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Elliot Volkin and Henry I. Kohn

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A FACTOR IN THE PLASMA OF THE IRRADIATED RAT WHICH CHANGES THE A/G RATIO

Elliot Volkin and Henry I. Kohn[†]

Following total-body X irradiation, the plasma or serum albumin-globulin ratio (A/G), measured by sodium sulfite fractionation, was found to increase in the guinea pig and rat (1, 2). In the rat (ID-50 per cent, 730 r), following exposures of 650 to 750 r, A/G increased from a normal level of 2 to about 2.3 within 1 day, reached a maximum of about 3 on the fourth day, and during the next 2 weeks maintained a level of about 2.5.

The rise in A/G was due most likely to some factor in the plasma which affected the measurement, rather than to a change in the proteins themselves. This conclusion followed from the fact that ether extraction of the abnormal plasma, just prior to fractionation, returned the elevated A/G to the normal level without affecting the total protein concentration (1, 2). Other evidence indicated that the factor was not a phospholipid or cholesterol.

The present experiments on the rat deal with a further analysis of this phenomenon by electrophoresis and ultracentrifugation. Ultracentrifugation of sera of irradiated rats showed an increase in the A/G ratio whereas there was no change by electrophoresis. Material (not protein) which may be the responsible

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factor has been extracted from the plasma. Addition of such material to normal plasma induced a rise in A/G.

Materials and Methods

All of the methods, excepting electrophoresis and ultracentrifugation, were as described previously (1, 2), and therefore require only brief mention. The four strains of rats that have been employed (Sprague-Dawley, Holtzman, Osborne-Mendel and Tumblebrook Farm hooded) behaved alike. Neither size nor sex influenced the results. Immaturity, hypophysectomy and adrenalectomy somewhat increased the rise in A/G that followed irradiation (3).

Irradiation was by means of a 250 kvp. source of X rays, of 0.4 mm. Cu half-value layer. The doses stated include the backscatter, and represent a mean for the entire animal.

Blood was obtained by cardiac puncture, with or without heparin. Serum was obtained from heparinized plasma by freezing, thawing, and centrifuging off the clot. Proteins were determined by Weichselbaum's modification of the biuret test (4). Globulin was separated from albumin by the temperature-independent sodium sulfite fractionation of Campbell and Hanna (5), the final concentration of sodium sulfite being 19.5 per cent. During the past year and a half, the A/G of heparinized plasma has averaged about 2, while that of serum has averaged 1.75.

To demonstrate the effect of ether extraction on A/G, one part of plasma was shaken for about 15 seconds with two parts of ethyl ether, and centrifuged. The layer of plasma was then used for analysis.

Electrophoresis at 2°, average current of 12 milliamperes, was performed in the Klett Tiselius apparatus using the Longsworth scanning method of photography (6). The sera were prepared by diluting them to about 3 gm. per cent of protein with buffer, and then dialyzing against buffer overnight (12 ml. of diluted serum against 2 l. of buffer). The electrode vessels were filled with the dialysate. In the first three experiments of Table I, the buffer, pH 7.4, contained 0.2 M sodium chloride and 0.025 M sodium phosphate; in the fourth experiment the buffer, pH 8.5, contained 0.1 M sodium diethylbarbiturate and 0.02 M diethylbarbituric acid. The relative concentrations of albumin and total globulin were calculated from the areas under the corresponding peaks, by the method of Tiselius and Kabat (7).

The operation of the ultracentrifuge (Specialized Instruments Company Model E) has been described by Schwert (8). The sera were prepared for each experiment, without dialysis, by dilution with buffer to a given total-protein concentration, thereby avoiding differences in A/G due to differences in total

TABLE I

Comparison of A/G Values for Rat Sera by the Methods of Sodium Sulfite Fractionation, Electrophoresis and Ultracentrifugation

(1)	(2)	(3)	(4)	(5) Sodium sulfite fractionation			(6) Other analyses		
Experiment	X-ray dose r	Days since exposure	Ether extraction[1]	Total protein conc. gm. per cent	A/G	Rise in A/G per cent	Total protein conc. gm. per cent	A/G	Rise in A/G per cent
							Electrophoresis		
1	0	35		6.95	1.82	--	3	2.16	--
	750	35		6.95	3.20	76	3	2.46	14
2	0	4		7.25	1.88	--	3	1.38	--
	675	4		5.95	2.45	30	3	1.50	9
3	0	60		7.4	1.74	--	3	2.12	--
	700	60		7.55	2.21	27	3	2.17	2
4	0	4		7.05	1.74	--	3	1.90	--
	750	4		5.35	2.53	45	3	1.96	3
							Ultracentrifugation		
5	0	4	-	7.05	1.75	--	3	2.67	--
	0	4	+	7.05	1.75	--	3	2.72	--
	750	4	-	5.35	2.68	53	3	3.81	43
	750	4	+	5.35	1.75	0	3	2.78	2
6	0	4	-	6.67	1.73	--	4	3.42	--
	0	4	+	6.67	1.70	--	4	3.88	--
	700	4	-	5.50	2.42	42	4	5.06	48
	700	4	+	5.50	1.75	3	4	3.86	0
7	0	4	-	6.67	1.99	--	3	1.94	--
	0	4	+	6.67	1.99	--	3	2.30	--
	650	4	-	5.00	3.54	78	3	4.25	118
	650	4	+	5.00	1.94	-2	3	3.16	37
	mg. residue per mg. serum protein								
8		0		4.65	1.69	--	2.0	2.0	--
		.37		4.65	2.72	61	2.0	2.42	21
9		0		5.00	1.70	--	2.5	3.41	--
		.52		5.00	2.48	46	2.5	4.00	17

1 = (+) indicates ether extracted, (-) indicates not ether extracted.

protein concentration *per se* [Pederson (9), MacFarlane (10)]. This resulted in diluting some sera with slightly different amounts of buffer in order to bring the sera of one experiment to the same total protein concentration. The possibility of different dilutions of buffer salt affecting the A/G ratio ["salt effect" (9)] was minimized by using a buffer system with a salt concentration similar to that of the sera, although no "salt effect" on the ultracentrifugal analysis of mammalian sera other than human has as yet been found. The buffer contained 0.2 M sodium chloride, 0.025 M monopotassium phosphate and 0.025 M disodium phosphate, pH 6.8. The relative concentrations of albumin and globulin were calculated by the method used for electrophoresis diagrams (7). The ultracentrifugal patterns contained only the albumin and globulin peaks, without evidence of a third component (e.g., X-protein). The very small amount of fast-moving inhomogeneous protein (9) present in all sera was ignored in the calculations.

It should be noted that the sera were dialized for electrophoresis, but not for ultracentrifugation. This difference may have contributed to the difference in the results found with the two methods.

EXPERIMENTAL RESULTS AND DISCUSSION

Measured by sodium sulfite fractionation, a significant rise in A/G ratio invariably followed irradiation (Table I, Expts. 1 to 7, Column 5). When the

serum was extracted with ether prior to analysis no rise occurred, thus confirming previous results (1, 2). Ether extraction did not affect the A/G of normal serum or the total protein content of any serum (Table I, Expts. 1 to 7). Analysis with the ultracentrifuge gave similar results (Table I, Expts. 5 to 7, Column 7), except in Experiment 7 where adrenalectomized animals were employed. Here ether extraction did not completely return the ultracentrifugal value of A/G to the preirradiation level, though it did cause a large shift in that direction.

It was not expected that the absolute values of A/G obtained by salt fractionation and ultracentrifugation would agree, since sodium sulfite incompletely separates albumin from globulin, and the conditions of the run in the ultracentrifuge (e.g., protein concentration) affect the calculated value (9). That both methods showed the same relative changes, however, was considered as good agreement and important confirmation.

In contrast, analysis by electrophoresis showed no significant change in A/G following irradiation (Table I, Expts. 1 to 4, Column 6). The patterns and the calculated mobilities of the globulin components and albumin were in excellent agreement with those reported by Moore _et al_. (11) for normal rat sera, and therefore are not repeated here.

The fact that extraction of the serum with ether abolished the rise in A/G without affecting the total protein concentration, or the normal value of A/G, suggested the existence of a nonprotein factor, which appeared in the serum after total-body irradiation. The hypothetical factor did not influence the electrophoretic analysis, although it did affect the methods of ultracentrifugation and salt fractionation.

An attempt was made to extract such a factor from the plasma or serum. The activity of the extracts was tested as follows. To 0.5 ml. of normal plasma or serum, 0 to 0.2 ml. of extract was added, and the volume made up to 0.7 ml. with 0.85 per cent sodium chloride. Sodium sulfite fractionation or ultracentrifugation (with larger volumes) was carried out after several minutes.

The best source of the factor was fresh plasma, though serum which had been preserved on ice was active. The most active plasma was taken from animals exposed to about 700 r 4 days previously. The crude isolation of the factor was carried out as follows. One part of plasma was precipitated with eight parts of alcohol-ether mixture (3:1), and centrifuged. The supernatant liquid, after filtration through a coarse paper, was evaporated to dryness on the water bath. The residue was extracted several times with warm petroleum ether, and the

petroleum ether discarded. The extracted residue was then dried in a desiccator, in which it retained activity for at least a month.

The yield of dried residue was about 6 mg. per ml. of plasma, both for normal and irradiated rats. The residue contained no phosphorus extractable with petroleum ether, and gave negative biuret and Lieberman-Burchard reactions. For testing, the residue was suspended in 0.85 per cent sodium chloride solution at a concentration of 60 mg. per ml. (occasionally higher), forming a light yellow, cloudy solution. When this solution was centrifuged at 2,000 r.p.m. for 15 minutes, a crystal-clear, yellow supernatant solution was obtained, which was inactive. When the precipitate was made up to the original volume, it was active, but not as much so as the original cloudy solution from which it had been obtained. The tests reported below deal with the original, cloudy solutions. The experiments had to be discontinued before further work on purification could be done.

Six residues were prepared according to the above procedure from six different batches of pooled plasma or serum, either fresh or frozen. The first and second residues were prepared at the same time from comparable groups of control and irradiated animals. The other four were prepared at various times from material expected to vary in activity, obtained from irradiated animals.

The first and second residues were compared several days after preparation, and again 3 weeks later. Table II shows that the residues from the irradiated animals were more active than those from the controls, that the residues retained their activity for 3 weeks, and that either serum or plasma could be used to test the activity of the residues. The addition of 6 to 12 mg. of residue produced for all practical purposes the maximum effect. The optimal concentration of residue in the test was confirmed, using the same procedure, with two other batches of residue (Residues 3 and 4). The greater activity of the extracts from irradiated animals' sera, when compared with controls, was confirmed in another experiment in which the preparation of the residues and the procedure had been somewhat different and therefore were not numbered in these series.

The activity of Residues 4 and 5 was tested by ultracentrifugation as well as by salt fractionation. Table I shows (Expts. 8 and 9, Columns 5 and 7) that some rise in A/G was evident in the ultracentrifuge after the addition of the residue, but this increase was much less than that measured by salt fractionation. The explanation for this difference was not apparent. Concentrations as high as 20 mg. of residue per ml. of serum had little or no measurable effect upon the total refractive increment of the sera as observed in the ultracentrifuge.

TABLE II

Effect of Plasma Extracts (Residues) from Control and Irradiated Animals upon the A/G Ratio of Normal Rat Serum and Plasma

The stated number of mg. of residue, suspended in 0.2 ml. of 0.85 per cent sodium chloride, were added to 0.5 ml. of normal serum or plasma, and A/G was then determined by sodium sulfite fractionation.

Residue			Test Medium	A/G when stated mg. of residue added					
No.	Age	Source		0	.6	1.2	3	6	12
	days								
1	3	controls	plasma	2.0	-	-	2.3	2.4	2.5
	24	controls	serum	1.7	1.7	1.8	1.9	2.0	2.2
2	3	650 r[a]	plasma	2.0	-	-	2.8	2.9	3.1
	24	650 r[a]	serum	1.7	1.9	2.1	2.2	2.8	3.0

[a] Exposed to 650 r 4 days before plasma was taken for extraction.

The sixth, and least active, residue was tested on sera of several species. Twelve milligrams of residue raised the A/G of 0.5 ml. of rat serum from 1.75 to 2.5, of human plasma from 2 to 2.5, and of guinea pig plasma from 3.1 to 4.4.

These experimental results with the residues were consistent with the hypothesis suggested above, that a nonprotein substance becomes active in the plasma following total-body irradiation, interfering with the determination of A/G by salt fractionation or ultracentrifugation. The residues obtained, however, could not have been identical with the active material in the plasma, though it seems possible that they contained a considerable amount of it. Ether extraction of the plasma before analysis brought the elevated A/G to normal, although no activity could be demonstrated in the ether extract itself. It was of interest that the active residues formed cloudy solutions, whose activity seemed to depend upon more than one component, and that some active material was extracted from a normal plasma. The latter finding recalls the situation in the guinea pig (1) where ether extraction decreased the A/G of normal plasma itself. Following irradiation A/G rose as in the rat, and ether extraction of such plasma returned the value of A/G to that of ether-extracted normal plasma.

Whether a similar rise in A/G occurs in other species is not known.

Electrophoretic studies on the serum of irradiated dogs showed a fall in A/G ratio, the albumin level falling and the globulin level rising. The changes were associated with the onset of fever and were thought to be associated with infection (12).

SUMMARY

Total-body X irradiation (250 kvp.) of the rat caused a rise in the serum or plasma albumin-globulin (A/G) ratio. The effect was maximal 4 days after exposure to about 700 r. The rise was found when the A/G ratio was determined by sodium sulfite fractionation or by the analytical ultracentrifuge, but not by electrophoresis. Ether extraction of the plasma just prior to analysis returned the elevated value of A/G to normal. A substance was obtained from alcohol-ether extracts of plasma or serum which raised the A/G ratio of normal rat serum or plasma *in vitro*.

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