THE ANTICOAGULANT AND ANTILYMPHOMA PROPERTIES OF ARSENIC AZOPROTEINS*

I. ANTICOAGULANT EFFECTS OF ARSENIC AZOPROTEINS IN VIVO AND IN VITRO: COMPARISON OF ARSENICALS AS ANTICOAGULANTS AND AS ANTILYMPHOMA AGENTS: MOLECULAR STRUCTURE IN RELATION TO ANTICOAGULANT AND ANTILYMPHOMA PROPERTIES

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The effects of arsenic on cells and organisms of various kinds depend to a considerable extent on the structure of the compound in which the arsenic is incorporated, as Ehrlich showed many years ago (1, 2). More recent studies have shown further that certain arsenicals act powerfully *in vitro* on microbial cells of various species and on several kinds of proliferating mammalian cells as well (3, 4); and that arsenicals sometimes inhibit temporarily and to a slight extent the growth of certain tumor cells (5, 6), though amounts of arsenicals that are readily tolerated by a given host usually have little or no enduring effect on its proliferating neoplastic cells (7–11). When arsenic is suitably combined with protein by means of the azo linkage, however, its effectiveness in inhibiting the growth of a number of transplanted mouse lymphomas, particularly Lymphoma 6C3HED, is greatly enhanced; indeed, small quantities of arsenic azoproteins will often bring about the complete regression of large 6C3HED lymphomas in adult C3H mice without proving notably toxic for the latter (12).

In further studies of arsenic azoproteins as antilymphoma agents, we have recently found that 4-arsonophenylazoproteins act as powerful anticoagulants under a variety of conditions *in vivo* and *in vitro*, while ordinary inorganic and organic arsenicals and other arsenic-containing compounds,—for example, those in which arsenic is joined to amino acids or peptides through the azo linkage, or to proteins through couplings other than the azo linkage,—are largely devoid of ability to inhibit the coagulation of blood. Further, arsenic-containing compounds that act strongly as anticoagulants also act strongly

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against Lymphoma 6C3HED cells *in vivo*, as direct comparisons have now shown; while conversely, arsenic-containing compounds that are devoid of anticoagulant activity are also devoid of ability to act upon the lymphoma cells *in vivo*.

The findings, given in detail in this paper, provide basis for the supposition that arsenic azoproteins may give rise to anticoagulant and antilymphoma effects through similar or related mechanisms. This possibility is explored further in an associated paper.

Materials and Methods

Azoproteins were prepared by standard methods as previously described (12, 13, 14). Diazotates were obtained from the following substances: arsanilic acid, *p*-aminophenylarsene oxide, sulfanilic acid, *p*-aminobenzoic acid, *p*-phenylenediamine (as described by Saunders, reference 15), and aniline. In most experiments these substances were conjugated with bovine albumin (Armour Pharmaceutical Company, Kankakee, Illinois, Cohn's fraction V) but on some occasions human albumin and human globulin (Merck and Company Inc., Rahway, New Jersey), were used.

By varying the proportions of diazotate and protein in the reaction mixture, azoproteins with different degrees of conjugation were obtained as shown by the analysis for arsenic and sulfur in the sulfonic groups (performed by Schwartzkopf Microanalytical Laboratories, Woodside, New York). To produce 4-arsonophenylazoproteins with 3 to 4 per cent As content 1 millimole of diazotate was used for each gram of protein. Conjugation was usually performed at pH 9.0 and 0–4°C. After 2 or more hours the pH of the reaction mixture was adjusted to 6.5 and the azoprotein was then freed from other reaction products by dialysis against water for 48 hrs.

In the case of compounds with a very high content of arsenic (more than 7 per cent), the azoprotein after dialysis was reacted with diazotate once or twice more. Final products were lyophilized.

The degree of conjugation in unsubstituted phenylazoprotein and 4-carboxyphenylazoprotein, could not be determined by direct analysis, but the substances used in the present experiments were prepared using similar amounts of reactants and similar conditions to those employed in preparing 4-arsonophenylazoproteins with 3 to 4 per cent As content. As described previously, the resulting azoproteins in solution gave similar extinction coefficients when optical density readings were made (14).

Arsenic protein conjugates were prepared in two other ways. First, bovine albumin was conjugated with *p*-chloracetyl aminophenylarsonic acid (kindly prepared by Dr. Charles C. Price of the Department of Chemistry, University of Pennsylvania by the method of Giemsa and Tropp, reference 16). 1 gm of this substance was dissolved in 100 ml 0.2 M PO_4 buffer at pH 8.0 containing 10 gm bovine albumin and 0.1 gm potassium iodide and the pH restored to 8.0 by the addition of 0.2 N NaOH. The solution was sterilized by passage through a Seitz filter and left at 37°C for 8 days, dialyzed against water for 72 hours, and the protein finally lyophilized. The potassium iodide acted as a catalyst, greatly increasing the reaction velocity as shown by estimation of the chloride ion liberated and by the content of arsenic in the final protein preparations.

Further arsenic-protein conjugates were obtained using *p*-(2-4 dichloro-*s*-triazinyl) aminophenylarsonic acid prepared according to Friedheim (17). Reaction conditions were similar to those employed with *p*-chloracetyl aminophenylarsonic acid but a 0.25 M bicarbonate-CO_2 buffer at pH 7.3 was used and the iodide catalyst omitted.

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4-Arsonophenylazopeptones were prepared in a manner similar to that used for the corresponding azoproteins, using commercially available peptones (Difco Laboratories, Inc., Detroit). Azopeptones were precipitated from the reaction mixture at pH 9.0 by the addition of 6 volumes of ethanol, dissolved in water, and reprecipitated, the process being repeated three times. The azopeptones were finally lyophilized from aqueous solution at pH 7.0.

The formation of 4-arsonophenylazo derivatives of amino acids has been described by Pauley (18) whose method has been followed in essentials in the present experiments. The structure of these derivatives has recently been discussed by Tabachnick and Sobotka (19). 4-Arsonophenylazotyrosine was prepared by slowly adding an equimolecular amount of diazotised arsanilic acid (pH 8.0) to a 1 per cent aqueous suspension of L-tyrosine (Merck and Co.). The pH of the reaction mixture was maintained at 9.5 and 10.0 by addition of N NaOH and the temperature at 0–6°C. L-Tyrosine rapidly and completely dissolved. After 2 hours, the solution was acidified to pH 3.0. A brown precipitate formed which was collected by centrifugation, and supernatant being discarded. The precipitate was dissolved by raising the pH to 9.0 and precipitated by acidification on two further occasions after discarding the supernatants. The material was finally dissolved at pH 7.0 and lyophilized.

4-Arsonophenylazohistidine was prepared from L-histidine (Nutritional Biochemicals Corporation, Cleveland, Ohio) in a similar manner.

Blood Coagulation Experiments.—Rabbit blood was obtained by cardiac puncture. Human blood was obtained by venipuncture and anticoagulated by adding 9 volumes of blood to 1 volume of 3.8 per cent sodium citrate solution. Plasma from freshly obtained blood was used in all experiments; it was separated by centrifugation at 3000 RPM for 15 minutes at temperatures of 3–4°C. Thrombin used was topical preparation of Parke Davis and Company, Detroit.

Blood clotting times were measured by the method of Lee and White (20). Plasma recalcification times were measured by modifications of the method of Biggs and McFarlane (21), as described in the text.

Implantation of Lymphoma 6C3HED in ZBC Mice.—The techniques used have been described previously (12, 22).

RESULTS

Anticoagulant Effects of 4-Arsonophenylazoproteins in Vivo.—In experiments undertaken for other purposes, rabbits were given intravenous injections of a 4-arsonophenylazoalbumin containing 6.5 per cent As. Several of the animals that received more than 10 mg As/kg died within 24 hours following the injections. Surprisingly, at autopsy 6 or more hours after death, the blood of these rabbits was everywhere uncoagulated; furthermore, fibrinolysis had plainly not occurred, since the blood eventually became fully coagulated when it remained for some time in contact with tissues cut in the dissection.

About this time, a similar anticoagulant effect was observed in mice. A group of 8 mice weighing approximately 25 gm were given a single intraperitoneal injection of 4-arsonophenylazoalbumin containing 6.5 per cent of arsenic, in a dosage of 1 mg As per mouse; following this a toe was cut for identification purposes in the manner used as routine in this laboratory. These animals bled profusely from the small wound produced,—indeed to the extent that all died exsanguinated in the following 24 hours. Untreated control mice remained healthy and as usual their toes ceased to bleed after a few minutes.

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Further experiments were performed to define the anticoagulant effect more precisely. First, estimations were made of whole blood clotting time in rabbits given the same arsenic azoprotein preparation as that used in the experiments already mentioned. Control blood samples were obtained from these rabbits by means of a clean cardiac puncture, and the clotting times were determined. The animals were then injected intravenously with various quantities of the azoprotein; after 5 minutes a further cardiac puncture was performed and the clotting time again measured. Table I shows the results; these clearly confirm the earlier *in vivo* observations. An anticoagulant effect was produced when 15.5 mg of the azoprotein/kg was given, and at higher dosage levels the anticoagulant effect increased progressively. However, it was not until 930 mg/kg was reached that the blood became completely incoagulable. No anticoagulant effect was observed in controls given arsanilic acid and protein mixtures, even though amounts of azoproteins with similar arsenic content were powerfully anticoagulant.

TABLE I

Delay in Whole Blood Clotting Time of Rabbits Given 4-Arsonophenylazoalbumin Intravenously

Rabbit	Dosage			Clotting time			
	Arsanilate*	4-Arsonophenylazoalbumin‡	As	Before treatment		5 min. after intraveneous injection of test substance	
	mg/kg	*mg/kg*	*mg/kg*	*min.*	*sec.*	*min.*	*sec.*
1	14.5	—	5.0	2	48	3	20
2	72.5	—	25.0	3	9	3	5
3	174.0	—	60.0	1	5	2	8
4	—	15.5	1.0	3	26	6	15
5	—	38.4	2.5	3	14	8	55
6	—	77.5	5.0	3		14	23
7	—	115.2	7.5	3	24	23	30
8	—	115.0	10.0		55	45	
9	—	310.0	20.0	1	30	Beginning at 3 hrs. complete by 18 hrs.	
10	—	620.0	40.0	1	56	Beginning at 6 hrs. complete by 24 hrs.	
11	—	930.0	60.0	2		Uncoagulated after 24 hrs.	

Market bought rabbits weighing 3.0 to 4.5 kg were used. Clotting times were measured by the method of Lee and White (20).

* 3 gm per cent arsanilate with 20 gm per cent bovine albumin, pH 7.0.

‡ 20 gm per cent aqueous solution of 4-arsonophenylazoalbumin M9 (As 6.5 per cent).

Anticoagulant Effects of 4-Arsonophenylazoproteins in Vitro.—Human plasma proved to be particularly sensitive to the anticoagulant effects of 4-arsono-

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phenylazoproteins. Table II shows that, with the same azoprotein preparation as that used in the previous experiment, an anticoagulant effect was demonstrable at a concentration as low as 0.1 mg azoprotein per ml, rising progressively above this. Other preparations of azoproteins with widely differing content of azo groups and of arsenic also produced anticoagulant effects, but according to a particular pattern. It will be seen from the experiment shown in Table III, that the anticoagulant effect of azoalbumins increased progressively with their content of arsenic, to approximately 7.4 per cent. Above this level, increased arsenic content had either a negligible effect in increasing anticoagulant activity, or indeed might actually produce a less effective substance, as for example,

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TABLE II

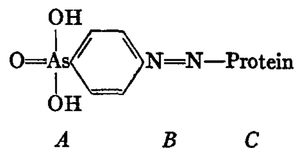
Delay in the Clotting Time of Human Plasma Produced by 4-Arsonophenylazoalbumin in Vitro

Clotting mixture: 0.4 ml plasma + 0.01 ml of azoprotein solutions producing the following concentrations	Clotting times after recalification with 0.25 ml of 0.05 M $CaCl_2$	
mg/ml	*min.*	*sec.*
Nil (control)	2	58
0.10	3	30
0.19	5	40
0.38	10	30
0.75	15	45
1.50	40	

The 4-arsonophenylazoalbumin M9 (6.5 per cent As) was used, dissolved in 0.9 gm per cent sodium chloride (pH 7.1).

material M8 containing 17 per cent As. It will be further seen that the two 4-arsonophenylazoglobulins were considerably less effective as anticoagulants than azoalbumins of corresponding arsenic content.

The Relationship of Structure to Anticoagulant Activity Amongst 4-Arsonophenylazoproteins and Chemically Related Substances.—The structure of a 4-arsonophenylazoprotein may be illustrated in the following way, where tyrosine in the protein forms the site of attachment of the azo group.



Azo groups are also joined to the protein molecule through the ring structures of tryptophane and histidine, through sulfhydryl groups, and possibly through other sites (Howard and Wild, reference 23).

Analogues of 4-arsonophenylazoprotein were prepared by varying the groups at *A*, *B*, and *C*. At *A* the arsonic group was replaced, (*a*) by acidic groups, namely, carboxylic, sulfonic and arsinoso (trivalent arsenic); (*b*) by the basic amino group, and (*c*) by hydrogen, forming unsubstituted phenylazoprotein. The azo group indicated at *B* was replaced by carbamoylmethyl and triazinyl groups in two further conjugates. These protein conjugates were prepared by alkylation, and hence free amino groups, phenolic hydroxyl groups, and sulf-

TABLE III

The Effectiveness of 4-Arsonophenylazoprotein Preparations with Differing Arsenic Contents as Anticoagulants in Vitro

Clotting mixture: 0.4 ml citrated plasma + 0.01 ml of the azoprotein solutions, each of 5 gm per cent.

Azoprotein solutions	Protein source, serum	Designation of conjugate	As content of conjugate	Clotting times after recalcification with 0.25 ml of 0.05 M $CaCl_2$	
			per cent	*min.*	*sec.*
Control, 0.9 gm per cent NaCl				2	50
4-Arsonophenylazo-albumins	Human	K11	0.2	2	40
	Bovine	K6	0.8	3	10
	Bovine	K13	1.9	4	23
	Bovine	K1	5.5	12	45
	Human	M11	7.4	17	
	Bovine	K3	15.7	19	30
	Human	M8	17.0	8	15
4-Arsonophenylazoglobulins	Human	M12	1.9	3	20
	Human	M13	7.5	8	10

Other tests performed using 0.01 ml of 5 gm per cent bovine albumin with arsanilic acid in concentrations of 1.0, 2.0, and 3.0 gm per cent (pH 7.0) produced no anticoagulant effect by comparison with the 0.9 gm per cent NaCl control.

hydryl groups of the protein would be expected to form sites of attachment (24). At *C*, instead of using whole protein, polypeptides, and amino acids were used.

Table IV shows the results of testing the effect of a number of the compounds described on the clotting times of recalcified human plasma. It will be seen that as before, 2 different preparations of 4-arsonophenylalbumin produced a clear anticoagulant effect, and so too, though less effectively in relation to its arsenic content, did a preparation of 4-arsinosophenylazoprotein. Other acidic azoproteins also produced a strong anticoagulant effect, indeed, a 4-sulfonophenylazoalbumin preparation gave the strongest anticoagulant effect of any of the substances tested; the 4-carboxyphenylazoalbumin produced an anticoagulant effect only slightly less strong that that of the 4-arsonophenylazoalbumin M9.

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None of the other substances tested showed anticoagulant activity, it being especially noteworthy that the protein conjugates of phenylarsonic acid which lacked the azo group were without effect. 4-Arsonophenylazo compounds of peptones were likewise devoid of anticoagulant effect, despite their high arsenic content. Clearly amongst the substances tested considerable structural specificity was required for anticoagulant activity. Active substances, it can be seen, (*a*) were proteins, (*b*) possessed a high negative charge, and (*c*) their acidic groups were linked to protein through azo bonds.

Structural Requirements for Antilymphoma Activity.—The general features of the inhibitory effects of 4-arsonophenylazoproteins on the growth of lymphomas *in vivo* have been fully described by Kidd (12). Lymphoma 6C3HED was shown to be particularly sensitive to these substances; using a sufficient dosage of 4-arsonophenylazoprotein, and one not appreciably toxic to the host, complete regression of fully established tumors 1 to 2 cm in diameter could be regularly produced.

In the present experiments, the antilymphoma activity of a number of different 4-arsonophenylazoprotein preparations and of other chemically related substances was assayed in a standardized way that gave a particularly sensitive and sharp end point. Small numbers of Lymphoma 6C3HED cells (50 thousand) were implanted into groups of ZBC mice subcutaneously in each flank. A suitable number of implanted animals were kept as controls, others were treated with the substances under test within 2 hours of implantation and on some occasions treatment was also given on the succeeding 2 days. In control animals, tumors appeared regularly on the 9th to 11th days following implantation. By careful daily examination, delay in tumor appearance brought about by treatment could be readily detected. As will be shown, small, non-toxic quantities of 4-arsonophenylazoprotein produced complete suppression of tumor growth.

The properties of a number of different 4-arsonophenylazoproteins in suppressing tumor growth are shown in Table V. It will be seen that 4-arsonophenylazoproteins with arsenic content from 0.2 to 5.5 per cent were all highly active in inhibiting tumor growth; single injections containing 0.5 to 0.75 mg As brought about complete tumor suppression. But, as with their anticoagulant activity, substances with higher arsenic content, had less tumor inhibiting activity. Thus, the azoalbumin K4, containing 7.6 per cent As, produced complete tumor suppression only in a dose of 1.0 mg As, while 2 other preparations containing 15.7 and 17.0 per cent As required to be given in doses of 3.0 mg As, before tumor suppression occurred. Again, azoglobulins were rather less effective as tumor inhibitory agents than were azoalbumins of equal arsenic content; for instance, it was necessary to inject azoglobulin containing 7.5 per cent As in a dosage of 2.0 mg As to produce complete tumor inhibition, whereas only 1.0 mg As was required for a similar azoalbumin. Analogues of 4-arsonophenylazoproteins containing trivalent arsenic (derived from 4-aminophenylarsene oxide) inhibited tumor growth, but, as shown in Chart 1, they did this considerably less

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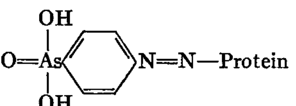
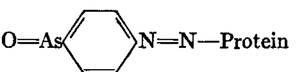
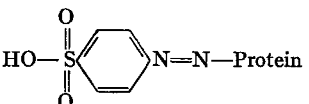
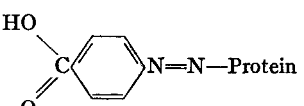
TABLE IV

Tests for the Effects of 4-Arsonophenylazoproteins and Their Analogues on the Clotting of Plasma in Vitro

Clotting mixtures: 0.4 ml citrated human plasma + 0.01 ml of solutions of the test substances*

Test substance		Preparation	Concentration of test substance in clotting mixture	Clotting time after recalcification with 0.25 ml of 0.05 M $CaCl_2$	
			mg/ml	*min.*	*sec.*
Control, 0.9 gm per cent NaCl			—	3	5
Acidic azoproteins					
4-Arsonophenylazoalbumin		L13, As 1.9 per cent	0.75	5	20
"		M9, As 6.5 per cent	0.75	19	30
4-Arsinosophenylazoalbumin		PPA, As 6.5 per cent	0.75	5	
			1.50	8	20
4-Sulfonophenylazoalbumin		S3, S in sulfonic groups 1.7 per cent	0.75	9	50
"		S2, S in sulfonic groups 3.4 per cent	0.75	48	
4-Carboxyphenylazoalbumin		PAB2	0.75	16	30

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Non-acidic azoproteins					
4-Aminophenylazoalbumin	$NH_2-C_6H_4-N{=}N-$Protein		0.75	3	6
Phenylazoalbumin	$C_6H_5-N{=}N-$Protein		1.50	3	
Protein derivatives lacking the azo group					
4-Arsonophenylcarbamoylmethyl-albumin	$O{=}As(OH)_2-C_6H_4-NHCOCH_2-$Protein	PCA 4, As 2.1 per cent	1.50	3	
2(*p*-Arsonophenylamino)-4,6-dichloro-*s*-triazine conjugates with albumin,‡ *e.g.*	$O{=}As(OH)_2-C_6H_4-NH-$triazine(Cl)$-$Protein	TC 4, As 1.9 per cent	1.50	3	3
Azo derivatives of non-protein materials					
4-Arsonophenylazopeptone	$O{=}As(OH)_2-C_6H_4-N{=}N-$Peptone	AAP$_2$, As 9.5 per cent	0.75	3	6
4-Arsonophenylazotyrosine		A73, As 17.0 per cent	0.75	3	10
4-Arsonophenylazohistidine		AH$_2$, As 15.0 per cent	0.75	3	14

* In 0.9 gm per cent NaCl pH 7.0.
‡ Chlorine atoms at positions 4 and 6 on triazine react with amino groups, but usually only one is replaced.

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powerfully in relation to their arsenic content than did 4-arsonophenylazoproteins. The tumor inhibitory activity of these substances thus corresponded closely with their effects on the coagulation process.

Further experiments soon made it clear that anticoagulant activity *per se* was not necessarily associated with the ability to inhibit tumor growth. Chart 2 shows that although 13 mg of a preparation of 4-arsonophenylazoprotein caused complete tumor supression in three animals, considerably larger quantities of 4-sulfonophenyl- and 4-carboxyphenylazoproteins, which were even more effective as anticoagulants (see Table IV), produced no significant inhibition of tumor growth. The doses of these substances were the maximum which could be given and were in several cases notably toxic. Phenylazoalbumin and 4-aminophenylazoalbumin were similarly ineffective. The essential role of arsenic in bringing about tumor inhibition is further shown by the ability of dimercaprol (BAL) to reverse the antilymphoma effects of 4-arsonophenylazoproteins.

TABLE V

The Arsenic Content of 4-Arsonophenylazoproteins in Relation to Their Tumor Inhibitory Activity

Identity of 4-arsonophenylazoprotein tested	As content of azoprotein	Curative dose of azoprotein	Curative quantity of arsenic
	per cent	*mg*	*mg*
Azo-bovine albumin K11	0.2	250.0	0.75
" " K6	0.8	62.5	0.50
" " K5	1.0	50.0	0.50
" " K17	2.0	25.0	0.50
" " K13	2.5	20.0	0.75
" " K1	5.5	9.1	0.50
" " K4	7.6	9.9	1.00
" " K3	15.7	19.1	3.00
Azo-human " M8	17.0	18.0	3.00
" globulin M12	1.9	26.3	1.00
" " M13	7.5	10.0	2.0

The method used was as follows: groups of 3 ZBC mice were implanted with 50,000 6C3HED cells in each flank. 30 to 60 minutes later these animals were injected intraperitoneally with azoprotein solutions to provide a dosage of 0.25, 0.50, 0.75, and 1.00 mg As. With K3 and M8 the dosages used were 0.5, 1.0, 2.0, 3.0 mg As. The curative dose represents the minimum dose which caused complete suppression of tumor growth in a group of three mice, estimated at 30 days following implantation. Tumors appeared in untreated control mice 9 days following implantation, causing death 16 to 20 days later.

The Effect of BAL on the Antilymphoma Activity of 4-Arsonophenylazoprotein.—Earlier experiments have shown that 0.25 to 0.5 mg arsenic contained in the most effective 4-arsonophenylazoprotein preparations brought about the complete suppression of tumors in animals newly implanted with cells of Lymphoma

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CHART 1

A comparison of the effects of trivalent and pentavalent arsenic in azoproteins on the growth of 6C3HED lymphoma cells in vivo

Experimental groups and materials *	As in prepn	Dosage	Outcome of implantations ‡ (3 test mice a, b, and c in each group)								
			Days following implantation								
			9			14			19		
			Mouse: a	b	c	a	b	c	a	b	c
	%	mg As									
1. Nil, untreated controls	--------	--------	● (1 cm)	●	●	●	●	●	●	●	●
2. 4- arsonophenylazoalbumin (K17)	2.0	0.25 x 1	n	n	●	●	●	●	●	●	●
3. " "	"	0.50 x 1	n	n	n	n	n	n	n	n	n
4. 4- arsinosophenylazoalbumin (TA3)	1.9	0.50 x 1	●	●	●	●	●	●	●	●	●
5. " "	"	1.00 x 1	n	●	●	●	●	●	●	●	●
6. " "	"	1.00 x 2	n	n	n	n	n	n	n	n	n

* 3 ZBC mice (22-27 gm) in each group implanted with 50,000 6C3HED cells in each flank. Mice treated with the materials shown within 2 hours of implantation and, where indicated, at daily intervals following this.

‡ To conserve space, only the tumor in the right side of each mouse is shown in the chart.

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CHART 2

The effect of various azoproteins on the growth of lymphoma 6C3HED in vivo

Experimental groups and materials *	Dosage (mg protein and No. of days given)	Outcome of implantations ‡ (3 test mice a, b, and c in each group)									
		Days following implantation	9			12			15		
		Mouse:	a	b	c	a	b	c	a	b	c
1. Nil; untreated controls	---------		● (1 cm)	●	●	●	●	●	●	●	●
2. 4- arsonophenylazoalbumin (As 1.8 %)	13.0 x 1		n	n	n	n	n	n	n	n	n
3. 4- sulfonophenylazoalbumin S3 (S in sulfonic group 1.7 %)	25.0 x 3		●	●	●	●	●	●	●	●	●
4. 4- sulfonophenylazoalbumin S2 (S in sulfonic group 3.4 %)	25.0 x 3		■ -	●	●	-	●	●	-	●	●
5. 4- carboxyphenylazoalbumin	20.0 x 3		●	●	●	●	●	●	●	●	●
6. Phenylazoalbumin	100.0 x 1		■ -	●	●	-	●	●	-	●	●
7. 4- aminophenylazoalbumin	50.0 x 1		■ -	■ -	●	-	-	●	-	-	●
8. "	25.0 x 1		●	●	●	●	●	●	●	●	●

* Implantation and method of treatment as in Chart 1.

‡ To conserve space, only the tumor in the right side of each mouse is shown in the chart.

■ Animal died on fourth day after implantation, presumably from drug intoxication.

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6C3HED. The effect of BAL simultaneously administered with azoprotein was examined in a number of experiments of which Chart 3 shows a representative result. It is clear that BAL while itself failing to influence the growth rate of the tumors is able to render entirely ineffective a quantity of 4-arsonophenylazoprotein containing 1.0 mg As. Alone, half this amount completely inhibited tumor growth and even one-quarter produced a detectable inhibition.

This experiment shows a further point which will be discussed more fully later, namely that since BAL reacts only with trivalent arsenic, the fact that it protects lymphoma cells against inhibition by 4-arsonophenylazoproteins, indicates that the As in these effective preparations must first be reduced before producing its cytotoxic effects.

The Dependence of Tumor Inhibitory Activity of Arsenic Protein Conjugates on the Azo Linkage and on Molecular Size.—Proteins to which phenylarsonic acid was joined through methylcarbamoyl or triazinyl groups were previously shown to lack anticoagulant activity. Similar results, and again in contrast to the azoproteins were found when these substances were tested against Lymphoma 6C3HED. Chart 4 shows that although 0.25 mg As given as azoprotein caused complete tumor suppression in each treated mouse, in other animals 3 daily injections each of 1 mg As from both carbamoylmethyl and triazinyl proteins had relatively little or no tumor inhibitory effect.

Preparations of 4-arsonophenylazopeptones were tested in the same manner by treatment of the hosts immediately following tumor cell implantation. It will be seen in Chart 5 that a preparation of azopeptone, containing 9.9 per cent As, produced no more than a slight delay in the appearance of the tumors, even though the dose of arsenic given, 2.5 mg, is the highest which toxicity tests have shown to be tolerated without a high mortality.

Preparations of azo derivatives of L-tyrosine and L-histidine were also largely devoid of inhibitory effect on the tumor when given in the maximum tolerated amounts; again the quantities of arsenic they contained were considerably in excess of those which as azoprotein bring about complete tumor suppression.

SUMMARY AND COMMENT

Experiments given in this paper have shown that 4-arsonophenylazoproteins possess marked anticoagulant activity both *in vivo* and *in vitro*. Mice and rabbits given moderate amounts of the arsenic azoprotein, for example, often bled to death from injuries that proved trivial in control animals, and their blood remained liquid during many hours' postmortem even when left in contact with transected tissues, fibrinolysis having no part in the outcome. So, too, the addition of minute amounts of 4-arsonophenylazoprotein to plasma procured from citrated rabbit or human blood greatly prolonged the time required for clotting after recalcification.

Other arsenic-containing compounds,—for example, those in which arsenic

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CHART 3

The reversal by B A L of the inhibitory effect of 4- arsonophenylazoprotein on the growth of 6C3HED tumors in vivo

Experimental groups and materials *	Dosage of 4- arsonophyll-azoprotein	Outcome of implantations ‡ (3 test mice a, b, and c in each group)								
		Days following implantation								
		8			10			17		
		Mouse: a	b	c	a	b	c	a	b	c
	mg As									
1. Nil, untreated controls	----------	● (1 cm)	●	●	●	●	●	●	●	●
2. 4- arsonophenylazoalbumin	0.25	n	n	●	n	●	●	●	●	●
3. "	0.50	n	n	n	n	n	n	n	n	n
4. "	1.00	n	n	n	n	n	n	n	n	n
Animals given B A L plus:										
5. Nil- untreated controls	----------	-	●	●	-	●	●	-	●	●
6. 4- arsonophenylazoalbumin	0.25	●	●	●	●	●	●	●	●	●
7. "	0.50	●	●	●	●	●	●	●	●	●
8. "	1.00	●	●	●	●	●	●	●	●	●

B A L was given subcutaneously in the axilla and back in 1.5 mg quantities. Treatment was begun 1/2 hour after tumor implantation. 12 hours later treatment was resumed and 4 injections were given at 4 hour intervals.

* 3 ZBC mice (21– 28 gm) were implanted with 50,000 6C3HED cells subcutaneously in each flank. The 4- arsonophenylazoalbumin (B4 - 2.5 % As) was given in a single intraperitoneal injection 1 hour later.

‡ To conserve space only the tumor in the right flank is shown. 1 animal in group 5 died of unknown cause 6 days after tumor implantation.

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CHART 4

Tests for effects of various arsenic containing proteins on the growth of lymphoma 6C3HED cells in vivo

Experimental groups and materials *	As in prepn	Dosage (mg As and No. of days given)	Outcome of implantations ǂ (3 test mice a, b, and c in each group) — Days following implantation: 9			14			19		
			Mouse: a	b	c	a	b	c	a	b	c
1. Nil, untreated controls	% -------	-------	● (1 cm)	●	●	●	●	●	●	●	●
2. 4- arsonophenylazoalbumin (M 9)	6.5	0.25 x 1	n	n	n	n	n	n	n	n	n
3. " (K 16)	1.8	0.25 x 1	n	n	n	n	n	n	n	n	n
4. 4- arsonophenylcarbamoylmethyl-albumin (PC 7)	2.1	1.00 x 3	n	n	●	n	●	●	●	●	●
5. 4- arsonophenylcarbamoylmethyl-albumin (PC 8)	1.2	1.00 x 3	●	●	●	●	●	●	●	●	●
6. 2- (*p* - arsonophenylamino) - 4, 6 - dichloro - *s* - triazine derivatives of albumin (TC 4)	1.9	1.00 x 3	n	n	●	●	●	●	●	●	●
7. 2- (*p* - arsonophenylamino) - 4, 6 - dichloro - *s* - triazine derivatives of albumin (TC 5)	0.5	1.00 x 3	n	●	●	●	●	●	●	●	●

* Implantation and method of treatment as in Chart 1.

ǂ To conserve space, only the tumor in the right side of each test mouse is shown in the chart.

(M 9), etc., designate particular laboratory preparations.

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CHART 5

The effect of 4 - arsonophenylazo derivatives of peptones and amino acids on the growth of 6C3HED tumors in vivo

Experimental groups and materials *	As in prepn	Dosage	Outcome of implantation ‡ (3 test mice a, b, and c in each group)								
			Days following implantation								
			11			13			17		
			Mouse: a	b	c	a	b	c	a	b	c
	%	mg As									
1. Nil, untreated controls	-------	-------	● (1 cm)	●	●	●	●	●	●	●	●
2. 4- arsonophenylazoalbumin (K 16)	1.8	0.25	n	n	n	n	n	n	n	n	n
3. 4- arsonophenylazopeptone §	9.9	2.50	n	n	●	●	●	●	●	●	●
4. 4- arsonophenylazotyrosine	10.0	2.50	n	●	●	●	●	●	●	●	●
5. 4- arsonophenylazohistidine	15.7	4.00	n	n	●	●	●	●	●	●	●

* Implantation and method of treatment as in Chart 1.

‡ To conserve space, only the tumor in the right side of each test mouse is shown in the chart.

§ Treated animals lost weight and showed prolonged toxic effects.

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was joined to amino acids or peptides through the azo linkage, or to proteins through couplings other than the azo linkage,—were largely devoid of anticoagulant and antilymphoma effects. The findings as a whole show clearly that the structural requirements for anticoagulant and antilymphoma effects are: (*a*) possession of negatively charged arsonic or arsinoso groups, (*b*) large molecular size (protein), and (*c*) linkage of arsenic-containing groups to protein through the azo bond.

Two acidic azoproteins that were devoid of arsenic,—namely 4-carboxyphenylazoprotein and 4-sulfonophenylazoprotein,—were also found to have marked anticoagulant effects *in vitro*, but they had no inhibitory action against cells of Lymphoma 6C3HED *in vivo*, even when they were given to mice in maximum tolerated amounts. The essential part played by arsenic in the antilymphoma activity of arsenic azoproteins was further emphasized by the action of dimercaprol (BAL) in preventing the antilymphoma effects of 4-arsonophenylazoprotein on Lymphoma 6C3HED cells *in vivo*.

In an associated paper the anticoagulant and antilymphoma effects of 4-arsonophenylazoproteins are studied further, and consideration is given to the ways in which these effects may be brought about.

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