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# ARMY MEDICAL RESEARCH LABORATORY

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REPORT NO. 76  
15 February 1952

AZORUBIN-BINDING CAPACITY OF SERUM ALBUMIN  
OF RATS SUBJECTED TO TOURNIQUET SHOCK AND  
TO TREATMENT WITH CARBON TETRACHLORIDE\*

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REPORT NO. 76

AZORUBIN-BINDING CAPACITY OF SERUM ALBUMIN  
OF RATS SUBJECTED TO TOURNIQUET SHOCK AND  
TO TREATMENT WITH CARBON TETRACHLORIDE\*

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FORT KNOX, KENTUCKY  
15 February 1952

\*Subtask under Environmental Physiology, AMRL Project No. 6-64-12-028, Subtask, Effect of Stress on Physicochemical Behavior of Blood Proteins.

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## ABSTRACT

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

#### OBJECT

In order to characterize certain biochemical changes occurring in traumatic shock, and to study the mechanism by which a decrease in the azorubin-binding capacity (ABC) of the serum albumin molecules is caused in vivo, the ABC of serum albumin was determined in rats under various conditions.

#### RESULTS

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. Electrophoretic studies demonstrated that the anionic dye azorubin is bound exclusively to albumin in rat serum, in a way similar to that previously observed with human serum. The ABC of serum albumin was decreased in rats subjected to tourniquet shock and after treatment with carbon tetrachloride. No correlation was found to exist between the lowering of the specific ABC and the albumin concentration.

#### CONCLUSIONS

In the rat subjected to tourniquet shock or to treatment with carbon tetrachloride, metabolic alterations take place which cause the ABC of the serum albumin to decrease. These metabolic changes are believed to be similar to those which are responsible for the lowering of the specific ABC values of serum albumin in humans observed under certain pathological conditions.

## RECOMMENDATIONS

On the basis of the present findings, the mechanism of the lowering of the specific ABC should be studied. Such an investigation may contribute to our knowledge of the metabolic alterations occurring under shock conditions. It may also result in an understanding of the cause of the subnormal ABC values of serum albumin observed in certain human patients.

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AZORUBIN-BINDING CAPACITY OF SERUM ALBUMIN  
OF RATS SUBJECTED TO TOURNIQUET SHOCK AND  
TO TREATMENT WITH CARBON TETRACHLORIDE\*

I. INTRODUCTION

A number of metabolic alterations have been observed in experimental animals subjected to traumatic shock (1). Using a standardized procedure to produce tourniquet shock, definite changes in the protein metabolism were found in the serum of rats (2, 3). In an attempt to characterize further the serum proteins in traumatic shock, the azorubin-binding capacity (ABC) of rats' serum albumin was determined.

Another aim of this investigation was to develop experimental conditions for elucidating the mechanism by which a decrease of ABC in the serum albumin molecules is effected in vivo. Subnormal ABC values have been observed previously in certain pathological cases in humans (4).

II. EXPERIMENTAL

A. Chemical Procedures.

The micro-Kjeldahl method was used for the determination of the protein and non-protein nitrogen. Essentially, the procedure of Hiller, Plazin, and van Slyke (5) was used, with the following modifications: Digestion was continued for two hours after the mixture became clear. To prevent bumping, brick chips heated at about 470°C for two hours were employed. Seven to eight ml of 10 N sodium hydroxide were added to the digested material prior to the distillation. Five drops of 0.1% methyl red in 95% ethanol were used as indicator in the titration flask. The end point was estimated by comparing the color with that of a solution containing the indicator in an equal volume of acetate buffer, the pH of which was adjusted to 5.31 (glass electrode). Two similar solutions of pH 5.21 and pH 5.41 were useful for this comparison.

The non-protein nitrogen was separated from the serum proteins by precipitation with uranyl acetate according to the procedure described by Neubauer (6). One ml of serum was diluted with 3 ml of water and 1 ml of 1.5% uranyl acetate was added, with stirring, to the solution. The protein was filtered off after 60 minutes, using a Whatman #50 filter.

In some preliminary experiments, a chemical determination of serum albumin was attempted by precipitation of the globulins with a

26.8% sodium sulfate solution (7,8). 0.2 ml of serum was mixed with 3.8 ml of the sulfate solution and, after about 18 hours, the mixture was filtered through a Whatman #50 filter. These operations were carried out in a covered jar, completely submerged in a constant temperature water bath of 37°C. The total nitrogen content in the filtrate was determined by the micro-Kjeldahl method and the globulin level was estimated by difference between total protein and albumin.

#### B. Electrophoretic Analysis.

In the electrophoretic analysis the veronal-acetate-sodium chloride buffer of Michaelis, pH 8.6,  $\mu = 0.1$ , was used as recommended by Wiedemann (9). The patterns obtained were practically identical with those observed in veronal buffer as used by Moore, *et al.* for rat sera (10). 0.8 ml of serum was placed in cellulose tubing of 8/32 inch diameter\* and dialysed for at least two days at 2°C on a shaking machine with repeated changes of the Michaelis buffer. The serum was then diluted with the buffer to a total volume of 2.4 ml. Turbid solutions were cleared by centrifugation at 12,000 g, 3°C. The electrophoretic analyses were carried out in the compact Tiselius apparatus (11)\*\*, employing the Longworth scanning mechanism and the 2 ml cell in an open system. The electrophoretic determinations were run for 120 to 150 min. at a temperature close to 0°C as provided by the ice-water bath. The potential gradient ranged from 5.8 to 7.5 volts per cm at an overall consumption of about 0.6 to 1.2 watts.

In some cases, the results were checked by an additional analysis of the sera in a standard size Tiselius electrophoresis apparatus\*\*\* using the long analytical cell (12). Practically the same patterns and the same albumin percentages were obtained.

The electrophoretic patterns of both the ascending and descending boundaries were photographed on the same films as used for the base lines. They were projected in a distortion-free way with a photographic enlarger and traced at a linear enlargement of approximately 3 times

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\* The Visking Corporation, Chicago 38, Illinois.

\*\* Model 38 of the Perkin-Elmer Corporation, Glenbrook, Connecticut.

\*\*\* Frank Pearson Associates, New York 12, New York.

original size. On the tracings, the areas attributable to albumin were defined according to Wiedemann (13) by superprojecting ideal Gaussian curves. The percentage of albumin was estimated by measuring the areas representative of this component and of total globulin with a compensating polar planimeter\*. The areas corresponding to the protein components were computed from both the ascending and the descending boundaries. The mean percentage values found were used for calculating Albumin concentrations from the total protein values obtained by the Kjeldahl analysis.

On the enlarged tracings the areas representing the various globulin components were defined by the method of Tiselius and Kabat (14) in which ordinates are drawn from the lowest point between each two components to the base line. The total globulin area was divided into three parts corresponding approximately to the alpha-, beta-, and gamma-globulin of human sera. Measuring the area attributed to the globulin components and calculating their percentage was done as described for albumin.

For the electrophoretic analysis of normal rat serum in the presence of azorubin, 4 ml of serum were dialysed against the Michaelis buffer of pH 8.6 and then mixed with 10 mg of purified azorubin (15) dissolved in 8 ml of the buffer last used for the dialysis. This equals a ratio of azorubin to albumin five times as high as the ABC of normal rat's serum albumin as determined by the chromatographic procedure. In some experiments a solution of 5 mg of azorubin in 8 ml of buffer was added to 4 ml of serum prior to the dialysis. The electrophoretic analysis of the interaction of azorubin with the proteins of rat serum was also carried out in phosphate buffers of pH 7.7 and pH 7.2  $\mu = 0.1$ . 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.

### C. Determination of ABC.

The ABC values of the sera were determined as described in a previous report (15). A correction was made for the small amount of albumin adsorbed on the aluminum oxide during the chromatographic

analysis. 0.13% of albumin was subtracted from the albumin concentration of the serum diluted 4:5 by addition of either the azorubin solution or of the 0.6% sodium chloride solution. If the albumin concentration of the 4.5-diluted serum was below 2.30%, 0.12% was subtracted. These figures were taken from Table 7 of a previous report (15). For the calculation of the specific ABC values, albumin concentrations as determined by electrophoresis were used throughout.

#### D. Animal Experiments.

White, male, Sprague-Dawley rats fed on Purina dog chow checkers were used for all studies. The average temperature of the animal room was kept at about 24°C. The rats were left without food and water for 24 hours before the experiment. Weights are given as those of the fasted animals. Tourniquet shock was produced as described in previous studies performed in this laboratory (2, 3); elastic rubber bands (Eberhard Faber No. 30) were applied in five tight turns to both hind legs, and generally left on for 4-1/2 hours. The blood was drawn by heart puncture 3 hours after removal of the tourniquets. The syringes and needles (Size No. 19) were coated with a thin layer of paraffine oil\* and rinsed thoroughly with distilled water and finally rinsed with saline.

The blood was kept in the refrigerator over night and then centrifuged at 2000g, 3°C. Since only about 2ml of blood and 0.5 ml of serum could be obtained from one rat in "shock", the sera of several animals had to be pooled for all experiments. The average yield per normal rat was 6 ml blood and 2.2 ml serum. In the analysis of the sera of the shocked animals no corrections were made for hemoconcentration.

The administration of carbon tetrachloride (C.P. Medicinal) was done by injecting intraperitoneally 0.5 ml per kg every second day. This procedure has been found to lead to a fairly reproducible degree of liver injury with only minor extrahepatic manifestations (16). Following this treatment the weight of the animals dropped considerably. Some rats which did not show much decrease in weight after several injections were subsequently given intraperitoneal injections of a 1:1 mixture of carbon tetrachloride and 95% ethanol (1 ml/kg). This solution which has been used for oral administration (17) affected the animals more severely, if the decrease of body weight was used as a criterion. In a number of rats the livers were examined histologically for the effectiveness of the carbon tetrachloride treatment. The sections showed toxic degenerative changes

\* White Mineral Oil, U.S.P., Viscosity 125/130.

in the liver cells 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.

### III. RESULTS

It is known from electrophoretic studies that azorubin added to normal or pathological human serum is bound exclusively to the albumin component (4, 18) even if the ratio of azorubin to albumin exceeds the ABC many times. A similar result was obtained in the present investigation using serum of normal rats. At pH 8.6 in Michaelis buffer, as well as in phosphate buffer at pH 7.7 and pH 7.2, the azorubin combined exclusively with the albumin. No dye interacted with any of the globulin components as could be seen at the descending side. If the azorubin was added after completion of the dialysis the presence of free dye, representing the excess over the binding capacity of the rat serum albumin, was demonstrated by a fast electrophoretic migration of protein-free azorubin in front of the ascending serum protein boundaries. The specific ABC of rat serum albumin was determined by dividing the ABC observed, by the albumin concentration estimated by electrophoresis.

An attempt was made to apply the fractionation with 26.8% sodium sulfate, successfully used in the albumin determination of human sera (7, 8), to the sera of normal rats. However, very low albumin concentrations were found by this method; the average value of the 6 sera shown in Table 1 was 1.74%, i. e., about 45% of the electrophoretic value. An electrophoretic analysis of the precipitated protein fraction, which is supposed to contain "euglobulin" plus "pseudoglobulin", revealed the presence of albumin in considerable quantities. Therefore, the 26.8% sodium sulfate precipitation cannot be used for the albumin determination in rat serum. This is in contrast to our observations with rabbit sera in which the albumin content, determined by this salt fractionation, was found to be in good agreement with the electrophoretic analysis (19).

The results of the studies in normal and "shocked" rats are given in Table 1, A and B. No significant differences are apparent between the two groups in the total protein and albumin concentrations of the sera. The NPN values of the sera (Table 1, B) were increased, indicating the severity of the shock. The specific ABC values of the serum albumin of the shocked rats were, on the average, 27% lower than those of the normal controls. Total protein and albumin values of the sera of the carbon tetrachloride-treated rats (Table 1, C) were lower than those of the

TABLE 1.  
ABC VALUES OF SERUM ALBUMIN OF NORMAL, SHOCKED  
AND CARBON TETRACHLORIDE -TREATED RATS.

1	2	3	4	5	6	7	8	9	10
Serum Pooled From No. of Rats	Average Weight Grams	CCl <sub>4</sub> Treatment Days	NPN mg%	Total Protein %	Albumin by Electrophoresis % of Total Protein	Albumin* %	Globulin** %	ABC Observed mg%	Specific ABC in 10 <sup>-5</sup> Mol Azorubin per g Albumin
<b>A. Normal Rats</b>									
6	396	-	38.3	6.31	63.9	4.06	2.25	37.3	2.38
4	411	-	37.8	6.81	54.9	3.74	3.07	38.2	2.66
4	408	-	30.0	6.82	58.2	3.97	2.85	34.3	2.24
3	389	-	35.3	6.52	50.4	3.29	3.23	30.7	2.45
4	280	-	33.3	6.66	65.0	4.33	2.33	36.7	2.20
4	255	-	38.0	6.72	60.6	4.07	2.65	46.5	2.96
Average Values***	357	-	35.5	6.64	58.9	3.91	2.73		2.48
<b>B. Rats Subjected to Tourniquet Shock</b>									
8	365	-	70.0	6.94	57.2	3.97	2.97	31.0	2.02
11@	346	-	138.0	6.97	58.5	4.08	2.89	20.9	1.33
13	344	-	75.5	6.86	70.6	4.84	2.02	31.9	1.70
13	322	-	78.5	6.51	51.6	3.36	3.15	27.9	2.17
23	266	-	83.5	5.43	58.4	3.17	2.26	22.1	1.83
12	276	-	84.5	5.57	63.8	3.56	2.01	24.6	1.80
Average Values***	320	-	88.3	6.38	60.0	3.83	2.55		1.81
<b>C. Rats Treated with Carbon Tetrachloride</b>									
4	317/248@@	7	41.8	6.19	51.1	3.16	3.03	16.9	1.40
4	318/264	9	43.5	5.67	54.5	3.09	2.58	22.4	1.91
5	314/223	11	46.5	5.71	53.8	3.07	2.64	23.9	2.04
4	292/251	10	43.0	6.17	59.8	3.69	2.48	24.3	1.72
4	310/241	15	49.0	5.28	56.9	3.01	2.27	21.2	1.85
5	307/270	16	48.8	4.87	59.2	2.88	1.99	13.9	1.27
4	345/305	7	43.8	6.71	57.3	3.84	2.87	21.1	1.43
Average Values***	315/257		45.2	5.80	56.1	3.25	2.55		1.66

\* Calculated from columns 5 and 6.

\*\* Calculated as difference between columns 5 and 7.

\*\*\* 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.

@@ Weights before and after treatment.

control animals. Whether this difference is significant cannot be concluded from the small number of rats used. The specific ABC of the serum albumin was significantly decreased by 33%.

In Table 2, the per cent values of the electrophoretic protein components are computed for the three groups of rats. Practically no differences were found in the average percentages of alpha-, beta-, and gamma-globulin between normal and shocked rats. A slight increase was observed in the average value of the alpha-globulin component of the rats treated with carbon tetrachloride. In accordance with previous observations (20), the electrophoretic analysis of the rats' sera did not result in patterns in which the different globulin components were as distinct as generally obtained with human sera. The procedure of separating the globulin area into the three components is, therefore, rather arbitrary, even more than in the differentiation of the globulins in human sera (21). This should be taken into account when considering the data of Table 2.

#### IV. DISCUSSION

The data of Table 2 indicate that no major changes occur in the protein composition of sera of rats subjected to tourniquet shock under the conditions described. Moore, *et al.*, have presented definite evidence of a shift of serum albumin from the circulating blood into the injured tissue of mice (22) and dogs (23) in traumatic shock. The present electrophoretic analysis of the sera of our shocked rats failed to reveal deviations in the albumin concentrations from normal values. Appearance of increased quantities of protein having a mobility similar to gamma-globulin, as observed in mice under certain conditions of severe traumatic shock (22), was not seen in the electrophoretic patterns of the sera of shocked rats (Table 2). It should be noted, however, that the present data do not exclude the possibility of producing, in the rat, serum protein changes similar to those described in mice and dogs by exposing the animals to different conditions of experimental shock. The finding of high amounts of alpha-globulin in the sera of carbon tetrachloride-treated rats is in accordance with similar observations in injured rats (20).

The present studies further demonstrate that it is possible to experimentally lower the ABC of serum albumin in laboratory animals. Under conditions of the chromatographic method employed, one Mol of albumin of normal rat serum was found to bind 1.71 Mol of azorubin; the values for serum of shocked and carbon tetrachloride-treated rats were 1.25 and 1.14 Mol of azorubin per Mol of albumin, respectively.

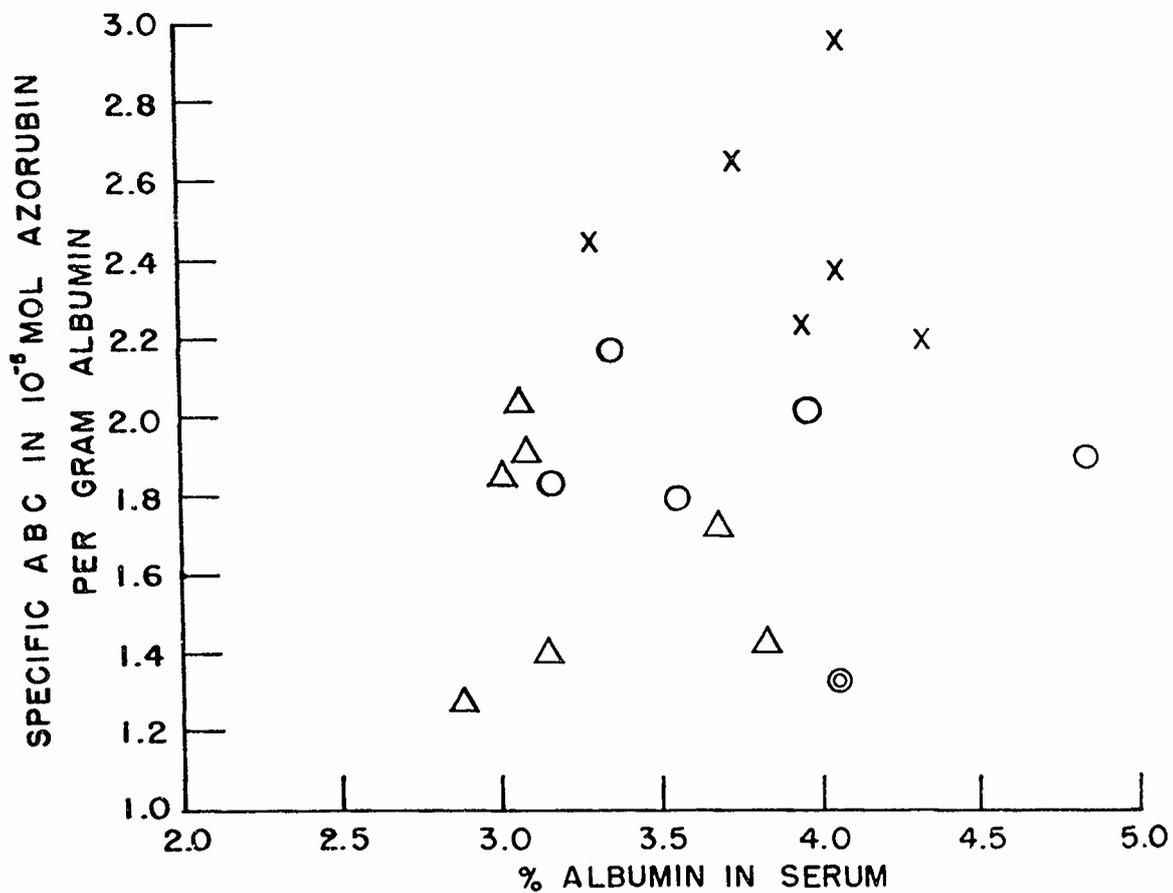
TABLE 2  
ELECTROPHORETIC ANALYSIS OF SERUM PROTEINS  
OF NORMAL SHOCKED AND CCl<sub>4</sub>-TREATED RATS.  
PROTEIN COMPONENTS IN PER CENT  
OF TOTAL PROTEIN

Rats Treatment	Total Protein %	Albumin	Globulin		
			Alpha	Beta	Gamma
Normal	6.31	63.9	9.9	17.3	8.9
..	6.81	54.9	10.0	22.0	12.9
..	6.82	58.2	14.5	17.7	9.2
..	6.52	50.4	18.5	20.2	10.7
..	6.66	65.0	12.5	14.8	7.4
..	6.72	60.6	8.1	14.8	15.9
Average	6.64	58.9	12.3	17.8	10.8
Tourniquet Shock	6.94	57.2	8.8	23.6	10.6
..	6.97	58.5	16.0	19.2	6.1
..	6.86	70.6	8.3	17.1	4.3
..	6.51	51.6	10.2	22.9	15.2
..	5.43	58.4	12.9	19.1	9.3
..	5.57	63.8	11.3	16.1	8.5
Average	6.38	60.0	11.3	19.7	9.0
CCl <sub>4</sub> -Treatment	6.19	51.1	22.0	19.5	7.0
..	5.67	54.5	19.2	16.4	9.3
..	5.71	53.8	24.4	14.8	5.8
..	6.17	59.8	17.3	16.7	6.2
..	5.28	56.9	14.7	19.1	9.0
..	4.87	59.2	14.5	18.3	7.8
..	6.71	57.3	15.9	18.3	8.2
Average	5.80	56.1	18.3	17.6	7.6

A decreased capacity of serum albumin to bind anionic dyes has been observed in sera of human patients by the method of equilibrium dialysis (24) as well as by means of the above chromatographic procedure (4). Huggins et al. (24) observed that sera of cancer patients, on the average, bound 22% less phenosulfonephthalein (PSP) per gram albumin than normal control sera. As can be calculated from the data given (24), 0.84 Mol of PSP were bound per Mol albumin, compared to 1.1 Mol in normal sera. In pregnant women, it has been shown that there is a decline of the PSP-binding capacity of serum albumin from a mean value (recalculated on a molar basis) of 0.96 Mol PSP during the fourth week of pregnancy to 0.58 Mol PSP per Mol albumin by the time for delivery (25).

It should be noted that numerical agreement between the binding values determined by the two methods cannot be expected for the following reasons: 1) the dye anions used are different; 2) the pH at which the binding capacity is measured with the chromatographic procedure is 6.1 (15), whereas a pH of 7.4 is employed in the equilibrium dialysis experiments (24); 3) in the chromatographic determination a certain part of the initially bound dye anion is detached, during the chromatographic analysis, from the albumin in a competitive reaction (4). The latter two features of the chromatographic method act in opposite directions: the anion-binding capacity is higher at lower pH values; the competitive adsorption of the azorubin on the aluminum oxide decreases the amount of dye estimated as finally bound to albumin.

The data obtained in the present experiments indicate that the lowering of the specific ABC is independent of the albumin concentration of the sera. Figure 1, computed from Table 1, shows the lack of correlation between the specific ABC values and the corresponding albumin concentrations. This seems noteworthy since attempts have been made by some investigators (26) to prove that a decrease in the specific ABC values of human serum albumin is caused exclusively by a lowering of the albumin concentration. The experimental data (obtained in a total of 13 sera including only 2 normal sera) do not seem to justify the conclusions drawn. The average specific ABC value of the 11 pathological sera was found to be 28% lower than the average specific ABC value of the two normal sera. The average of the five lowest of these 11 ABC values, observed in cases including liver cirrhosis, tuberculosis, and nephritis, was 45% below the average of the two normal sera (26). The albumin levels in these five pathological sera were in the range between 1.5 and 2.6%. At these concentrations the chromatographic method has been found (15) to give a specific ABC value for a given albumin, 4-5% lower than that found at the normal serum albumin concentration of 4%. The observed decrease of 45%, therefore, cannot be explained by the dependency of the specific ABC values on the albumin concentrations. It is hard to see how the experimental data obtained by the above investigators (26) can be interpreted as disproving a lowering of the specific ABC in pathological sera.



X 25 RATS, NORMAL  
 O 69 RATS, SHOCKED FOR 4 1/2 / 3 HRS.  
 ⊙ 11 RATS, SHOCKED FOR 5/17 HRS.  
 Δ 30 RATS, TREATED WITH CCl<sub>4</sub>

SPECIFIC ABC VALUES AND CONCENTRATION OF SERUM ALBUMIN IN RATS

## V. SUMMARY AND CONCLUSIONS

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.

2. Electrophoretic studies demonstrated that, in rat serum, the anionic dye azorubin is bound exclusively to albumin in a way similar to that previously observed with human serum.

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

4. No correlation was observed between the lowering of the specific azorubin-binding capacity and the albumin concentration.

5. The metabolic alterations which cause the azorubin-binding capacity of the serum albumin to decrease are believed to be similar to those which are responsible for the lowering of the azorubin-binding capacity of serum albumin in certain human pathological cases.

## VI. RECOMMENDATIONS

The mechanism of the lowering of the specific azorubin-binding capacity should be studied. Such an investigation may contribute to our knowledge of the metabolic alterations occurring under shock conditions. It may also result in a better understanding of the cause of the subnormal ABC values of serum albumin observed in human patients.

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