# The Binding of Phenolsulfonephthalein by Serum and by Albumin Isolated from Serum in Cancer*

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It is recorded in this paper that the binding capacity of albumin in the serum of cancerous patients for phenolsulfonephthalein (PSP) is often reduced below that of normal individuals and that it is restored in the albumin fraction prepared by the method of Cohn et al. (5) The binding capacity of proteins, a measure of the availability of certain cationic centers in the molecule, and the iodoacetate index are correlated in the present study.

The binding of PSP by serum albumin was first observed by Grollman (10). Since then many cases have been observed where albumin forms complexes with organic and inorganic anions, many with considerable physiologic and pharmacologic significance. Serum albumin is outstanding among native proteins for its binding capacity. Albumin and dye or other anions combine on the basic side of the iso-electric point although each carries a negative charge.

The method of studying anion binding by proteins include: the penetration of dyes added to serum into gelatin (2); attempts at removal of free dye by charcoal adsorption after mixing dye and serum, with estimation of the unadsorbed protein-bound dye (9); electrophoresis (21); changes in the absorption spectrum of dyes caused by association with the protein (18, 24); and equilibrium dialysis (6, 19); the last method was employed in the present study.

There is good evidence that anions are bound to proteins both by electrostatic attachment and by molecular attraction of the van der Waals type. Electrostatic fixation apparently occurs through cationic residues of the basic amino acids (21, 23), of the protein particularly of the $\epsilon$-ammonium group of lysine (18) and less importantly to the guanidinium group of arginine (15, 19). Acetylation of albumin with a consequent decrease of available cationic groups of lysine greatly reduces but does not eliminate the binding of anions (17) suggesting that the guanidinium group of arginine participate to some extent in the binding. Salmine (87 per cent arginine) binds methyl orange (15). Mere possession of these cationic groups seems to be inadequate for anion binding since certain proteins such as $\beta_2$ and $\gamma$-globulins cannot bind anionic dyes although they contain substantial amounts of lysine and arginine. Guanidination of albumin does not change its affinity for methyl orange indicating that the $\epsilon$-amino group of lysine is not essential as such but only because it furnishes a + charged nucleus (17). As evidence for non-electrostatic molecular attraction is the fact that there is no interaction between anionic dyes and the free amino acids arginine and lysine (15). There is evidence (16) that in binding significance electrostatic attraction outweighs van der Waals action. The relative position of $N^+$ in the protein structure is a critical factor (17) although the nature of the topology in the protein molecule is still obscure.

In clinical material Bennhold (2) using as a measure the penetration of dyes into gelatin found that the serum albumin of patients with hepatic and renal diseases had decreased binding capacity; these findings were confirmed with the charcoal adsorption method (9). With the latter method Ehrström (7, 8) observed low binding values in 10 patients with tumor cachexia and presented the hypothesis that hypoproteinemia in cancer occurring as a result of increased protein destruction disturbs the synthesis of plasma proteins with the result that proteins with defective adsorptive capacity are fabricated.

A deficiency of -SH groups in the serum in human cancer has been known for some years to occur commonly in cancer based on the following evidence: a) Activation of enzymes. Certain enzymes require free -SH groups for their activity; the activity of dialyzed (inactive) papain (22) or methyl glyoxylase (27) is restored to a greater extent by the addition of blood from normal than from can-

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cerous patients. b) Polarography. The height of the catalytic wave in the polarogram of cobalt, caused by the presence of sulfhydryl compounds, is less with cancer serum than with normal serum (4). c) The reducing power of serum in cancer is frequently decreased (3, 11, 25).

## EXPERIMENTAL

*Equilibrium dialysis.*—Stock solutions containing 100 mg. of PSP were prepared by dissolving the dye in 100 ml. of M/15 phosphate buffer pH 7.4. A calibration curve was made for solutions containing 25 to 100 μgm. of PSP per 100 ml., 1 ml. of 5N NaOH being added just before diluting the solution to 100 ml. The development of color of PSP and its stability has been considered elsewhere (14). An Evelyn photoelectric colorimeter with a 540 mμ filter and matched tubes were used throughout.

Dialysis experiments were done in triplicate. 1 ml. of serum (or weighed protein dissolved in 1 ml. of phosphate buffer) and 1 ml. of stock solution of PSP were placed in a bag of cellulose casing 1.75 cm. ($\frac{2}{3}\frac{2}{2}$ inch) in diameter and 15 cm. long; the bag, knotted at both ends, was placed in a 125 ml. Erlenmeyer flask containing 98 ml. of water and the flask rotated 12 times per minute in a cylindrical rotator for 48 hours at 5° C. After warming to room temperature 10 ml. of the dialysate was diluted to 100 ml. with water with the addition of 1 ml. of 5 N NaOH just before the final volume was reached. Triplicate determinations of the color of the dialysates were made.

To determine the amount of PSP which was bound by the globulin fraction of the serum, one end of the cellulose casing was then cut off and the bag inserted in a graduated 15 ml. centrifuge tube, the closed end of the bag being fastened firmly to the top of the tube by adhesive cellulose tape. By centrifuging for 10 minutes at about 1500 r.p.m. the contents of the bag were transferred quantitatively to the tube where the water-insoluble globulins were thrown to the bottom. The volume of the liquid was measured. The supernatant liquid having been discarded, the precipitate was stirred up in a few ml. of water and again centrifuged. The globulins were dissolved in 0.15 M NaCl and transferred to a 10 ml. volumetric flask, 0.1 ml. of NaOH was added and the color determined colormetrically after filling to the mark.

It was found experimentally that equilibrium between protein solution and dialysate was reached in 40 hours under the conditions described. Although cellulose bags adsorbed larger quantities of PSP, when 1 mg. of PSP was used as in these experiments, less than 10 μgm. was retained by the bag.

A weak Donnan effect operates in the dialysis system; the contents of the bag were 0.18 to 0.36 pH units more acid than the dialysate. The Donnan effect in equilibrium dialysis has been discussed by Klotz and Urquhart (16).

The reproducibility of the results in triplicate determinations was within ±2 per cent. The amount of PSP dye bound by serum albumin was linear with protein concentration in the range 12.5 to 75 mg. (Fig. 1) under the conditions of the experiment. Naturally occurring albumin values in serum greater than 60 mg. per 1 ml. of serum were not encountered in the observations.

Estimations of the albumin content of serum and the iodoacetate index with respect to total protein were made as reported in a previous paper (13).

The results were expressed in forms of a ratio, B/A, expressing the amount of PSP bound per 10 mg. of albumin

$$B/A = \frac{\mu gm.\ of\ PSP\ bound\ by\ 1\ ml.\ of\ serum}{Gm.\ of\ albumin\ per\ 100\ ml.\ of\ serum}$$

*Purification of proteins.*—Albumin was isolated from approximately 300 ml. quantities of 6 samples of normal serum and 3 sera of patients with cancer by the method of Cohn et al. (5). The albumins obtained all showed a single sharp electrophoretic peak with mobility of 6.37 ± 0.15, characteristic of albumins; no traces of other protein fractions were evident[1]. Micro-analyses for carbon, hydrogen, nitrogen, and sulfur were made on the various albumin fractions. Aliquots of albumin[2] were dissolved in phosphate buffer and the following items were determined: PSP binding; iodoacetate index; amino groups by the method of Van Slyke (26); -SH groups[3] by amperometric titration (1, 20). Similar tests had been made on these sera before isolation of the albumins.

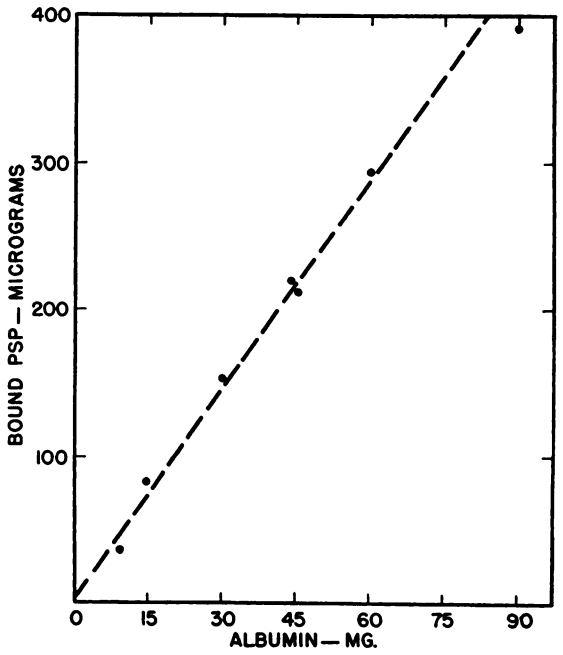


FIG. 1.—Linearity in binding of PSP by serum with an albumin content between 12.5 and 60 mg.

[1] Electrophoretic analysis was carried out by Mr. Bernard Udin, Department of Biochemistry, the University of Chicago.

[2] Microanalyses of purified albumins were carried out by Dr. J. F. Alicino.

[3] We are indebted to Dr. S. G. Weissman for the sulfhydryl determinations.

## RESULTS

*PSP binding by globulins.*—50 mg. lots of plasma proteins[2] prepared by Method 6 of Cohn et al. (5) were tested for PSP binding. The approximate composition of the fractions and the binding values follow: Fraction V, 95 per cent albumin, bound 300 μgm.; Fraction IV-4, 16 per cent albumin and 84 per cent globulins, bound 70 μgm. No PSP was bound by any of the following fractions: II + III, 4 per cent albumin and 91 per cent globulins; IV-1, 100 per cent α-, β- and γ-globulins; a fraction containing over 95 per cent γ-globulin.

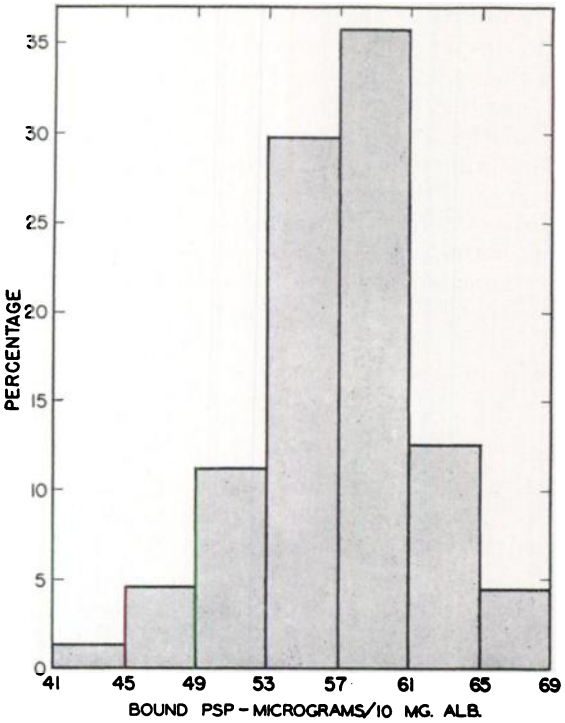


FIG. 2.—Histogram of PSP binding ratio (B/A) of the serum of 156 normal human adults.

The water insoluble globulins of serum bound no dye in any case except in certain myelomas. In 2 cases of multiple myeloma a glassy precipitate, very red with bound dye, was observed; the binding of PSP was 16 and 22 μgm. per 10 mg. of protein respectively. On electrophoresis of these sera the abnormal component migrated slightly slower than or with the γ-globulin peak. In 8 other multiple myeloma sera this binding globulin was not observed.

*PSP binding by human serum.*—The serum was examined for PSP binding in 418 cases distributed in 3 categories: 156 normal adults; 139 persons with non-malignant disease; 123 persons with cancer. The range of dye bound in the whole series of sera examined varied from 10 to 345 μgm. per 1 ml. of serum.

Relating the bound PSP to 10 mg. of albumin (B/A ratio), the sera of normals (Fig. 2) had higher mean and median values (Table 1) and a smaller standard deviation than serum from cancer patients. Only 2 persons believed to be healthy had a B/A ratio less than 45; the value 45 was accordingly selected as an arbitrary lower limit for the binding of normal human serum.

In the entire series, 81 persons (19.4 per cent) had B/A ratios less than 45. These comprised: 2 in the normal group; 55 cancer patients; 24 persons with diseases other than cancer. The non-malignant diseases associated with these lower values comprised: liver disease, 9; infection, 6; uremia, 3; rheumatoid arthritis, 3; other conditions, 3.

The sera from 123 cancer patients had a median B/A ratio of 46.4. Fifty-five (44.7 per cent) of the patients with cancer had ratios less than 45. While the neoplasms were in an advanced stage in most of the cancer series, some cases of metastatic cancer were encountered in which all of the properties of the serum albumin including PSP binding were normal.

TABLE 1

STATISTICAL ANALYSIS OF PSP BINDING OF SERUM

| PSP index: Bound PSP in μgm / Gm. albumin in 100 ml. | No. of patients in series | Range | Median | Mean | Standard deviation |
|---|---|---|---|---|---|
| (1) Normals | 156 | 41.0–68.9 | 57.4 | 57.07 | 5.79 |
| (2) Cancer | 123 | 2.8–65.6 | 46.4 | 44.61 | 11.91 |

The difference between the serum of normal and cancerous persons is more apparent when the binding-albumin ratio of 50 is taken as a critical level: 15 members of the group of 156 normals (9.6 per cent) had B/A ratios of 50 or less while 88 patients in the group of 123 cancerous individuals (71.5 per cent) were in that range.

*Correlation of PSP binding with the iodoacetate index.*—The iodoacetate index (13) and PSP binding were determined simultaneously on the sera of

[4] We are indebted to Dr. John T. Edsall, Department of Physical Chemistry, Medical School of Harvard University for the gift of some of these fractions.

47 cancer patients. In all of the cancer sera when the B/A ratio was less than 45, the iodoacetate index was also in the pathologic range; whenever the B/A ratio was larger than 50, the iodoacetate index was also normal. There were 12 cases where the B/A ratio ranged from 45 to 50; 9 of these sera had iodoacetate values in the pathologic range and 3 of them in the normal distribution.

*Effect of surgical operation on PSP binding and the iodoacetate index.*—Both the binding of PSP and the iodoacetate index often decrease markedly in persons with normal original values, subjected to trauma. These phenomena have been most frequently observed in post-operative states and they occur frequently even when a major surgical operation has been carried out with little blood loss and with minimal manipulation of the tissues. Marked lowering has occurred without decreases of total protein or albumin content of the serum and within 8 hours after the operation; the rapidity of the decrease is significant.

*Dialysis and fractionation of albumin from normal and cancer serum.*—Sera with low PSP binding values from patients with cancer were dialyzed for 2 days against water or 0.15 M NaCl; PSP was then added to the cellulose bag and the binding capacity of the proteins determined in the usual way. No differences were observed on dialysis with water or sodium chloride solutions; in both cases dialysis caused a slight improvement in the binding capacity of the proteins, increases of the order of 3 to 5 per cent being observed, but these values were considered insignificant.

The values given in Table 2 indicate that the relatively large differences in the iodoacetate index of thermal coagulability and in dye binding which exist between the proteins of normal and cancer sera are not in evidence in the albumin fraction purified by the cold ethanol fractionation technique (5). Moreover there are no significant differences in the composition of the albumins isolated from the 2 types of sera as indicated by their content of carbon, nitrogen, hydrogen, and sulfur as well as free amino and -SH groups.

TABLE 2

COMPARISON OF SERUM PROTEINS WITH ISOLATED ALBUMINS

| | Original Serum | | Isolated Albumin | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Serum Source | Iodoacetate index | Dye binding μgm/10 mg. albumin | Iodoacetate index | Dye binding μgm/10 mg. albumin | SH μm/g | $NH_2$ μm/g | %C | %H | %N | %S |
| *Normal* | | | | | | | | | | |
| P-2 | | | | 69.8 | 11.2 | 950 | 51.8 | 6.98 | 16.3 | 1.91 |
| P-3 | | | 19.4 | 71.0 | 11.4 | 986 | 52.9 | 6.93 | 15.9 | 1.85 |
| P-4 | | | 19.6 | 71.7 | 9.5 | 921 | 52.6 | 7.02 | 15.9 | 1.81 |
| H-5 | 10.9 | 58.2 | 18.6 | 73.2 | 10.4 | 928 | 53.7 | 7.58 | 15.7 | 2.29 |
| J-6 | 11.1 | 52.0 | 17.5 | 68.8 | 11.5 | 878 | 52.8 | 6.96 | 15.8 | 2.09 |
| W-7 | 9.8 | 63.0 | 18.4 | 69.5 | 11.2 | 907 | 53.0 | 7.02 | 16.1 | 1.88 |
| *Cancer* | | | | | | | | | | |
| P-1 | 6.0 | 25.0 | 15.6 | 49.5 | 7.9 | 1157 | 53.1 | 6.97 | 15.8 | 2.11 |
| W-2 | 6.5 | 26.0 | 18.6 | 72.3 | 10.1 | 1035 | 52.8 | 6.97 | 16.1 | 1.85 |
| W-3 | 6.5 | 38.2 | 16.8 | 66.6 | 10.0 | 914 | 52.6 | 6.98 | 16.2 | 1.94 |

## DISCUSSION

Except with myeloma serum and protein fractions containing globulins all of the values reported in this paper are related to weight of albumin.

In this series an abnormally low capacity (B/A less than 45) for binding PSP was encountered in 44.7 per cent of the sera from cancer patients compared with 17.3 per cent of the sera of patients with a wide variety of serious non-cancerous diseases; all of these cancer sera had low iodoacetate indices. On fractionation of the serum albumin from normal and cancerous persons these defects are eliminated; the refined albumin fractions from both normal and cancer patients resemble one another in the properties examined.

According to current theory the iodoacetate index is dependent on the availability of thiol and certain amino groups (12) in the protein molecule while the binding of dye anions is related to cationic centres with specific, but as yet ill-defined, configurational relationships. The same sera in general show similar defects in both coagulation and dye-binding properties; refinement of the albumin by the cold ethanol procedure largely eliminates both defects. It would appear that the defects are not intrinsic in the albumin molecule, and it is postulated that they arise because of associated groups, possibly of anionic type which prevent the normal reaction of protein groups. To explain the results obtained the binding must be of such a nature that the bound substance is not removed by dialysis but is removed by the cold 40 per cent ethanol of the fractionation procedure.

The speed with which normally reactive albumin acquires defects in PSP binding and thermal

coagulation after a surgical operation is evidence supporting a superficial rather than an intrinsic change in the albumin molecule.

## CONCLUSIONS

By equilibrium dialysis it was demonstrated that there was defective binding of anionic dyes in the serum of 44.7 per cent of patients with cancer in this series. Dialysis against water or sodium chloride caused only an insignificant improvement in binding. On refinement of serum albumin from cancer patients by cold ethanol fractionation this defect and also deficiency of thermal coagulation were eliminated. No significant differences were found on elementary analysis or on determination of thiol or amino groups between fractionated normal or cancerous serum albumin.

A marked defect in the binding of PSP and a decrease of the iodoacetate index of thermal coagulability of serum can occur within 8 hours after a surgical operation.

Globulins in refined plasma protein fractions did not bind PSP. The water insoluble globulins of serum likewise failed to bind PSP except in 2 cases of multiple myeloma where a gelatinous precipitate of water insoluble globulins occurred following dialysis; these binding globulins migrated electrophoretically with $\gamma$-globulin.

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