++±E
Average					0.633		
				Strain 13			
				Chickens with both sarcoma and leukosis			
8086 P	10	47.8	0.48	1.0	0.48		+E
7992 P	7.5	42.8	0.56	1.3	0.75		+E
7573, 7689 P	27.5	140 *ca.*	0.91*	0.6	0.33	1.3×10^{-5}	++E ++E
7675, 7692 P	9	45 *ca.*	0.47	1.0	0.52	2×10^{-3}	+E +E
7569, 7593 S	17	90 *ca.*	0.51	0.6	0.30	1.2×10^{-3}	+±E ++±E
Average					0.476		
				Chickens with sarcoma only			
8106 S	5.5	29.6	0.15	0.5	0.27		Slight anemia
8155 S	4.0	19.6	0.09	0.5	0.23		Anemia
				Chicken injected with strain 13 but free from leukosis and sarcoma			
8308 P	13.0	73.3	0.92	1.3	0.71		—
				Normal chickens			
8173 S	7	37	0.07	0.2	0.10		
8246 P	24	159	0.55	0.3	0.23		
Pooled P	14		0.30		0.21		
Pooled S	14		0.34		0.22		
8345 S	7	32.2	0.06	0.2	0.09		
8349 I S	14	79	0.84	1.1	0.60		
8349 II† S	6	42.1	0.17	0.4	0.29		
8352 I P	10.5	59	0.43	0.7	0.41		
8352 II‡ S	12	76	1.28	1.7	1.07		
8352 III‡ S	9	65.4	0.17	0.3	0.19		
Average					0.34		

* 2.5 mg. of the 2.9 mg. of deposit and 0.79 mg. of the 0.91 mg. of deposit were soluble in saline.

† Bled 19 days later.

‡ Second and third bleeding 20 days and 39 days after the first.

The technique is essentially that described for tumor extracts. All sera were given a preliminary centrifugation at 8000 R.P.M. for 15 minutes. The relative severity of erythroleukosis (E) or myeloid leukemia (M) as indicated by blood smears immediately before bleeding is tabulated in the last column of the table.

An average of 0.34 mg. of N sedimentable at high speed per 10 ml. of serum was found in normal chicken sera. The difference noted for three specimens of serum of the same chicken obtained at different times indicates that there is considerable variation in the amount of heavy material present in sera of an apparently healthy animal. The average amount of heavy material per 10 ml. of leukotic serum or plasma of strain 1 was almost twice as high as the average for normal sera, while the average for erythroleukotic sera produced by strain 13 showed only slightly more heavy material than did normal sera.

High speed centrifugation of human plasma or serum made under similar conditions gave the following values.

Plasma or serum	Number of samples	N per 10 ml.		
		Maximum	Minimum	Average
		mg.	*mg.*	*mg.*
Leukemic	4	0.05	0.00	0.02
Hodgkin	1	—	—	0.01
Normal	3	0.07	0.00	0.03

Heavy Material in Virus-Free Tissues.—In order to evaluate the significance of heavy material in tumors, experiments were undertaken to determine the amounts of material sedimentable at high speed in normal chicken spleens and in different leukemic human and mouse tissues that have not been shown to contain a filterable transmitting agent (Table III). These materials were found to contain much larger quantities of heavy substances than the best tumor preparations. Thus the mere presence of large amounts of heavy materials in tumors is no indication of its relation to the virus, though the size of both is of the same order of magnitude.

Relation of Cells to Virus.—In order to learn about the association of virus with blood cells, experiments were carried out with both strain 1 and strain 13 on the effect of washing leukemic cells with saline. It will be noted (Table IV) that both plasma and cell residue are much more active than the washings in producing disease. The second washings contain only very small amounts of virus, indicating that the virus is firmly bound to blood cells as Pentimalli (11) found. Irradiation of washed cells with 15,000 r did not significantly reduce the ability of the washed cells to produce leukosis.

TABLE III

Amounts of Material Sedimentable at High Speed in Different Tissues

Source	Material centrifuged: Amount	Volume	Total N	High speed sediment	
	gm.	*ml.*	*mg.*	*mg. N*	*per cent*
Chicken: Normal spleen	26	84	250	14.1*	5.7*
" " "	64	112	450	25*	5.6*
Man: Leukemic marrow	14	77	154	17.8	11.6
" " spleen	16	91	193	12.9	6.7
Mouse: Normal spleen	2.3	19	27	3.2	12
" Leukemic spleen	4	28	42	3.6	8.5
" " liver	13	42	142	21.6	15.2
" " tumor	4	28	49.3	8.1	16.4

* Part soluble in saline. The residue was not analyzed.

TABLE IV

Relative Activity of Plasma, Cell Washings, and Washed Cells

Material	Strain 1: Activity, Dilution 1:500	Strain 1: Activity, Dilution 1:5	Strain 1: Amount of high speed deposit	Strain 13: Activity, Dilution 1:250	Strain 13: Activity, Dilution 1:5	Strain 13: Amount of high speed deposit
			mg. N			*mg. N*
Plasma	3/5	3/4	0.48	1/5	3/5	0.56
Washings						
First	3/5	2/4	0.00	1/5	1/5	0.02
Second	0/4	—	0.00	0/5	1/5	0.01
Washed cells	4/5	3/3	—	1/5	4/5	—
				Dilution 1:500	Dilution 1:5	
Plasma	3/5	4/4	Large disc	0/4	4/4	0.47
Washings						
First	1/5	1/2	Very small disc	0/4	3/4	0.02
Second	0/4	2/3	Trace	0/6	1/6	0.01
Spleen extract	2/3	2/3	Large disc	—	—	—
Liver extract	0/5	0/3	—	—	—	—

Stability of Agent.—The stability of crude tumor extracts and of solutions of high speed sediment were studied under various conditions. Previous workers found that reducing agents had a stabilizing effect on the activity of Rous filtrates (*cf.* 12) and could reactivate partially inactivated solutions.

It is seen from the first two experiments in Table V that under the conditions studied neither cysteine-cobalt (12) nor sodium hydrosulfite (1:1000) increased the stability of the tumor extracts. Crude extracts showed but little loss of activity after one month in the ice box, but solutions of high speed deposits deteriorated rapidly. Freezing in solid CO_2 at $-60°C.$

TABLE V

Survival of Agent in the Ice Box and at −60°C. with and without Reducing Substances

Material	Dilution in saline		Dilution in saline + cysteine-cobalt	
	1:100	1:1	1:100	1:1
Crude extract (No. 7421)				
Immediate	2/4	2/4	1/4	2/4
Ice box, 1 wk.	1/3	1/3	1/3	1/3
Ice box, 2 wks.	1/3	2/3	1/3	2/3
High speed sediment				
Immediate	2/4	4/4	3/4	2/4
Ice box, 1 wk.	0/3	0/3	0/3	1/3
Ice box, 2 wks.	0/3	0/3	0/3	0/3
			Dilution in saline + sodium hydrosulfite 1:1000	
Crude extract (No. 7423)				
Immediate	0/6	4/6		
Ice box, 14 hrs.			0/2	2/2
37°C., 2 hrs.	0/2	2/2		
Ice box, 14 days	2/4	4/4	0/6	1/6
−60°C., 14 days	3/6	4/6		
−60°C., 3 mos.	0/8†	5/8		
	Dilution in saline		Dilution in saline	
	1:250	1:5	1:250	1:5
Crude extract	2/4	2/4		
High speed deposit	Portion soluble		Portion insoluble in saline	
Immediate*	0/3	1/3	0/3	0/3
−60°C., 4 days	3/6	6/6	2/6	6/6
−60°C., 3 mos.†	1/8	1/8	0/8	3/8
−60°C., 6 mos.	4/10	8/10		

* Many animals in this series died with intercurrent disease.

† White Leghorn chicks used instead of Barred Rocks.

preserved both for periods as long as 6 months. After addition of merthiolate, crude extract was found to be inactive after one month in the ice box in dilution 1:5, whereas the same extract without merthiolate was active in dilution 1:500. High speed deposits kept at $-60°C.$ for 36 days also became inactive if merthiolate was present.

Experiments were also carried out to find a substance which would prevent the deterioration of the high speed deposits. The results were as follows:

Solution added to saline extract	Ice box 7 days Dilution		Ice box 29 days Dilution	
	1:250	1:10	1:250	1:10
0	3/4	3/4	0/4	2/4
5 per cent saturated Na_2SO_4	4/4	4/4	2/4	2/4
30 per cent saturated Na_2SO_4	4/4	4/4	1/4	1/4
5 per cent saturated $MgSO_4$	4/4	4/4	2/4	3/4
30 per cent saturated $MgSO_4$	3/4	3/4	0/4	3/4
High speed supernatant	4/4	4/4	1/4	1/4
Tumor polysaccharide 1:2000	4/4	4/4	0/4	1/4
Gum acacia 1:200	3/4	4/4	0/4	2/4
Original activity	6/8	6/8		

After 66 days in the ice box all activity had disappeared.

These data show that the addition of 5 per cent of saturated Na_2SO_4 or of $MgSO_4$ solution slightly delayed deterioration. In another experiment that will not be fully described the untreated extract became entirely inactive after 49 days in the ice box but the extract containing added 5 per cent Na_2SO_4 retained some activity. Neither gum acacia, tumor polysaccharide (6), nor the supernatant from high speed centrifugation of a crude tumor extract had any preservative effect on the agent.

Concentration of Agent by Sodium Sulfate Precipitation.—Most of the tumor-producing activity was precipitated by one-third saturation with sodium sulfate (Table VI). This precipitate contained about 12 per cent of the nitrogen of the crude tumor extract but almost all of the material centrifugable at high speed. Nevertheless, only about 75 per cent of the saturated Na_2SO_4 precipitate could be redissolved. A small amount of material centrifugable at high speed was found in the fraction precipitated between one-third and one-half saturation with sodium sulfate but the supernatant was free from material centrifugable at high speed. Parallel phosphorus and nitrogen analyses showed that the N:P ratios in the insoluble fraction of the Na_2SO_4 precipitate were approximately the same as that of two fractions obtained by high speed centrifugation of the soluble portion of the Na_2SO_4 precipitate (Table VI).

The activity value for crude tumor extract (Table VI) is calculated from the total amount of high speed sediment of fraction A and the values shown represent the highest dilution tested; end values were not obtained. The

figures in parentheses indicate the ratios of successful to total inoculations. Tumors developed most rapidly in chickens injected with crude extract.

Immunological Studies with Material from Normal and Leukemic Chickens Sedimentable at High Speed

Experiments were undertaken to determine if precipitins or complement-fixing antibodies directed against the agent of leukosis or sarcoma can be detected in sera of chickens. As antigens, crude tumor extracts, leukotic plasma, high speed deposits containing agent 1 or 13, and supernatant from high speed deposits were used. The sera were obtained from chickens that

TABLE VI

Effect of Purification of Tumor Extracts on Nitrogen and Phosphorus Ratios and on Activity

Material	Total volume	Nitrogen per ml.	Phosphorus per ml.	N:P ratio	Activity
	ml.	*mg.*	*mg.*		*mg. N*
Crude tumor extract	275	1.92	0.191	10:1	(4/6) 3.1×10^{-5}
Precipitate obtained by ½ volume saturated Na_2SO_4					
Fraction soluble in H_2O (A)	65	1.26	0.047	27:1	
Insoluble residue	10.6	1.69	0.089	19:1	
High speed sediment of fraction A					
Saline soluble fraction (B)	25	0.37	0.019	19.3:1	(1/6) 3×10^{-5}
Insoluble residue	10	1.02	0.055	18.3:1	
High speed sediment of fraction B					
Soluble in saline	10	0.08			(1/6) 6×10^{-6}
Insoluble	—	1.83 (total N)			

had chronic sarcoma 13 or resisted repeated injections of agents 13 or 1, or received repeated injections of high speed deposits containing the agents. The complement fixation tests with chicken sera like those of Greppin (13) were uniformly negative in dilutions 1:5 to 1:80 and will not be described in detail.

Rabbits were immunized with crude tumor extract, high speed deposits and supernatant of sarcoma 13, high speed deposits of leukotic plasma (strain 1), normal buffy coat of chicken blood, and high speed deposits of normal spleen. The sera gave strong complement-fixing reactions, particularly those prepared with high speed deposits, but cross reactions disclosed no difference between the heavy substances containing the agent

and those obtained from normal spleen (Table VII). In these tests the supernatant behaved as a weaker material both in the production and fixation of antibodies.

TABLE VII

Complement Fixation Test

Serum dilutions	Antigen			
	Crude extract	High speed deposit from		Supernatant from tumor extract
		Tumor extract	Normal spleen extract	
Serum prepared with crude extract				
1:50	0	0		0
1:150	0	0		0
1:450	c	st		c
1:1350	c	c		c
Serum prepared with high speed deposit from tumor				
1:50	0	0	(1:100) 0	0
1:150	0	0	(1:200) 0	0
1:450	ac	0	(1:400) st	c
Serum prepared with high speed deposit from normal spleen*				
1:100		0	0	0
1:200		0	0	tr
1:400		0	0	st
1:800		st	st	ac
Serum prepared with tumor high speed supernatant				
1:50	0	0		tr
1:150	st	0		c
1:450	c	c		c
Normal rabbit serum				
1:50	c	c		c
1:150	c	c		c

0 = complete inhibition, tr = trace, st = strong, ac = almost complete, c = complete hemolysis. All controls showed complete hemolysis.

* Made at different times.

Precipitin tests (Table VIII) yielded similar results. The high speed supernatant gave relatively stronger precipitation than complement fixation reactions. In attempt to disclose possible slight differences between heavy materials from normal spleen and sarcoma, precipitin reactions were carried

TABLE VIII

Precipitation Test

Antigen solutions	Sera				Saline
	Anti-A	Anti-B	Anti-C	Anti-D	
A. Crude tumor extract					
1:500	+++±	+	++	++	−
1:2000	++±	+	+±	+±	−
1:10,000	+±	+	+	±	−
B. High speed deposit of tumor extract					
1:2000	++	+	+±	++	±
1:10,000	+	+	+	+	
C. High speed supernatant					
1:500	+++±	+	++	++	±
1:2000	++±	+	+±	+±	−
1:10,000	+±	+	+	+	−
D. High speed deposit of normal spleen extract					
1:2000	+	+	+	+±	−
1:10,000	+	±	±	±	−
Saline	−	−	−	−	−

TABLE IX

Precipitin Absorption Test

Antigen dilutions	Serum against high speed deposit from						Antigen control
	Tumor extract (T)			Normal spleen extract (S)			
	Unabsorbed	Absorbed with		Unabsorbed	Absorbed with		
		T	S		T	S	
Supernatant							
1:1000	+	−	−	++	−	−	−
1:5000	+	−	−	+±	−	−	
High speed deposit from tumor extract							
1:1000	+++±	±	++	++++	+±	+	+
1:5000	++	−	+	++±	−	−	−
High speed deposit from normal spleen extract							
1:1000	++±	−	−	++	±	±	−
1:5000	±	−	−	+	−	−	
Serum controls: negative							

out, using diminishing amounts of serum in one experiment and diminishing amounts of antigen in another, but no difference was observed.

Precipitin absorption tests (Table IX) were carried out by adding to the sera homologous or heterologous antigen until no further precipitation occurred. There was a parallel decrease of antibody for both sedimentable material from normal spleen and from tumor irrespective of which of these antigens was used in the absorption.

The high speed deposit from spleen never reacted as strongly as that from tumor. This explains why the serum against high speed deposit from tumor that had been absorbed with high speed deposit from normal spleen still reacted strongly with tumor deposit but not with spleen deposit (Table IX). Furthermore, the spleen antiserum after partial absorption with tumor still reacted more strongly with tumor than with spleen high speed deposit. In other experiments the supernatant showed a different specificity from the high speed deposits.

Tests of the absorbed sera by means of complement fixation reactions yielded similar results indicating that the bulk of the heavy substances from normal spleen and tumor are probably identical. Buffy coat from normal chickens was likewise found to be capable of absorbing the antibodies directed against the heavy tumor material. Antibodies prepared against high speed deposits from leukotic plasma gave powerful reactions with high speed deposits from tumors. Many immune sera also agglutinated sheep red corpuscles.

DISCUSSION

The filterable agents of leukosis and sarcoma of fowls have thus far not been obtained in a state approaching purity. This is due mainly to their lability, especially in the partially purified state, and to the difficulty of securing large enough quantities of material. The studies with the ultracentrifuge (4, 5) have shown that one of the agents is of high molecular weight. Stern and Duran-Reynals (14) and Stern and Kirschbaum (15) obtained values for s_{20} of 550×10^{-13} for the average sedimentation constant of the heavy fraction obtained from Rous sarcoma and 583×10^{-13} and 532×10^{-13} for similar materials from fowl leukosis and normal chick embryo extracts respectively. These data suggest that the molecular weight is approximately 140,000,000 though the preparations were not homogeneous. The large size suggests that the agent is protein in nature though it probably constitutes only a small percentage of the products thus far isolated.

The results of attempts to correlate the activity and chemical properties

of material centrifugable at high speed are shown in Table I. The last two columns of the table show that the activity per milligram of high speed centrifugable nitrogen is the same within the limits of experimental error in the original extract as in the redissolved high speed sediment, indicating that the tumor extracts do not contain substances which enhance or inhibit the tumor-producing activity of the agent. Amounts as small as 10^{-5} mg. N produced tumors. In the enzyme-treated preparations larger amounts of material centrifugable at high speed were found in the second extract than in the first. This may be due to a better extraction of the tumor after the breakdown of the polysaccharide. Claude and Murphy (3) observed that the second extract of Rous sarcoma was more potent than the first. They ascribed this to the presence of an inhibitor in the first extract but as already stated there is no definite indication of the presence of an inhibitor in our material.

The carbohydrate-splitting enzyme itself has no effect on the activity of the agent nor did it affect the rate of deterioration of the crude extract in the ice box. High speed deposits obtained from enzyme-treated extracts were no less active than control preparations. The reduction of the viscosity of the tumor extracts has made possible the use of larger quantities of tumor material and has made manipulation of the extracts much easier.

Plasma and serum of chickens with leukemia produced by strains 1 and 13 have been found to contain varying quantities of agent and of material sedimentable at high speed (Table II). There is in chicken sera or plasma a considerable amount of material centrifugable at high speed of which the nitrogen content varies from 0.19 to 1.07 mg. per 10 ml. (Table II). In leukemia produced by strain 1 the average for 10 sera was 0.633 mg. N as compared with an average of 0.476 mg. N for 5 leukotic sera produced by strain 13 and 0.34 mg. N per 10 ml. for 10 normal sera. The severity of blood changes could not be correlated with the amount of material sedimentable at high speed. Human leukemic and normal sera contain negligible amounts of these substances. The total amount of heavy material obtainable from chicken plasma or serum is much less than that obtainable from tumor extracts.

Normal chicken and mouse spleen, as well as leukemic tissues from man and mice contain very large amounts of material centrifugable at high speed (Table III) but a virus producing leukemia in man or mice has thus far not been demonstrated. The finding of Stern and Kirschbaum (15) that normal chicken bone marrow contained only small amounts of heavy material can be explained by the predominantly fatty character of the femoral marrow of normal chickens. If the heavy material is a nucleo-

protein as suggested by Claude, it is probable that it is derived from the cells, perhaps chiefly from the nuclei. The heavy substance found in adult tissues is probably similar to that isolated by Claude from chick embryos (16). The available data suggest that both tumor extracts and leukemic plasma of chickens contain large amounts of normal heavy substance (Table II).

Earlier experiments showed that the agent rapidly deteriorates in the ice box. The present experiments show that high speed deposits rapidly deteriorate in the ice box but crude extracts could be kept for about a month without apparent loss of activity. Cysteine-cobalt had no noticeable preservative effect under the conditions studied. In sealed tubes submerged in solid carbon dioxide-alcohol mixture, no loss of activity of high speed deposits or crude extracts occurred even after 6 months (Table V).

In order to purify the agent, precipitation by one-third saturation with sodium sulfate can be combined with high speed centrifugation. At each stage of the procedure considerable quantities of the high speed sediment from both tumor extracts and normal chicken spleens remain insoluble.

The immunological studies failed to disclose any specific antibody directed against the virus in immune sera prepared by high speed deposits from tumor extract and leukotic serum. The failure to demonstrate a difference by immunological methods between the matter centrifugable at high speed from tumor and from normal spleen extracts suggests the presence of large amounts of normal heavy material in virus preparations. If the agent producing leukosis or sarcoma is different from that of normal heavy material it is present in our preparations in small amounts only, or it is a less effective antigen than the normal heavy substance. Another possibility, namely that the agent and the normal heavy material are related chemically, has been suggested by Claude, but the evidence available thus far does not warrant a definite conclusion.

SUMMARY

The activity of the agent producing sarcoma or leukosis in material deposited by high speed centrifugation is the same as that of the original crude extracts. Material sedimentable at high speed containing approximately 10^{-5} mg. N produces tumors at the site of injection.

Small quantities of material sedimentable at high speed are present in normal chicken sera, and about twice as much in leukemic sera (strain 1). Normal chicken and mouse spleens and all other human and mouse tissues examined contain large amounts of material sedimentable at high speed.

Extraction of leukemic blood cells with saline yields little additional virus.

The washed cells readily produce leukosis even after irradiation with amounts of x-rays sufficient to destroy the leukemic cells but not the virus.

Freezing at −60°C. preserves the activity of the high speed deposits for at least 6 months.

Addition of 5 per cent of saturated Na_2SO_4 solution slightly delays deterioration of high speed deposits in the ice box.

Most of the agent measured by inoculation of chickens and the fraction sedimentable at high speed measured by its nitrogen content is precipitated by one-third saturation with sodium sulfate.

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BIBLIOGRAPHY

1. Ellermann, V., and Bang, O., *Centr. Bakt., 1. Abt., Orig.*, 1906, **46,** 595.
2. Rous, P., *J. Exp. Med.*, 1911, **13,** 397.
3. Claude, A., and Murphy, Jas. B., *Physiol. Rev.*, 1933, **13,** 246.
4. Ledingham, J. C. G., and Gye, W. E., *Lancet*, 1935, **1,** 376.
5. Claude, A., *Am. J. Cancer*, 1937, **30,** 742; *J. Exp. Med.*, 1937, **66,** 59.
6. Kabat, E. A., *J. Biol. Chem.*, 1939, **130,** 143.
7. Meyer, K., Dubos, R., and Smyth, E. M., *J. Biol. Chem.*, 1937, **118,** 71.
8. Stubbs, E. L., and Furth, J., *J. Exp. Med.*, 1935, **61,** 593.
9. Furth, J., *J. Exp. Med.*, 1932, **55,** 465.
10. Bauer, J. H., and Pickels, E. G., *J. Exp. Med.*, 1936, **64,** 503.
11. Pentimalli, F., *Z. Krebsforsch.*, 1934, **40,** 166.
12. Pirie, A., and Holmes, B., *Brit. J. Exp. Path.*, 1931, **12,** 127. Engelbreth-Holm, J., and Frederiksen, O., *Acta path. et microbiol. Scand.*, 1938, suppl. 37, 738.
13. Greppin, J., *Bull. Assn. franç. étude cancer*, 1937, **30,** 232.
14. Stern, K. G., and Duran-Reynals, F., *Science*, 1939, **89,** 609.
15. Stern, K. G., and Kirschbaum, A., *Science*, 1939, **89,** 610.
16. Claude, A., *Proc. Soc. Exp. Biol. and Med.*, 1938, **39,** 398.