JCI The Journal of Clinical Investigation

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J Clin Invest. 1952;31(12):1064-1068. https://doi.org/10.1172/JCI102700.

Research Article

AZORUBIN-BINDING CAPACITY AND PROTEIN COMPOSITION OF SERUM OF RATS SUBJECTED TO TOURNIQUET SHOCK AND TO TREATMENT WITH CARBON TETRACHLORIDE

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(Submitted for publication July 28, 1952; accepted September 17, 1952)

The cause of a decreased azorubin-binding capacity (ABC) of serum albumin, previously observed in humans under certain pathological conditions (1), is not known. The present paper is part of an experimental study into the mechanism of a lowering of the ABC (2). It describes the production of decreased ABC values of serum albumin of rats by subjecting them to tourniquet shock or to treatment with carbon tetrachloride.

In addition, the serum protein composition of these experimental rats was analyzed, since changes in the protein metabolism have been observed in traumatic shock (3, 3a).

EXPERIMENTAL

Chemical procedures. For the determination of the protein and non-protein nitrogen, the micro-Kjeldahl method of Hiller, Plazin, and van Slyke (4) was used, with some modifications which are described elsewhere (5). The non-protein nitrogen was separated from the serum proteins by precipitation with uranyl acetate according to the procedure of Neubauer (6). One ml. of serum was diluted with 3 ml. of water, and 1 ml. of 1.5 per cent uranyl acetate was added, with stirring, to the solution. The protein was filtered off after 60 minutes, using a Whatman No. 50 filter.

Since preliminary experiments indicated that a chemical determination of serum albumin with a 26.8 per cent sodium sulfate solution (7, 8) gave low values (for details see [5]), all albumin determinations were done by electrophoretic analysis.

Electrophoretic analysis. The veronal-acetate-sodium chloride buffer of Michaelis, pH 8.6, $\mu = 0.1$, was employed (9). Practically identical patterns were obtained in veronal buffer as used by Moore, Levin, and Leathem for rat sera (10). All pH measurements were done at room temperature with a glass electrode. The electrophoretic analyses were carried out in the compact Tiselius apparatus[1] (11), employing the Longsworth scanning mechanism and the 2 ml. cell in an open system. Some sera were also analysed in a standard size Tiselius electrophoresis apparatus[2] using the long analytical cell (12); practically the same patterns and the same albumin percentages were obtained. The albumin concentrations were defined according to Wiedemann (13) and determined from both the descending and ascending boundaries. The globulin area was divided, by the method of Tiselius and Kabat (14), into three parts corresponding approximately to the alpha-, beta-, and gamma-globulin of human sera. More details on the electrophoretic analysis are given elsewhere (5).

For the electrophoretic analysis of normal rat serum in the presence of azorubin, a solution of 5 mg. purified azorubin (for purification see [15]) in 8 ml. buffer was added to 4 ml. of serum and dialysed. The buffers used were Michaelis buffer pH 8.6 and phosphate buffers of pH 8.6, 7.7, 7.2, and 6.1, $\mu = 0.1$. The patterns were recorded on Kodak Ektachrome Color Film, using the optical system of Philpot-Svensson and a diagonal bar in the standard size electrophoresis apparatus.

Determination of ABC. The chromatographic method for estimating the ABC (16) was modified so that only 1 ml. of serum was needed for the single chromatographic runs. A total of 4 or 3 ml. of serum was required, therefore, for each analysis, which consisted of a blank run and triplicate, or at least duplicate, runs of the serum-azorubin mixtures (see below). The chromatographic tubes employed were about 20 cm. long, with an inner diameter of 5 mm., and terminated in a 0.5 mm. capillary of 2 to 3 cm. length. A funnel was sealed to the upper end. The columns were formed using a slurry of 500 mg. of anionotropic aluminum oxide in freshly prepared, twice-distilled water. Methods for preparation and deactivation of anionotropic aluminum oxide have been described previously (15, 16). The length of the column obtained was about 25 mm. The average total flow time for the serum-azorubin mixture described below was 9.5 minutes.

For each run, 1 ml. of serum and 0.25 ml. of an azorubin solution (mostly 0.5 per cent) in 0.6 per cent sodium chloride (pH 7.8) were combined at least 30 minutes prior to the analysis. The azorubin concentration in the mixture should be at least twice as high as the "observed ABC" (see below). For the blank run, 0.25 ml. of 0.6 per cent sodium chloride was added to 1 ml. of serum. The mixtures were poured on the column immediately after the water (used to wash in the aluminum oxide) ceased to run out of the capillary. The first 1.0 ml. of the filtrate was discarded and 2 dilutions (1:20) of the last 0.25 ml. (pH 6.1) were made with M/15 phosphate

[1] Model 38 of the Perkin-Elmer Corporation, Glenbrook, Conn.

[2] Frank Pearson Associates, New York 12, N. Y.

buffer, pH 7.7 (0.1 ml. + 1.9 ml. buffer). The optical density was determined in a Coleman Junior Model 6A spectrophotometer at 515 mμ using 10 × 75 mm. tubes. The azorubin concentrations were read in mg. per cent from standard curves prepared as described elsewhere (15). The value obtained is the "ABC observed." This modified procedure has been found (15) to give the same results as the previous method (16).

The "specific ABC" was calculated in moles azorubin per gram of albumin, according to

$$ABC_{spec.} = \frac{ABC_{obs.} \cdot 10^{-3}}{(0.8\ C - A)\ 502}$$

where $ABC_{obs.}$ = ABC observed, in mg. per cent;

C = albumin concentration of serum, in grams per cent;

A = 0.13 for values of C > 2.9%;
0.12 for values of C < 2.9%.

The correction factor "A" accounts for the amount of albumin adsorbed by the aluminum oxide (see reference 15, Table 7). Studies on reproducibility and reliability of the chromatographic procedure for the estimation of the ABC have been described (15).

Animal experiments. Male Sprague-Dawley rats, fed on Purina Dog Chow checkers, were used. The average temperature of the animal room was about 24° C. The rats were left without food and water for 24 hours before the experiments. Weights given were those of the fasted animals. Tourniquet shock was produced as in previous studies (17). The rubber bands were applied to both hind limbs for 4½ hours and the blood was drawn by heart puncture 3 hours after release of the tourniquets. The average yield per rat in "shock" was about 2 ml. of blood and 0.5 ml. of serum, whereas 6 ml. of blood and 2.2 ml. of serum were obtained from the normal rats.

The carbon tetrachloride (C. P. Medicinal) was injected intraperitoneally (0.5 ml. per kg. every second day for from 7 to 16 days) (18). Some rats that did not lose weight after several injections were then given intraperitoneal injections of a 1:1 mixture of carbon tetrachloride and 95 per cent ethanol (1 ml./kg). The treatment with this solution (19) caused the weight of the animals to decline rapidly. The histological examination of the livers of a number of rats showed toxic degenerative changes which were consistent with the known picture of carbon tetrachloride poisoning. The amounts of blood and serum obtained from the carbon tetrachloride-treated rats were approximately the same as those from normal animals. Since the amount of serum obtained from one rat was not sufficient for the various analyses, pooled samples were used in all groups, as indicated in Table I.

TABLE I

Azorubin-binding capacity (ABC) of serum albumin of normal, shocked, and carbon tetrachloride-treated rats

1 Serum pooled from No. of rats	2 Average weight gm.	3 NPN mg. %	4 Albumin %	5 Globulin %	6 Specific ABC 10^{-5} moles azorubin per gm. albumin
A. *Normal Rats*					
6	396	38.3	4.06	2.25	2.38
4	411	37.8	3.74	3.07	2.66
4	408	30.0	3.97	2.85	2.24
3	389	35.3	3.29	3.23	2.45
4	280	33.3	4.33	2.33	2.20
4	255	38.0	4.07	2.65	2.96
Average Values*	357	35.5	3.91	2.73	2.48
B. *Rats Subjected to Tourniquet Shock*					
8	365	70.0	3.97	2.97	2.02
13	344	75.5	4.84	2.02	1.70
13	322	78.5	3.36	3.15	2.17
23	266	83.5	3.17	2.26	1.83
12	276	84.5	3.56	2.01	1.80
11†	346	138.0	4.08	2.89	1.33
Average Values*	313	78.4	3.78	2.48	1.91
C. *Rats Treated with Carbon Tetrachloride*					
4	317/248‡	41.8	3.16	3.03	1.40
4	318/264	43.5	3.09	2.58	1.91
5	314/223	46.5	3.07	2.64	2.04
4	292/251	43.0	3.69	2.48	1.72
4	310/241	49.0	3.01	2.27	1.85
5	307/270	48.8	2.88	1.99	1.27
4	345/305	43.8	3.84	2.87	1.43
Average Values*	315/257	45.2	3.25	2.55	1.66

* All average values were calculated as arithmetical means of the figures obtained, not considering the varying number of animals in the groups.

† Tourniquets applied unilaterally to the left fore and hind legs for 5 hours, blood drawn 17 hours after removal. Not included in average values.

‡ Weights before and after treatment.

RESULTS

The electrophoretic analysis of normal rat sera in the presence of azorubin demonstrated that this dye is bound exclusively to the albumin component, at pH values between 6.1 and 8.6.

The results of the serum analysis and the ABC values are given in Table I. The average specific ABC values of the serum albumin of the shocked and carbon tetrachloride-treated rats were 23 per cent and 33 per cent, respectively, lower than those of the normal controls. In one group of rats which were subjected to more severe conditions of tourniquet shock, a 46 per cent decrease of the ABC was observed.

Table II shows the percentage values of the electrophoretic protein components for the three series of rats.

TABLE II

Electrophoretic analysis of serum proteins of normal, shocked, and CCl_4-treated rats

Protein components in per cent of total protein

Rats, treatment	Total protein %	Albumin	Globulin		
			Alpha	Beta	Gamma
Normal	6.31	63.9	9.9	17.3	8.9
Normal	6.81	54.9	10.1	22.1	12.9
Normal	6.82	58.2	14.6	17.9	9.3
Normal	6.52	50.4	18.6	20.3	10.7
Normal	6.66	65.0	12.6	14.9	7.5
Normal	6.72	60.6	8.2	15.1	16.1
Average	6.64	58.9	12.3	17.9	10.9
Tourniquet Shock	6.94	57.2	8.7	23.5	10.6
Tourniquet Shock	6.97	58.5	16.1	19.3	6.1
Tourniquet Shock	6.86	70.6	8.2	17.0	4.2
Tourniquet Shock	6.51	51.6	10.2	23.0	15.2
Tourniquet Shock	5.43	58.4	13.0	19.2	9.4
Tourniquet Shock	5.57	63.8	11.4	16.2	8.6
Average	6.38	60.0	11.3	19.7	9.0
CCl_4-Treatment	6.19	51.1	22.1	19.7	7.1
CCl_4-Treatment	5.67	54.5	19.5	16.6	9.4
CCl_4-Treatment	5.71	53.8	24.9	15.3	6.0
CCl_4-Treatment	6.17	59.8	17.3	16.7	6.2
CCl_4-Treatment	5.28	56.9	14.8	19.2	9.1
CCl_4-Treatment	4.87	59.2	14.6	18.4	7.8
CCl_4-Treatment	6.71	57.3	16.0	18.4	8.3
Average	5.80	56.1	18.5	17.7	7.7

DISCUSSION

Analyzing the data of Table II, it should be pointed out that the electrophoretic analysis of the rat sera did not result in patterns in which the different globulin components were as distinct as generally seen with human sera. This is in accordance with observations of Gjessing and Chanutin (20). The separation of the globulin area into the three components is, therefore, rather arbitrary, even more than in the differentiation of the globulins in human sera (21).

In spite of this limitation, Table II shows that subjecting rats to tourniquet shock, under the conditions described, did not change markedly the concentration of albumin or of the globulin components in the serum. The results did not indicate any shift of serum albumin from the circulating blood into the injured tissues as demonstrated by a different experimental procedure by Moore and his associates in mice (22) and dogs (23) in traumatic shock. The electrophoretic patterns of the sera of "shocked" rats (Table II) did not reveal the appearance of increased quantities of a protein with a mobility similar to gamma-globulin as observed in mice under certain conditions of severe traumatic shock (22).

The average absolute and relative albumin values of the carbon tetrachloride-treated rats were lower than those obtained in the normal animals. A slight increase in the average alpha-globulin value was obtained in the rats that received carbon tetrachloride. High values of this globulin component have been found under various pathological conditions in several species, among others in injured rats (20).

The specific ABC values were determined on the basis of the finding that azorubin is bound exclusively to albumin in rat serum. A similar result was obtained previously with human serum (1, 16). Assuming a molecular weight for rat serum albumin similar to that of human albumin, 1 mole of serum albumin of normal rats was found to bind 1.71 moles of azorubin; the values for "shocked" rats were 1.32 and 0.92 moles, depending on the severity of the shock produced. In the serum of carbon tetrachloride-treated rats, 1.14 moles of azorubin were bound per mole albumin. It may be pointed out in this connection that the average specific ABC value obtained with the chromatographic procedure in humans was 5.8 moles of azorubin per mole of albumin in normal persons, whereas values down to about 1 mole were observed in pathological cases (1).

The present studies demonstrate that it is possible to lower experimentally the ABC of serum albumin in rats. This decrease of the specific ABC values is considered to be typical of the albumin of the experimental rats, and not to be caused by a lower concentration of "normal" albumin in the treated animals. Previous studies on the chromatographic method have shown (16) that the specific ABC value of a given serum albumin is lowered by an extreme reduction of the albumin concentration of the test solution. However, an investigation into the quantitative side of this effect (see reference 15, Fig. 2) has shown that the specific ABC value of a 3.25 per cent solution of human albumin is less than 0.2 per cent lower than that of a 3.91 per cent solution of the same albumin. Assuming the binding of azorubin to rat serum albumin and that to human serum albumin to be essentially similar, a difference of about 0.2 per cent would be expected between the specific ABC values of the sera of normal and those of carbon tetrachloride-treated rats (albumin concentration 3.91 and 3.25 per cent, re-

spectively, see Table I), if such a difference were caused solely by the concentration effect described above. It seems improbable, therefore, that decreases in the specific ABC 100 to 200 times as great (23, 33 or 46 per cent) as observed between the normal and experimental groups (Table I) could be explained on the basis of differences in the albumin concentrations.

The degree of decrease in the specific ABC values of the "shocked" rats was proportional to the severity of their condition (Table I, last group of "shocked" rats compared to the other groups). Analyzing the single data of all groups of rats studied (Table I), no proportionality was found between albumin concentration and the degree of lowering of the specific ABC values. These observations are considered to be additional evidence for the conclusion that the albumin concentration does not play an essential role in the lowering of the specific ABC values obtained in the experimental animals. Disregarding the species difference, this is in contrast to the claim of Bennhold, Ott, and Kallee (24) that a decrease in the specific ABC values of human sera is caused exclusively by a reduced albumin concentration.

An analysis of the mechanism of a lowering of the ABC of serum albumin is believed to be of interest since a decrease in the ABC is considered to be but one example of the general phenomenon of a lowered capacity of serum albumin to bind anions. Decreased binding values of serum albumin for another anion, phenolsulfonephthalein, have been observed in cancer patients (25) and in pregnant women (26).

It is believed by the present authors that the decrease in the ABC of serum albumin, observed in different species under various conditions, is not caused by chemical differences in the albumin molecules, but rather by the binding of certain anions, *e.g.*, fatty acids, to the albumin. A firm binding of such anions would block the binding sites for azorubin and thus reduce the ABC. This conclusion is based on the following findings:

No proportionality was observed between the specific ABC values of the albumin fractions isolated by the cold alcohol fractionation procedure from sera giving normal and sera giving decreased specific ABC values. No chemical differences in the protein composition were observed either between the albumin fractions obtained from normal ABC-sera and those from low ABC-sera (1). The significance of these negative results is limited, however, since alterations during the preparation of the albumin fractions cannot be excluded.

Any degree of decrease in the specific ABC of serum albumin could be produced by addition of various amounts of certain fatty acids and other anions to albumin solutions and normal sera (2). This effect was exerted by very small amounts of anions, *e.g.*, one mole of myristic acid per mole of human albumin decreased its specific ABC by 26 per cent, and 5 moles of stearic acid per mole of albumin lowered the ABC value of a human serum by about 30 per cent. It may be noted that in all these experiments the albumin concentrations of the test solutions containing the anions were equal to those of the control solutions.

Experiments are in progress to find out whether the albumin present in low ABC-sera contains greater amounts of firmly bound anions (higher fatty acids?) than that present in normal sera. The results of these studies may aid in an understanding of the relationship between the decreased specific ABC values and the pathological conditions under which they occur.

SUMMARY

1. The protein composition of the serum of rats subjected to tourniquet shock was found not to be significantly different from that of normal rats. Administration of carbon tetrachloride caused a slight elevation of the serum alpha-globulin level, and some decrease in the average albumin concentration.

2. Electrophoretic studies demonstrated that in rat serum the anionic dye azorubin is bound exclusively to albumin.

3. The azorubin-binding capacity of serum albumin of rats was decreased in tourniquet shock and after treatment with carbon tetrachloride.

4. This lowering of the specific azorubin-binding capacity was not caused by and not related to a decrease in the albumin concentration.

5. A possible mechanism of a decrease in the azorubin-binding capacity of serum albumin is suggested.

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