types of ACTH preparations. In 6 of the preparations treated with acid and heat as indicated above, pressor effects were absent in the 100 μg dose levels tested, whereas it was uniformly present in the nontreated preparations. In the instance of the pressor tests, no effort was made to assay for total original contamination. It was thought to be of importance that antidiuretic and pressor activities were found to parallel each other, both in qualitative and in quantitative tests insofar as these were conducted.

The assumption is made in these experiments that the anti-diuretic and pressor activities noted in these ACTH preparations is of posterior lobe origin. No note was made for the 90-minute intervals reported, of any distinct difference in the form of the urinary excretion curves obtained after injection of: (1) ACTH test substances; (2) pitressin; or (3) ACTH tested with added pitressin. No tests have been carried out routinely for oxytocic or chloruretic activity.

The acid and heat treatment described does not inactivate the ACTH activity of these preparations as tested by their capacity to produce lowering of the ascorbic acid level of the adrenals of the hypophysectomized rat(6). Under certain conditions, this activity may be actually enhanced by this treatment (7).

*Summary.* Treatment of sheep ACTH protein or peptide with 0.2 N hydrochloric acid in the boiling water bath for at least one hour, under the conditions described, produces a substantially complete reduction in antidiuretic and pressor activities originally present.

6. Sayers, M. A., Sayers, G., and Woodbury, L. A., *Endocrinol.*, 1948, v42, 379.

7. Li, C. H., *J. Am. Chem. Soc.*, 1950, v72, 2815.

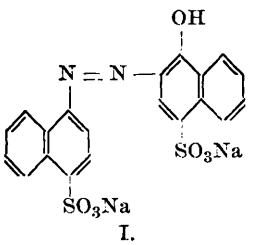
Received April 2, 1951. P.S.E.B.M., 1951, v76.

## Azorubin-Binding Capacity of Normal and Pathological Sera.* (18649)

ULRICH WESTPHAL† AND PETER GEDIGK.‡ (Introduced by H. Jensen)
*From the Chemical Department, Medizinische Klinik, University of Tuebingen, Germany.*

The ability of serum proteins to bind certain organic dyestuffs, has been studied by means of various methods, and certain differences have been found between normal and pathological sera(1-6). A chromatographic method for the quantitative determination of the capacity of serum albumins to bind azorubin, an acidic dye, has been described by Westphal, Gedigk and Meyer(7). In the present study, this method has been used to compare the azorubin-binding capacity (ABC) of normal with that of pathological sera.

*Methods.* (For details see reference(7)). The azorubin (formula I) preparation used




I.

* The experiments described in this paper were carried out with the assistance of Mr. O. Geiss and Mr. H. Gropper.

† Present address: Army Medical Service Field Research Laboratory, Fort Knox, Ky.

‡ Present address: Medizinische Klinik der Universitaet Marburg, Marburg, Germany.

1. Bechhold, H., *Biochem. Z.*, 1907, v6, 379.

2. Friedemann, U., Oppenheimer's *Handbuch der Biochemie*, 1909, vIII, 2, 282; cf. de Haan, J. and van Creveld, S., *Biochem. Z.*, 1921, v124, 174.

3. Bennhold, H., *Z. f. d. ges. exp. Med.*, 1926, v49, 71; *Ergebn. Inn. Med. und Kinderheilk.*, 1932, v42, 273; Bennhold, H., Kylin, E., and Rusznyak, St: *Die Eiweisskoerper des Blutplasmas*, Steinkopf, Dresden, 1938, p220.

4. Ehrström, M. Ch., *Acta Med. Scand.*, 1936, v90, 427.

5. Gottlieb, H., and Ludwig, H., *Z. f. klin. Med.*, 1937, v131, 358.

6. Huggins, C., Jensen, E. V., Player, M. A., and Hospelhorn, V. D., *Cancer Research*, 1949, v9, 753.

in these studies was obtained from Hollborn und Soehne, Leipzig, Germany. Two ml of serum were mixed with azorubin, dissolved in 0.5 ml of 0.6% sodium chloride, and after standing 30 min., the mixture (pH 7.8 to 8.0) was chromatographed on aluminum oxide (preparation of E. Merck, Darmstadt, Germany, standardized according to Brockmann), which was made anionotropic by the method described previously(7). The amount of dye used was in excess of the binding capacity of the serum analysed. Three parallel runs (in some cases two), using 3 different concentrations of azorubin, were made with each serum. The azorubin concentrations in the final mixtures to be chromatographed were 100, 200, 300 or 400 mg %. During the chromatographic process, the excess azorubin, not bound to the proteins, is adsorbed in a narrow zone while that part of the dye which is combined with the proteins is carried through the column and can be estimated colorimetrically in the filtrate. All of the azorubin in the filtrate is bound to proteins, its concentration representing a relative measure of the azorubin-binding capacity of the serum tested. Using an anionotropic aluminum oxide preparation of constant activity under the same conditions, reproducible results can be obtained. In 13 triplicate and 4 duplicate experiments on human sera, for instance, using 200, 300 and 400 mg % azorubin in the mixture to be chromatographed, the observed average values ranged from 145.2 to 193.6 mg %; the maximal deviation from the average values was −3.9%, and the average maximal deviation 2.0%(8). The concentration of azorubin found in the filtrate after the chromatographic analysis will be designated "ABC observed." This value, given in mg %, refers to the serum diluted with the dye solution in the ratio of 4 : 5. As pointed out previously(7), azorubin is bound exclusively, in normal serum, to albumin as shown by electrophoretic analysis. This observation is in agreement with the finding of the ability of this serum component to combine with various acidic compounds(3,9). Electrophoretic studies demonstrated that even in sera giving extremely low ABC values (7.1 mg %, in a case of nephrotic stage of chronic glomerulonephritis) the globulin fractions did not contain any azorubin although the concentration of the azo dye in the serum mixture was 100 mg % before chromatographic adsorption(10). In order to characterize the albumins of different sera, the observed ABC values were divided by the albumin concentration. The values thus obtained will be designated "specific ABC" and will be expressed in moles of azorubin per gram albumin. It should be emphasized that these values are only relative since they are obtained by a competitive reaction between the aluminum oxide and the serum albumin, and they cannot be compared directly, therefore, with the capacity of albumin to bind acid compounds, as determined by other methods (*e.g.*, dialysis, electrophoresis)(7). Since circumstances did not permit the electrophoretic determination of the albumin concentration, the Howe procedure was employed. It is known that this latter method gives higher albumin values than the electrophoretic analysis, especially in certain pathological cases of low albumin concentration such as nephrosis(11). The pH at which the binding of azorubin to albumin is estimated, is that of the chromatographic filtrate (pH 6.2).

*Experiments and results.* As can be seen from Table I (Group 1), all sera of *normal persons* studied gave ABC values above 100 mg % (Class 1); values under 80 mg % (Class III) were found in pathological cases only. No definite evaluation can be made for the range from 80 to 100 mg % (Class II). For the "specific ABC" values, an arbitrary value of $6.0 \times 10^{-5}$ moles of azorubin per gram albumin was chosen as borderline between normal and pathological sera. Normal ABC values were obtained also for the sera of

7. Westphal, U., Gedigk, P., and Meyer, F., Hoppe-Seyler's *Z. f. physiol. Chem.*, 1950, v285, 36.

8. Geiss, O., *Inaugural-Dissertation.* Tübingen, 1948.

9. Klotz, I. M., Walker, F. M., and Pivan, R. B., *J. Am. Chem. Soc.*, 1946, v68, 1486, 2299.

10. Ott, H., unpublished experiments.

11. Gutman, A. B., *Advances in protein chemistry*, 1948, v4, 155, 182.

TABLE I.

| Group | Diagnosis (No. of patients in parentheses) | Class and range of ABC (mg %) | | ABC observed (mg %) | Specific ABC (moles × $10^{-5}$ per g albumin) |
|---|---|---|---|---|---|
| 1. Normal | Normal persons (26) | I | >100 | 106 to 195 | 6.4 to 9.8 |
| 2. Normal as to serum proteins | Essential hypertension (16), mostly fully compensated; light cases of gastritis (5), and peptic ulcer (3); sciatica (1); colloid goitre (1) | I | >100 | 107.6 to 182 | 7 to 10.6 |
| 3. Nephrosis | Nephrotic stage of chronic glomerulonephritis (3); amyloid disease of kidney (2); lipoid nephrosis (1) | III | <80 | 3.2 to 78.9 | 1 to 8.3* |
| 4. Liver disease | Infectious hepatitis (9); chronic infectious hepatitis (2); cirrhosis (2); enlargement of liver, splenomegaly (1). Bilirubin: 0.6 to 31.6 mg % | I | >100 | 101 to 163.4 | 7.2 to 8.3 |
| | Infectious hepatitis, cholangitis, cholecystitis (1); chronic hepatitis (1). Bilirubin: 5.2, 2.9 mg % | II | 80–100 | 81.8 to 88.2 | 5.7 to 6.2 |
| | Homologous serum hepatitis (1); infectious hepatitis (1); coma hepaticum (1); severe hepatitis of unknown etiology (1); gastric carcinoma, liver metastases, obstructive jaundice (1). Bilirubin: 8.8 to 29.7 mg % | III | <80 | 16.9 to 75.7 | 1.3 to 4.2 |
| 5. Infectious diseases | T.b. of various locations (10); cystitis due to *E. coli* (2); pyelonephritis (1); Boeck's sarcoid (1) | I | >100 | 103.1 to 171.7 | 6 to 9.8 |
| | T.b. of lungs (3) | II | 80–100 | 89.4 to 95.1 | 6 to 6.8 |
| | T.b. of various locations (5); broncho- and lobar pneumonia (5); brucellosis (1); chronic tetanus (1) | III | <80 | 22.6 to 76.7 | 2.5 to 6.2 |
| 6. Subacute bacterial endocarditis | Subacute bacterial endocarditis, combined aortic and mitral insufficiency (1) | II | 80–100 | 87.8 | 6.5 |
| | Subacute bacterial endocarditis, combined aortic and mitral insufficiency (3); diffuse glomerulonephritis (2); aortic insufficiency (1); mitral insufficiency (1) | III | <80 | 31.3 to 79.9 | 2.3 to 7.3 |
| 7. Neoplasm | Astrocytoma (1); cavernous hemangioma of lung (1); clear cell carcinoma of right kidney (1) | I | >100 | 102.2 to 132.2 | 6.5 to 8.5 |
| | Chronic myelogenous leukemia (3); hypernephroma, liver metastases (1); melanosarcoma (1) | III | <80 | 46.1 to 73.1 | 3 to 6.9 |
| 8. Myeloma | Multiple myeloma (1) | I | >100 | 107.5 | 10.2 |
| | $\beta_2$—plasmocytoma (1) | III | <80 | 46.6 to 69.9 | 4 to 7.9 |
| | $\gamma$—plasmocytoma (2) | | | | |

* Specific ABC value of 8.3 found during recovery; see Table II.

*patients* whose *serum protein patterns* are generally considered to be *"normal"* (Group 2, Table I).

In all cases of *nephrosis* (Group 3), ABC values were found to be in the lower range, and the specific ABC values were also found to be extremely low. These results confirm earlier findings of the low binding capacity of serum albumins in nephrosis(3,4). A case of chronic glomerulonephritis of primary inflammatory origin, which resulted in a nephrotic stage, was followed over a period of 33 months (Table II). Definite clinical improvement during 8 months of hospitalization was reflected by a gradual increase in the ABC. After 2 years, typical symptoms of the glomerulonephritis were found, again accompanied by a decreased specific ABC value.

TABLE II. ABC Values in a Case of Chronic Glomerulonephritis, Nephrotic Stage.

| Time after admission | Clinical picture | ABC observed (mg %) | Specific ABC (moles × $10^{-5}$) |
|---|---|---|---|
| 0* | Severe edema; ascites | 15.1 | 3.0 |
| 6 wk | | 37.4 | 4.3 |
| 3 mo. | Retrogression of edema | 56.2 | 6.6 |
| 8 mo. | No edema, marked improvement of clinical picture | 78.9 | 8.3 |
| 33 mo. | Edema on both lower legs | 72.0 | 4.6 |

* Admission.

In the cases of *liver disease* (Group 4, Table I), a wide range of ABC values was found. No correlation could be established between ABC and the bilirubin level, Takata-reaction or sedimentation rate. However, a definite parallelism was observed between the severity of the disease and decrease in ABC, since all 5 patients of Class III showed very heavy damage of the liver parenchyma confirmed by autopsy§ in 3 fatal cases. Among the 14 cases with normal ABC values (Group 4, Class I), on the other hand, only one patient, suffering from severe infectious hepatitis, was suspected of having major liver damage; the ABC values of this serum were found to be 103.0 mg %, 7.3 moles x $10^{-5}$. No patient of Class I and II, Group 4, died.

Since the bilirubin level has been found generally to be elevated in these cases, experiments were performed in order to see whether the increased bilirubin concentration could account for the decrease in ABC values. Addition of crystalline bilirubin to normal serum resulted in a lowering of the ABC values. This effect is probably due to a combination of bilirubin with the same groups of the albumin molecules to which azorubin attaches itself(12). The amounts of bilirubin added were of the same order as observed for the sera of Class III (Group 4, Table I). The decrease in ABC, however, was found to be much smaller than the deviation of the ABC values from the borderline of 80 mg %. Only a part of the lowering effect can, therefore, be accounted for by the elevated bilirubin level.

In the group of *infectious diseases* (Group 5, Table I), parallelism was observed between lowered binding capacity and the severity of the clinical picture. The patients of Class III, 6 of whom died, again had the more unfavorable diagnosis, and the lowest ABC values were found in the sera of 4 patients tested 1 or 2 days before death. Most patients of Class I were lighter cases; clinical improvement took place in all of them, as well as in the patients of Class II. The results in this group indicate that abnormal ABC values were found whenever the disease had reached a severe "toxic" phase. In 7 out of 8 cases of *subacute bacterial endocarditis* (Group 6, Table I), low ABC values were observed. In 4 of these, liver damage, and in 3, nephrotic symptoms were found. Six patients of this group died.

In 10 cases of *neoplasms* (Group 7, Table I), ABC values were found to overlap into all 3 classes. In all patients of Class III, the illness was far advanced and severely affected their general condition. However, advanced phases of these neoplastic diseases did not coincide regularly with low ABC, since they occurred also in sera of patients of Class I and II. Death occurred in 4 patients of Class II and III. Lack of correlation between the clinical picture and ABC values was found also in 4 cases of *myeloma* (Group 8, Table I).

Examination of the sera of 21 patients with *endocrine diseases* did not reveal any abnormality in the ABC. This group included 4 cases of moderate diabetes, 5 of tetany, 2 of myxedemas, one of hyperthyroidism, one of slight adrenal insufficiency, 3 of hypophyseal metabolic disorders, and several patients suffering from obesity or emaciation of endocrine origin. In none of these patients was the course of disease alarming. No abnormality

§ The autopsies were done by the Pathological Institute, University of Tuebingen, Tuebingen, Germany.

12. Westphal, U., Ott, H., and Gedigk, P., *Hoppe-Seyler's Z. f. physiol. Chem.*, 1950, v285, 200.

in ABC values could be observed either in 11 out of 13 cases of *heart and circulatory diseases*; the 2 patients with lowered ABC suffered from liver congestion. In other sera tested, normal ABC also was observed in 2 cases of thrombocytopenic purpura (thrombocytes 21,000 and 45,000, respectively) and in a patient with pernicious anemia treated with liver extract (campolon). Decreased ABC values (51.0 mg %; 3.7 moles x $10^{-5}$) were found in the serum of a patient who died from porphyria during the second month of pregnancy. Severe damage of the liver parenchyma, as based on clinical tests, was verified by autopsy.

*Discussion.* The determination of the ABC is not supposed to serve as a tool of clinical diagnosis, although, in certain cases, the test may perhaps be useful for the characterization of sera as to binding function of their albumins. The estimation of the ABC is rather intended to obtain information on the quality of serum albumin, and to aid in the elucidation of the problem whether or not there are chemical differences in the serum albumin molecules of normal and pathological sera. The ABC of albumin apparently is not altered by admixture of nonbinding proteins: The observed and specific ABC values of a 1.2% solution of crystalline human albumin¶ in 0.15 m NaCl, to which 4.65% γ-Globulin was added, were the same as those of the globulin-free 1.2% albumin solution.

It was mentioned previously that the Howe procedure will overestimate the amount of albumin, particularly at low albumin concentrations as in nephrosis. The albumin values in the sera of our cases of nephrosis (Table I, Group 3) ranged from 0.9 to 4.1%, 5 out of 6 cases being 1.6% and below. The albumin concentration in the filtrate corresponding to the lowest specific ABC value observed (1.0 x $10^{-5}$ mol) was found to be 0.6%. Electrophoretic albumin concentrations of 5 nephrotic sera have been reported to range from 0.2 to 2.1% (11). It is possible, therefore, that the lowering of the specific ABC values found in nephrosis is partly due to the error inherent in the Howe method. Another factor which may add to the unreliability of the specific ABC values observed in nephrotic sera, is the observation that under the conditions of the competition reaction in the chromatographic adsorption, the specific ABC will decrease somewhat with decreasing albumin content (7). Lowering of the albumin concentration from 4.41 to 0.88% (before 4:5-dilution) resulted in a 23% decrease of the specific ABC (7). Experiments using crystalline human albumin¶ in NaCl solutions of various concentrations have shown likewise that a decrease of the albumin content from 4.2 to 1% is paralleled by a maximal lowering of the specific ABC of about 20%. At an albumin concentration of 0.3%, the specific ABC was decreased about 45%. In case of an extremely low albumin concentration in a nephrotic serum, this phenomenon may be an additional cause of a low specific ABC. However, in all the sera studied in the present paper, except those from nephrosis patients, albumin concentrations were higher: 4.0 to 5.6% in Groups 1 and 2, Table I; 2.0 to 4.9% in Groups 4 to 8, Table I. In the above experiments, the specific ABC of a 2% solution of crystalline human albumin was only 5% lower than that of a 4.2% solution. Decrease of specific ABC, as found in the sera of Classes II and III of Groups 4 to 8, Table I, therefore cannot be due to low albumin concentrations.

In contrast to nephrosis, in none of the other diseases studied (Table I, Groups 4 to 8) has the Howe method been found to give results similarly deviating from the electrophoretic values (11). In hepatitis serum, a moderate decline in serum albumin has been observed by the Howe method as well as in the electrophoretic analysis. In cirrhosis, excellent agreement was found between the two methods at low albumin levels. Electrophoretic values for tuberculosis sera have been described from 2.7 to 3.9% (11). The albumin values found in the sera of the tuberculosis patients of Classes II and III, Group 5, Table I, ranged from 2.3 to 4.0%.

¶ We are greatly indebted to Dr. J. T. Edsall and Dr. L. D. Wojcik, University Laboratory of Physical Chemistry, Harvard University, Boston, for kindly supplying the albumin and γ-globulin preparations.

The question whether differences in the ABC values of normal and pathological sera are caused by changes in the albumin molecules cannot be answered at the present time. The studies of Huggins and associates(6) are of interest in this respect. These authors, using equilibrium dialysis, found a decreased binding of phenolsulfonephthalein with the serum protein in 44.7% of the sera of 123 cancer patients (compare results listed in Groups 7 and 8, Table I, of the present paper). They correlated this change with a deficiency in the thermal coagulability of the albumin. Both of these abnormalities could be eliminated, however, on purification of the serum albumins by cold ethanol fractionation. The albumin fractions thus obtained from serum of cancer patients or normal serum, did not show significant differences in elementary composition, or in the content of thiol and amino groups.

In our own studies(13), the sera of 16 patients suffering from various diseases were used, showing normal as well as abnormal "observed" and "specific" ABC values (50.4 to 145.0 mg %; 3.2 to 8.2 moles x $10^{-5}$). The albumin fractions were isolated, as in the studies of Huggins and co-workers, by the method of Cohn and associates(14). No proportionality was found between the specific ABC values of these albumin fractions and of the corresponding sera. No differences in the composition (arginine, histidine, tyrosine, tryptophane, and amino nitrogen) of the isolated albumin fractions could be found between sera giving normal and sera giving low ABC values. The specific ABC values for these separated albumins were found to be lower than in the original sera (2.6 to 5.6 moles x $10^{-5}$). This decrease probably is due to a removal of salts by dialysis; diminishing the salt concentration has previously been shown to lead to a lowering of the ABC values in the test employed(7).

*Summary.* The relative capacity of normal and pathological sera to bind the acid dye, azorubin, was determined by means of a chromatographic method, using anionotropic aluminum oxide as absorbent. Subnormal azorubin-binding capacities (ABC) of serum albumins were demonstrated, in varying degrees, in nephrosis, diffuse damage of liver parenchyma, and severe toxic states of infectious diseases. In some cases of malignant neoplasms abnormally low ABC values were also observed.

13. Westphal, U., and Leube, I., unpublished experiments (1947-1950).

14. Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, 1946, v68, 459.

Received April 2, 1951. P.S.E.B.M., 1951, v76.

## Effect of C-Reactive Protein on Normal Human Leukocytes.* (18650)

HARRISON F. WOOD. (Introduced by Maclyn McCarty)

*From the Hospital of The Rockefeller Institute for Medical Research, New York City.*

Tillett and Francis described the occurrence of a reaction between the sera of patients acutely ill with pneumococcal pneumonia and the somatic C polysaccharide of the pneumococcus(1). This reaction was obtainable only with acute phase serum, and was generally not demonstrable after the onset of convalescence. In later studies by Avery and Abernethy, and Avery and MacLeod, the reactive substance was shown by chemical and immunological methods to be a specific abnormal serum protein which reacted selectively to form a pre-

* The author wishes to thank Dr. Cynthia H. Pierce for her active interest in the work presented in this study, and takes pleasure in acknowledging the able technical assistance of Miss Helen H. Babcock.

1. Tillett, W. S., and Francis, T., Jr., *J. Exp. Med.*, 1930, v52, 561.