BINDING PROPERTIES OF NORMAL AND CANCEROUS LIVER PROTEINS

by

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INTRODUCTION

Growth is a universal phenomenon, but the biochemist and physiologist are overwhelmed by its complexities. There is no complete explanation as to why the process starts, how it is coordinated during its course or why it stops at the definite point which characterizes adult development.

As the adult organism is formed, the function of tissue changes from that of growth to a more subordinate position of maintenance and balance of the organism as a whole. It is quite conceivable that a tissue in a localized area may grow out of proportion, but as long as it remains responsible to the body and does not affect a sensitive organ, no serious consequences may result.

Animals are faced with another type of tissue growth which appears to be completely independent of body control. Greenstein (8) has characterized it as being, "frequently unlimited, continuous . . . . . far in excess of the normal, without consideration for neighboring tissues which it progressively invades, destroys and replaces." The result of this abnormal, independent type of growth is a neoplasm which is familiarly called a tumor or cancer.

Cancer is predominently a problem in growth, a general characteristic of malignant tissue being its capacity for uncontrolled sustained growth. An indispensable part of the

growth process is protein and protein synthesis. For a long time workers have not been sure whether malignant growth is associated with actual changes in protein composition. Much of the available evidence speaks against a very great difference between "normal protein" and "cancer protein." The suggestion that structural changes exist between the two types of protein has occurred repeatedly in the literature, but no conclusive evidence has been presented.

It is accepted that the corresponding nucleoprotein fractions of normal and carcinogenic hepatic tumors are nearly identical in the proportion of most amino acids. (7) Sauberlich and Baumann (36) found the amount of amino acids in the tumor of rats fed dimethylaminoazobenzene (DAB), were similar to those in the normal rat tissue and to those in ordinary cuts of pork or beef. It is possible however that the molecular configuration of the protein may be different.

Investigators (39) have reported that the concentration of nucleic acids in tumor-bearing animals is greater than in normal animals. Schneider (37,38) found that the ribosenucleic acid content of rat livers and hepatomas were essentially the same with an increase in the desoxyribosenucleic acid content of hepatomas. He states that this increase is due mainly to an increase in the number of cells in the tumor tissue, rather than to an increase in the amount per nucleus. But the workers at Wisconsin (21) claim that liver tumors have a higher nuclear density than normal liver and therefore contain more desoxy-

pentonucleic acid per gram of tissue than ordinary liver.

Kahler and his co-workers (11) found a higher acidity in cancer liver tissue (pH=6.99), than in normal liver (pH=7.39). The increased nucleic acid content of the malignant tissue is perhaps the most likely explanation of this difference.

Mann and Walker (22) state that their immunological observations indicate that malignant tissue protein differs from the proteins of normal tissue.

Swedish workers Casperson and Santesson (3) are convinced that in cancer there is a universal shift toward what are more probably simpler protein, which are considerably richer in diamino-acids than the average protein.

Mayer & Barrett (23) reported that rats under the influence of carcinogens produced cathepsins that differed serologically from those of normal liver.

Lavik and his co-workers (20) on the basis of their experiments with carcinogenic hydrocarbons, postulated that carcinogenesis is essentially an accumulation of abnormal protein within the cell. Potter (32) theorized that this "abnormal protein" consists of an autosynthetic cancer virus, which he felt was similar to a normal enzyme and therefore competitively blocks its action. This theory of the mechanism of carcinogenesis has been widely acclaimed in recent studies.

The first significant evidence that a qualitative difference in protein may exist between the liver and the tumor has come from the workers at Wisconsin. In 1947 Miller and Miller

(24) observed that the livers of rats fed DAB contain amino-azo-dyes bound tightly to a cellular constituent which they presumed to be protein. No bound dye was found in rat tissues such as the small intestine, kidney, spleen, lung and heart in which DAB does not induce tumors. The bound dyes were found only in the liver, the site of tumor formation. The Millers (33) found also that the bound dyes were not found in the liver tumors. The binding of the carcinogenic dye first occurred in the liver in four days and the bound dye was found to be at a maximum at the end of four weeks. Although the dye was continually ingested, it was found that the amount of dye in the liver slowly diminished to a negligible amount at twenty weeks. Miller (25) contends that binding of the dyes to the protein took place at the $-N(CH_3)_2$ group or possibly at the ring to which this group is attached. The amount of bound dye was reduced when the level of riboflavin, a protective substance in the diet, was increased.

The Millers (25) propose the theory that the azo-dyes initiate the carcinogenic process by combining with proteins in the liver that are responsible for the control of growth but not for life. They imply that cells may result which have been depleted of those factors that control normal growth and the tumor may be the eventual outcome.

A great deal of work has been devoted to the plasma proteins of rats administered carcinogens. Lanurande, et al. (19) found the concentrations of plasma proteins to vary during liver damage. The protein composition of the liver, and ultimately

the composition of the plasma protein, seems to be affected by such damage. Electrophoretic studies by Luck, et al. (9) have revealed definite changes in the composition of serum protein during azo-dye carcinogenesis.

Relatively little is known concerning the liver protein during azo-dye carcinogenesis. This paper deals with the examination and comparison of normal, pre-cancerous, cancerous and tumor proteins of rat livers. The albumin, globulin, ribonucleoprotein and desoxyribonucleoprotein of rat livers were studied.

Configurational differences, if any, between the respective protein and their state of being were obtained through the binding of organic ions by the different proteins. Spectral changes indicated qualitative binding and the dialysis-equilibrium method as developed by Klotz (15) and his co-workers was used for a more quantitative determination of the degree of binding.

A dye such as methyl orange has a highly specific absorption spectra. When the ion of this dye is bound by a protein molecule it shows a spectral absorption curve distinctly different from that of the free ion. The change in absorption of the dye is a reflection of the state of the protein molecule since the structure of the protein determines to a large degree the extent of binding. (16)

Evidence (17) seems to point to the fact that the cationic center on the protein molecule is involved in the binding process. Klotz compared the degree of binding of differently

charged pairs of compounds of practically identical structure. He found that the negatively charged structure bound bovine serum albumin more strongly than the analagous compound without a negative charge. It seemed likely that the protrusion on the protein which attracted the negative ion was positively charged.

Any factor that disturbs the positive charge from these cationic loci or the arrangement, number and kind of the basic amino acid residues of the protein, would markedly affect the binding of anions such as methyl orange and be reflected in a spectral change.

It is quite conceivable that when two proteins for example, normal albumin and cancerous albumin, produce a remarkably different absorption spectra with the same dye (anion) under essentially identical conditions, there must be at least a difference in configuration around the cationic loci on the protein molecule or one can assume that the arrangements of amino acid residues are different.

The normal, precancerous, cancerous and tumor protein was prepared under exacting similar circumstances and their spectrum and degree of binding compared.

BASAL RATION

Young, mature albino Sprague-Dawley rats, 200-240 grams in weight, were fed 0.06 per cent of meta'-methyl dimethylaminoazobenzene (m'-Me DAB) for a period of twelve weeks. Inbred rats are a necessity in experiments involving the use of carcinogens. In 1911 it was shown (30) that female mice in whose ancestors cancer of the breast had occurred, were more liable to develop the disease than mice in whose ancestors the disease was more remote.

The animals were kept in individual screen bottom cages, food and water were given "ad libitum."

The dye was incorporated in the basal ration by dissolving it with heat in the corn oil of the diet. The rations were mixed in amounts sufficient for two to four weeks and stored at 0° C.

The basal diet was essentially the same as that of the Wisconsin Group (35) and consisted of the following:

Casein (vitamin free)	12%
Glucose	79%
Corn Oil (Mazola)	5%
Salts Mixture	4%

The supplements added per kilogram of ration were:

Thiamine Chloride	3.0 mg.
Riboflavin	2.0 mg.

Pyridoxine Hydrochloride	2.5 mg.
Calcium Pantothenate	7.0 mg.
Choline Chloride	30.0 mg.

Halibut Liver Oil was administered one drop per rat each month.

*The salts mixture was composed of:

	Parts by Weight
NaCl	1470
Ca_3PO_4	2086
$MgSO_4 \cdot 7H_2O$	558
KCl	1680
$CaCO_3$	2940
$FePO_4 \cdot 4H_2O$	206
KH_2PO_4	4340
$MgCO_3$	672
KI	1.2
$MnSO_4 \cdot H_2O$	3.2
$K_2Al_2(SO_4)_2 \cdot 12H_2O$	1.2
NaF	7.4
$CuSO_4 \cdot 5H_2O$	5.4

Another group of rats was maintained on the basal ration for the same period of time (twelve weeks).

To compare any distinction in protein structure and binding in reference to the type of diet fed, another group of rats was maintained on "Purina Lab Chow", a standard feed preparation.

The average amount of ration consumed daily per rat was 14.8 grams.

It appears to be well established that the incidence of tumors caused by feeding m'Me DAB depends upon the character of the diet during the precancerous period. Factors in the ration that have been reported (28) to retard tumor development include liver, yeast, grain, the combination of protein and the "B" vitamins and the combination of cysteine and choline. Although it is well recognized that rats which have been fed azo dyes, develop tumors at rates that depend upon the diet fed with the dye, the mechanism by which this accomplished is unknown. Clayton and Baumann (4) suggest that pertinent diets alter the metabolism of the dye and thus alter the concentration of effective carcinogen.

Workers (34) have demonstrated that male inbred rats which were fed m'Me DAB developed liver tumors more readily than females. This observation was noted periodically over a five-year period and has been demonstrated in controlled experiments.

The aminoazobenzenes that show carcinogenic activity have at least one methyl group attached to the carbon of the ring or to the nitrogen of the amino group. For example, aminoazobenzene is inactive, but N-methylaminoazobenzene, 4-dimethylaminoazobenzene and o-aminoazotoluene (31) are active. Neubauer (31) suggests that these apparently essential methyl groups may merely repel electrons and increase the activity of some other

part of the molecule or they react directly with some cell constituents. Boyland (1) presents evidence indicating that for the last ten years, consideration of the essential nature of the carcinogenic hydrocarbons has shown that carcinogenic activity was usually associated with the presence in the molecule of a region of high electron density.

The method of operation of carcinogenic agents has brought forth many theories. Haddow (10) suggests that they function by producing interference with certain normal functions of the cell in such a way to "to induce variation."

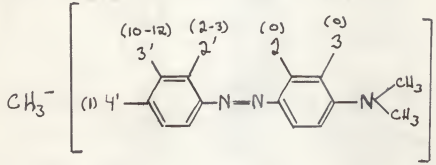
Because the carcinogens produce specific damage to chromosomes and chromosomes contain desoxyribosenucleic acid, workers (2,40) have found it tempting to assume that the carcinogens produce abnormalities in the nucleoprotein. Such abnormalities might be induced in different ways. It is theorized however that the most direct way in which damage in the nucleic acid might produce the observed effect in the chromosomes is by cleavage of the nucleic acid chains.

Many agents (5) have been employed successfully in the experimental induction of tumors in several species of animals.

m'-Me DAB was used for producing cancer in the rats since it has proved to be the most potent carcinogen azo dye reported for the liver of the rat. Miller, et al. (6) reported that for an equivalent concentration of dye, rats fed the m'-Me DAB invariably lost more weight, developed a more severe cirrhosis

and formed large hepatic tumors more rapidly than when any other compound had been fed.

Weber, et al. (25) has recorded the observed carcinogenic activities of various monomethyl ring substituted derivatives of 4-dimethylaminoazobenzene. The parent compound was assigned an activity of 6, and the activities of the derivatives that were studied are given in parentheses next to the position numbers on the ring.



A group in Germany (18) believes that the carcinogenicity of the azo dyes depends upon the ultimate oxidation product, benzo-quinone or its methyl derivative. Kensler, et al. (12) thinks that it is not the azo dye that initiates the carcinogenic process, but an enzyme poison; the split product formed in the breakdown of the parent azo compound in the body. On the other hand the Wisconsin Group (26) take the attitude that the determining factor in carcinogenesis with azo dye is the concentration of azo dye in the liver, rather than the stability of a hypothetical cleavage product. Their experimental data indicates

that no tumors resulted in ten months when the split products of DAB were fed at levels equivalent to 0.06 per cent of the parent azo dye.

Kensler's "split theory" of carcinogenesis by azo dye is attacked by Miller, et al. (27) on the grounds that it can only be valid if cleavage precedes demethylation. Miller shows that DAB can be reversibly demethylated in the rat to form P-monomethylaminoazobenzene prior to reduction at the azo linkage. Much controversy still remains in regard to the mechanism of carcinogenesis.

PREPARATION OF meta'METHYL DIMETHYLAMINOAZOBENZENE

Meta'-methyl dimethylaminoazobenzene was prepared as follows (6). Fifty-four grams of m-toluidine was dissolved in a mixture of 115 ml. of concentrated hydrochloric acid and 250 ml. of water. The solution was cooled to 0° C. in an ice bath, stirred mechanically, and the diazotization affected by adding, dropwise, a cold solution of 34.5 grams of sodium nitrite in 150 ml. of water. The temperature of the reaction was not permitted to rise above 3° C. Sixty-one grams of N-dimethylanaline and 85 grams of anhydrous sodium acetate were then dissolved in 1500 ml. of 70 per cent ethyl alcohol and the solution cooled to 20° C. The diazonium salt was added all at once with stirring to the solution of the amine. The precipitate of the azo compound was filtered and recrystallized from ethyl alcohol-water.

The yield was approximately 100 grams of recrystallized product whose melting point is 119-120° C.

LIVER FRACTIONATION

The animals were anesthetized with ether and their livers removed. It had been observed (9) that low temperature storage of liver aids the extractability of certain of the proteins. Best results were obtained when the livers were either kept at -15° C. for five to ten days before extraction or if extraction was to take place soon after removal of the livers, the livers were frozen in a dry ice - acetone mixture for ten to fifteen minutes.

The various groups of livers were fractionated into four protein components (albumin, globulin, ribonucleoprotein and desoxyribonucleoprotein) on the basis of differential solubility in solutions of sodium chloride and ammonium sulfate.

Fractionations were made as follows:

(1) Normal (basal ration / Purina Lab Chow) twelve weeks.

(2) Rats that had ingested the carcinogen for four to five weeks and died. These livers are designated as "precancerous". If autolysis had taken place before the liver could be removed, it was discarded.

(3) Hepatomas or tumors - rats on the carcinogen twelve weeks.

(4) The liver the hepatoma evolved from - to be known as "cancer liver".

The livers from five to seven rats usually constituted one group for the fractionation procedure.

The procedure as originated by Mirsky and Pollister (29) with modifications by Luck, et al. (9) was as follows:

To the liver was added 3.5 parts by weight of neutral 0.4 M NaCl and the mixture was homogenized for two minutes in a Waring Blender. The entire fractionation procedure was carried out in a room maintained at 2-3° C. After 10 to 15 minutes of stirring, the homogenate was centrifuged for 15 minutes at 3200 RPM (International Centrifuge No. 2). The supernatant fluid (A) was removed and the residue again extracted with a volume of 0.4 M NaCl equal to that of the above supernatant fluid. Following this extraction the remaining residue was stirred with 1.5 volumes of 1.0 M NaCl and centrifuged for one hour at 3200 RPM as above. The supernatant fluid (B) was removed and the residue discarded.

Albumin, globulin and ribonucleoprotein (RNP) were obtained from the initial 0.4 M NaCl extracts and the 1.0 M NaCl extract (B) contained the desoxyribonucleoprotein (DNP).

The extract (A) was reduced to a pH of 5.0, allowed to stand for one hour and then centrifuged for one hour at 3200 RPM.

The residue (C) contained the RNP. The supernatant fluid containing the globulin and albumin was dialyzed overnight with

running distilled water. The precipitated globulin was centrifuged off. The further addition of solid ammonium sulfate to 4.6 M and adjustment of the pH to 6.5 precipitated the albumin fraction.

The residue (C) was stirred in water and the pH adjusted to 8.0 to dissolve the RNP. Adjusting the pH to 5.0 and repeating the process precipitated a relatively pure fraction of RNP.

The DNP was precipitated from the 1.0 M extract (B) by changing the molarity of the NaCl to 0.14 M. At this concentration the protein appears in a fibrous state and can be removed with a stirring rod. The fibers were then redissolved in 1.0 M NaCl. This procedure, repeated three times resulted in a pure preparation of DNP. Acetic acid and NaOH were used to adjust the various pHs.

All the proteins were dialyzed for twelve hours and then lypholized (freeze-dry) by means of acetone - dry ice. This gave a dry pure preparation of the various proteins.

The rats that were subjected to the addition of M'-Me DAB to the basal diet did not gain weight normally as the rats on the basal ration.

Many of the hepatomas produced weight as much as 55 grams. The average liver weight of the rats on the basal ration was 9.25 grams.

BINDING TECHNIQUE

The spectrum of the dye - protein complex was obtained in the following manner.

First the spectrum of the methyl orange was found using buffer (phosphate, pH=6.8) as the blank. The concentration was calculated by the formula $C=\frac{O.D.}{\xi}$. C= concentration, O.D.= optical density and ξ the extinction coefficient. The extinction coefficient was provided by Dr. R. K. Burkhard. By keeping the dye concentration, the same as above, the introduction of protein (resultant protein concentration = .1%) produced the dye-protein spectrum and indicated if binding had taken place. Protein plus buffer was used as the blank.

The graph of the spectrum was obtained by plotting ξ vs. the wavelength. The spectrum was found by employing a Beckman Spectrophotometer (model DU) and using 1-cm. corex cells.

The dialysis - equilibrium method was used as a quantative estimation of the degree of binding. The method consisted essentially of immersing a casing bag with a known concentration of protein (.1%) into a solution of methyl orange. The tube was placed on a mechanical shaker for eighteen hours in order for the system to reach equilibrium. The temperature was maintained at 0° C. with a crushed ice-water bath. The concentration of free dye was determined spectrophotometrically on a Beckman Spectrophotometer (model DU) using 1-cm. corex cells. A control to establish the approximate quantity of free dye

which is present in the absence of protein was accomplished by placing the buffer in the casing and putting this in the methyl orange under exactly the same conditions. All solutions were buffered in a phosphate buffer, pH=6.8, made from reagent grade salts. The methyl orange was a commercial sample of reagent grade, that had been recrystallized from water and dried.

An investigation of the absorption spectra of the various proteins was also conducted. The absorption of light by the proteins (.2% concentration) was determined with the Beckman Spectrophotometer using one-centimeter corex cells. For the determinations the protein was dissolved in phosphate buffer, pH=6.8.

RESULTS AND CONCLUSIONS

Methyl orange complexes with the various proteins are shown in Figures 5 - 6. The maximum absorption of the dye was lowered by both albumin (Fig. 5) and globulin (Fig. 6). There is a decided spectral shift in the peak of cancerous and precancerous albumin.

The marked differences in the absorption spectra of the dye complexes with normal, precancerous, cancerous and tumor albumin convey the impression that structural differences exist among the various types of albumin. The spectra indicate a resemblance between the cancerous and precancerous albumin since both show an identical shift toward a higher wavelength.

The spectral complex of both the normal and tumor albumin are also remarkably similar.

The addition of the albumin in all cases lowered the maximum extinction of the dye. While the displacement is not as great in the case of cancerous and precancerous albumin as in the normal and tumor albumin, there is a distinct lowering of the maximum absorption and a shift of the peak by approximately 15 mμ.

One may assume that the spectral displacements are due to binding, as methyl orange obeys Beer's Law, at these concentrations. There is a linear relationship between the optical absorption and concentration (13).

Quantitative binding data (Fig. 9) appears to confirm the resemblance between tumor - normal and precancerous - cancerous albumin. The normal and tumor albumin seem to have practically the identical affinity for methyl orange.

The cancerous and precancerous albumin bind much more than does either the normal or tumor albumin. It is quite possible that the proteins have changed structurally and new sites have been made available on the protein molecule for binding to occur.

The absorption spectra of normal, cancerous, precancerous and tumor albumin (Fig. 1) also substantiate the normal - tumor and precancerous - cancerous similarities.

It is interesting to note the appearance of the livers

themselves when first removed from the animal. The tumor and normal livers appeared very much alike, whereas both the precancerous liver and cancerous liver to which the tumor was attached resembled each other. Both were severely changed, grossly cirrhotic and hobnailed. The cancerous liver, since it was on the carcinogen three times as long as the precancerous liver, was more damaged.

Miller, et al. (25) believes that the carcinogens combine with the protein in the liver that are responsible for the control of growth. He proposes the theory that cells are formed which have been so changed that they no longer can control normal growth and these changed protein eventually produce the tumor. The results obtained from this investigation seem to support Miller's premise. Possibly during carcinogenesis the albumin of the liver becomes changed or damaged, or in some way modified that it no longer may control growth. From this damaged liver evolves the tumor. Since the tumor evolves from the liver it shall be like liver - normal liver, and the albumin of the tumor will be nearly the same as the albumin of normal liver.

The carcinogen alters the albumin of the precancerous liver in the same manner as the cancerous albumin. However owing to the limited time involved (four weeks), the carcinogen has not been able to produce a tumor.

Miller, et al. (33) has shown also that the bound dye (carcinogen) is not found in the tumor but in the cancerous liver to which the tumor is attached. It appears therefore

that the tumorous growth is not affected in any way and might be very much the same in protein configuration as normal liver, with alteration of the cancerous and precancerous protein (more specifically the albumin).

An examination of the various absorption spectra of the globulin (Fig. 2), of the globulin-methyl orange complex (Fig. 6) and the affinity of globulin for the dye (Fig. 10) does not indicate any remarkable difference between the normal, precancerous, cancerous and tumor globulin. The absorption spectra of the different types of globulin are practically identical.

The methyl orange - globulin complex illustrate the same trend as noted previously with the albumin. The normal and tumor globulin have seemingly the same spectra when complexed with methyl orange.

The quantitative data showing the degree of binding of the globulins (Fig. 10) indicates that although the normal, precancerous, cancerous and tumor globulin bind approximately the same, the cancerous globulin binds the most. These results parallel those obtained with the albumin protein (Fig. 9).

The normal, cancerous, precancerous and tumor ribo and desoxyribonucleoproteins have approximately the same protein-dye spectra (Figs. 7, 8). The absorption spectrum of these proteins are practically identical (Figs. 3, 4) and both proteins do not appear to bind quantitatively to any reasonable extent. This can be readily explained. If we consider that the anion of the dye is attracted largely by positive loci on the protein

in order that the complex may materialize, it would be expected that any factor which would affect the positive charge on these loci would markedly reduce the extent of binding. With the nucleoproteins we have the following situation. The electrostatic repulsion of the negatively charged phosphate groups that are quite abundant in nucleoproteins never permit the dye anion to complex or bind with the protein.

Another factor should also be mentioned. Although the absorption spectra of the normal, precancerous, cancerous and tumor nucleoproteins - methyl orange complexes are for all intensive purposes alike (Fig. 7, 8), this is not necessarily conclusive evidence that absolutely no protein-dye interaction occurs. It is quite conceivable that more than one type of binding that has a tendency to take place might cancel each other. This seems remote in the case of the nucleoprotein due to the negatively charged phosphate groups.

The comparison of the binding abilities of albumin, globulin, ribonucleoprotein and desoxyribonucleoprotein (Figs. 9, 10) indicate very clearly that albumin regardless of whether it is normal, precancerous, cancerous or tumor albumin has a far greater affinity for methyl orange than globulin. The nucleoprotein do not show binding at all.

This was also found to be the case with serum albumin and serum globulin. Klotz and his co-workers (14) have found that serum albumin binds more than serum globulin.

Four different lots of each type of protein were employed

to determine the degree of binding and results indicate that they can be reproduced quite satisfactorily.

The proteins from rats that were maintained on "Purina Lab Chow" and from the group on the basal ration behaved in their binding like other proteins. It appears therefore that the type of diet fed does not affect the protein structure.

Explanations of the differences in the protein-dye complexes and the degree of binding of the various kinds of proteins in terms of configurational differences still remains as a major problem.

When the detailed atomic arrangement of the crystals of amino acids are determined, when more information is available concerning the number and kind of amino acids in the proteins under consideration, and other configurational data is obtained sometime in the future, then the "binding technique" will be of immense value in determining the amino acid residues and configurational changes involved during carcinogenesis and eventually the mechanism of the cancer process itself.

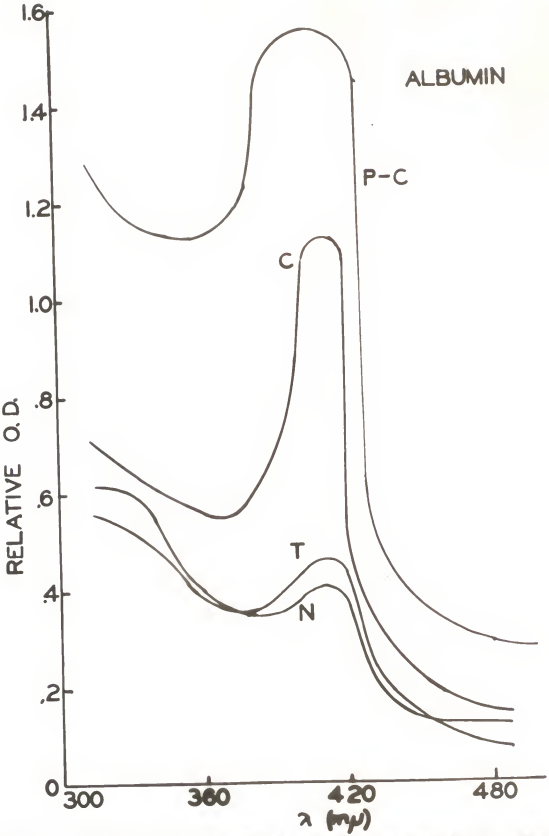


Fig. 1. Absorption spectra of normal, pre-cancerous, cancerous and tumor albumin at pH 6.8.

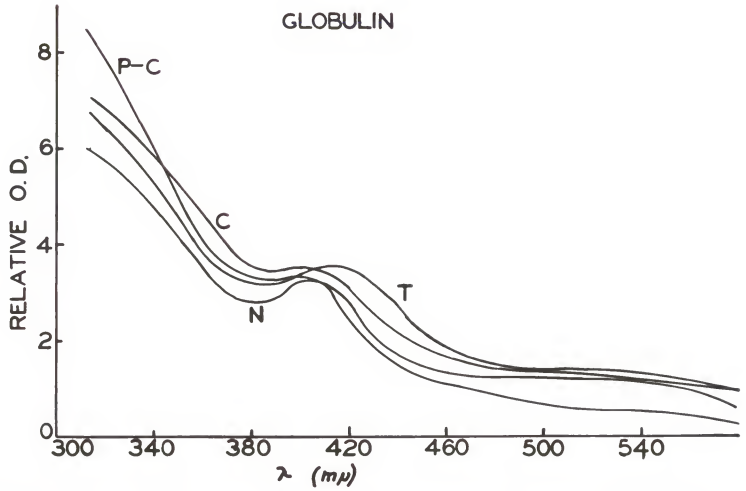


Fig. 2. Absorption spectra of normal, pre-cancerous, cancerous and tumor globulin at pH 6.8.

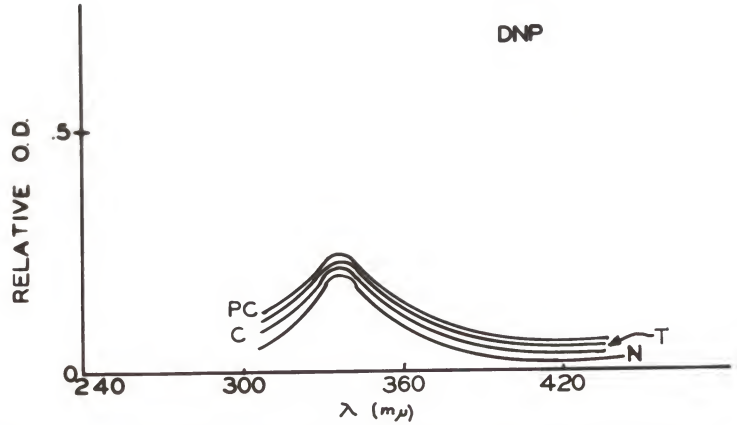


Fig. 3. Absorption spectra of normal, pre-cancerous, cancerous and tumor desoxyribonucleoprotein at pH 6.8.

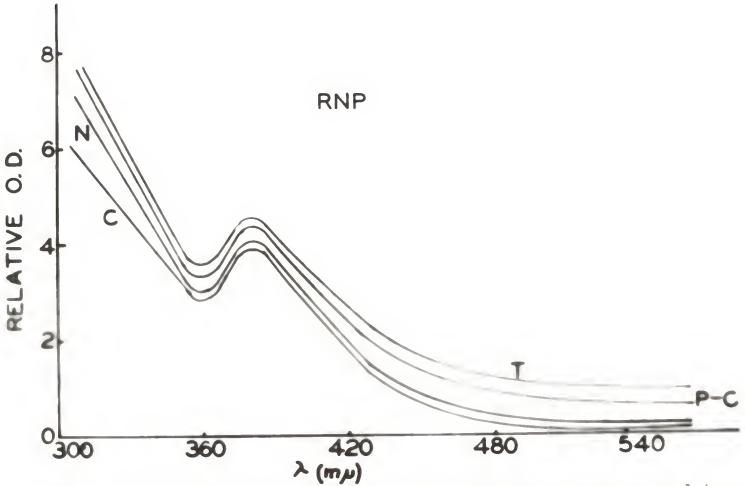


Fig. 4. Absorption spectra of normal, pre-cancerous, cancerous and tumor ribonucleoprotein at pH 6.8.

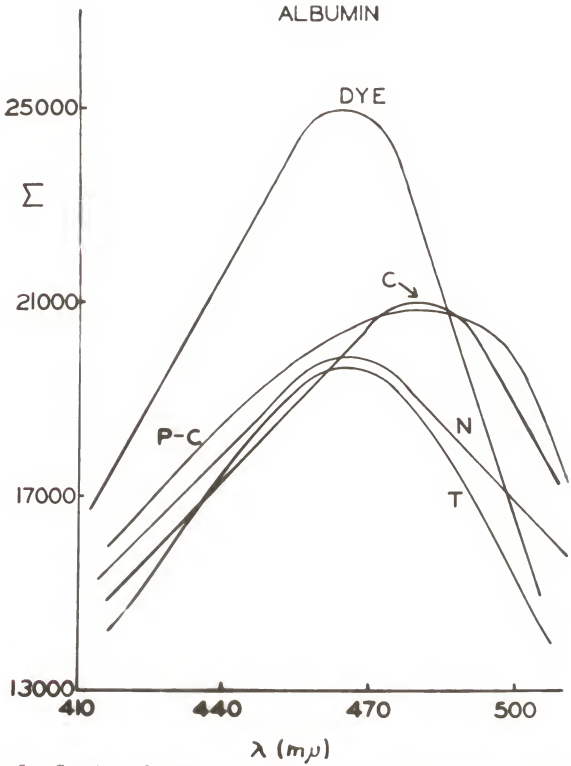


Fig. 5. Spectra of methyl orange and its complexes with normal, pre-cancerous, cancerous and tumor albumin at pH 6.8.

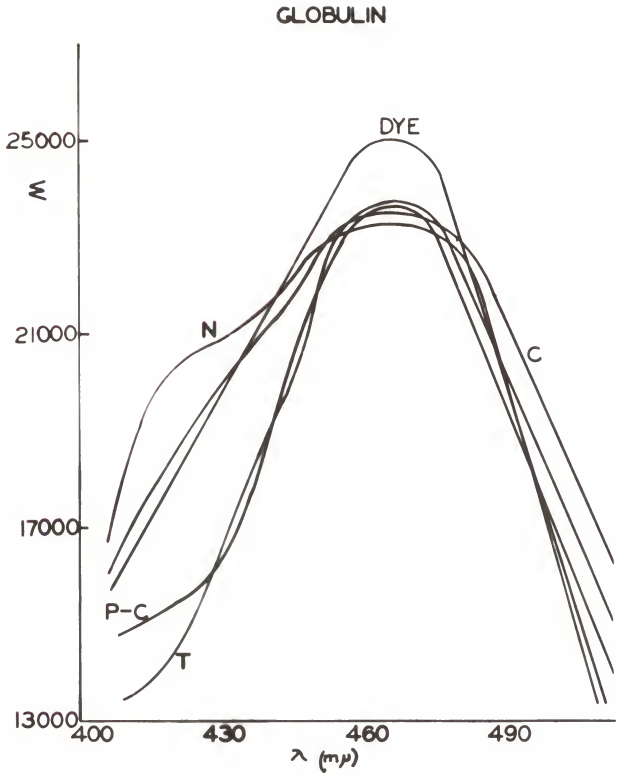


Fig. 6. Spectra of methyl orange and its complexes with normal, pre-cancerous, cancerous and tumor globulin at pH 6.8.

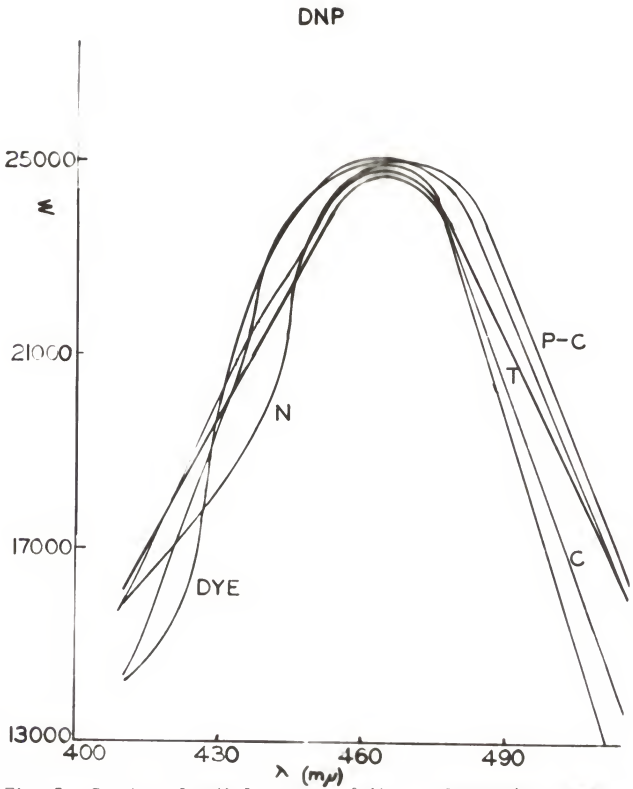


Fig. 7. Spectra of methyl orange and its complexes with normal, pre-cancerous, cancerous and tumor desoxyribonucleoprotein at pH 6.8.

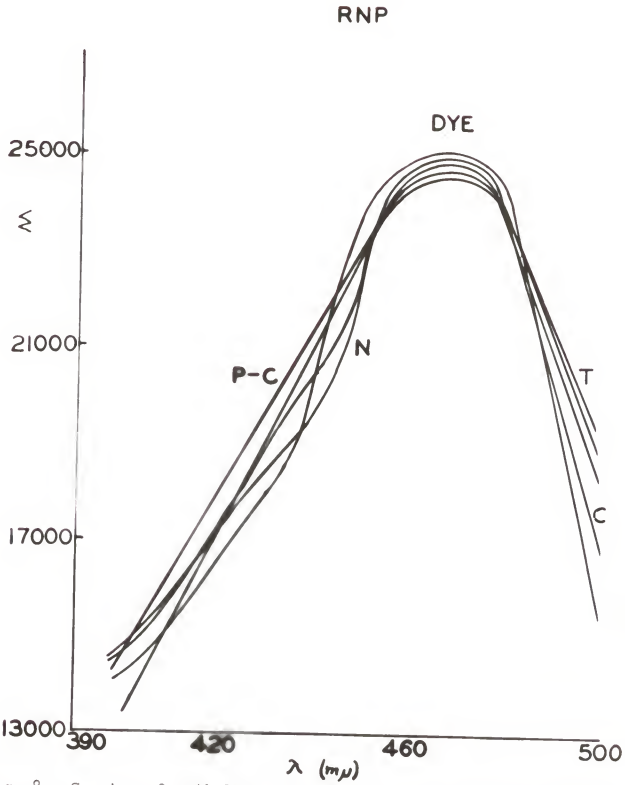


Fig. 8. Spectra of methyl orange and its complexes with normal, pre-cancerous, cancerous and tumor ribonucleoprotein at pH 6.8.

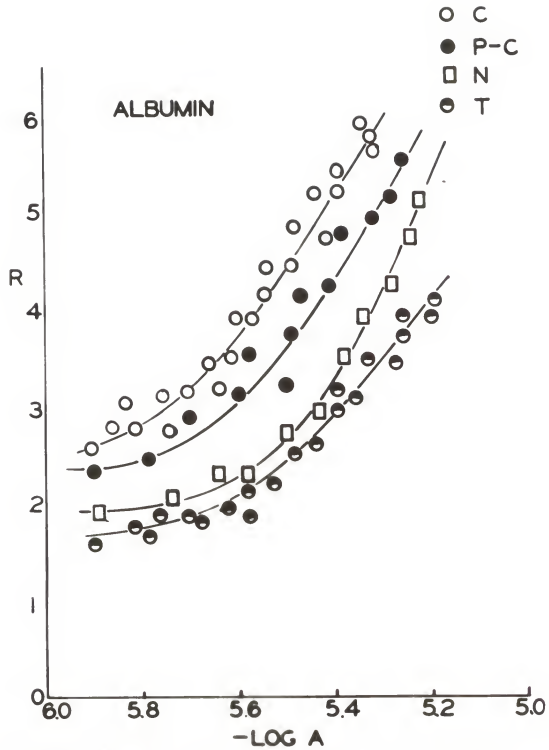


Fig. 9. Binding of methyl orange with normal, pre-cancerous, cancerous and tumor albumin at 0° C and pH 6.8.

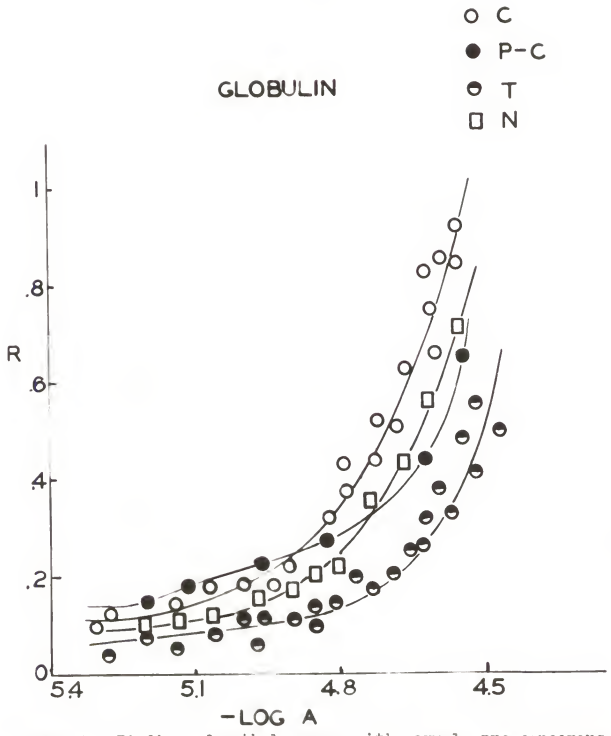


Fig. 10. Binding of methyl orange with normal, pre-cancerous, cancerous and tumor globulin at 0° C and pH 6.8.

SUMMARY

The absorption spectrum, protein-dye complex and the degree of binding of normal, precancerous, cancerous and tumor albumin, globulin, ribonucleoprotein and desoxyribonucleoprotein have been studied. The technique used to produce quantitative evidence concerning the bonding of the various proteins was the equilibrium - dialysis method.

The degree of binding, absorption spectra, and protein-dye complex, indicates that there was a substantial resemblance between normal and tumor albumin and precancerous and cancerous albumin.

The spectra of the protein-dye complex for the various types of globulin also indicated normal-tumor similarities.

The different kinds of nucleoprotein did not bind to any considerable extent and this was attributed to the negatively charged phosphate groups which would have a tendency to repel the negatively charged dye anion.

Albumin, regardless of its condition, normal, precancerous, cancerous or tumor, had a far greater affinity for the methyl orange than any of the other proteins investigated.

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APPENDIX

NORMAL ALBUMIN

Temp. 0° C.

pH. 6.8 LOT # I

TUBE #	CONC. M/L (10^{-6})	CONC. M (10^{-8})	AMT. BOUND: M (10^{-8})	MG. PROTEIN:	r (10^{-8})	-LOG A
Ia	5.23	13.10	9.93	5	1.99	5.896
I	1.27	3.17				
2a	7.22	18.05	11.50	5	2.30	5.581
2	2.62	6.55				
3a	9.65	24.18	14.93	5	2.99	5.432
3	3.70	9.25				
4a	12.27	30.95	19.52	5	3.90	5.341
4	4.56	11.43				
5a	13.61	34.17	21.05	5	4.21	5.281
5	5.24	13.12				
6a	16.10	40.50	25.30	5	5.10	5.217
6	6.07	15.20				
			LOT # II			
Ia	5.21	13.00	9.25	5	1.85	5.825
I	1.50	3.75				
2a	5.93	14.83	10.31	5	2.05	5.742
2	1.81	4.52				
3a	6.91	17.27	11.52	5	2.30	5.639
3	2.30	5.75				
4a	8.63	21.45	13.51	5	2.70	5.499
4	3.17	7.94				
5a	11.22	28.80	17.50	5	3.51	5.380
5	4.17	11.30				
6a	15.13	37.77	23.56	5	4.70	5.245
6	5.69	14.21				

PRE-CANCEROUS ALBUMIN

Temp. 0° C.

pH. 6.8 LOT # I

TUBE: #	CONC. M/L (10^{-6})	CONC. M (10^{-8})	AMT. BOUND: M (10^{-8})	MG. PROTEIN:	r (10^{-8})	-LOG A
Ia	5.95	14.85	11.75	5	2.35	5.907
I	1.24	3.10				
2a	7.76	19.39	14.40	5	2.88	5.701
2	1.99	4.99				
3a	8.66	21.66	15.45	5	3.09	5.605
3	2.48	6.21				
4a	11.59	28.84	20.50	5	4.10	5.478
4	3.33	8.34				
5a	14.62	36.54	24.55	5	4.91	5.320
5	4.79	11.99				
6a	16.35	40.98	27.50	5	5.50	5.261
6	5.49	13.48				
			LOT # II			
Ia	6.64	16.55	12.45	5	2.49	5.797
I	1.64	4.10				
2a	9.75	24.27	17.75	5	3.55	5.583
2	2.61	6.52				
3a	10.63	26.60	18.70	5	3.74	5.496
3	3.19	7.99				
4a	12.22	30.60	21.05	5	4.21	5.418
4	3.82	9.55				
5a	13.71	34.32	23.90	5	4.78	5.379
5	4.18	10.42				
6a	15.62	39.12	26.00	5	5.20	5.280

CANCEROUS ALBUMIN

Temp. 0° C.

pH. 6.8 LOT # I

TUBE: #	CONC. M/L (10^{-6})	CONC. M (10^{-8})	AMT. BOUND: M (10^{-8})	MG. PROTEIN:	r (10^{-8})	-LOG A
Ia	6.42	16.06	13.02	5	1.23	5.911
I	1.23	3.04				
2a	7.10	17.76	14.01	5	2.81	5.824
2	1.50	3.73				
3a	8.30	20.76	15.91	5	3.18	5.713
3	1.94	4.85				
4a	11.38	25.95	19.40	5	3.88	5.582
4	2.62	6.55				
5a	12.56	31.40	22.51	5	4.50	5.450
5	3.55	8.89				
6a	14.39	35.97	26.02	5	5.21	5.401
6	3.98	9.95				
			LOT # II			
Ia	7.00	17.46	14.01	5	2.80	5.859
I	1.38	3.45				
2a	9.08	22.70	17.25	5	3.45	5.661
2	2.18	5.45				
3a	10.32	25.80	19.52	5	3.90	5.601
3	2.51	6.28				
4a	13.00	32.49	24.21	5	4.84	5.480
4	3.31	8.28				
5a	16.04	40.57	28.65	5	5.73	5.321
5	4.77	11.93				

CANCEROUS ALBUMIN

Temp. 0° C.

pH. 6.8 LOT # III

TUBE: #	CONC. :M/L (10^{-6})	CONC. :M (10^{-8})	AMT. BOUND: M (10^{-8})	MC. : PROTEIN:	r (10^{-8})	-LOG A
Ia	7.24	18.06	13.61	5	2.72	5.749
I	1.78	4.45				
2a	9.44	23.57	17.52	5	3.50	5.617
2	2.42	6.05				
3a	11.20	27.99	20.79	5	4.16	5.541
3	2.88	7.20				
4a	13.24	33.07	23.55	5	4.71	5.419
4	3.81	9.52				
5a	14.88	37.24	27.04	5	5.41	5.390
5	4.08	10.20				
6a	15.20	37.95	27.93	5	5.59	5.318
6	4.81	12.02				
			LOT # IV			
Ia	16.60	41.46	29.46	5	5.89	5.319
I	4.80	12.00				
2a	14.00	34.98	25.96	5	5.19	5.442
2	3.61	9.02				
3a	11.72	29.26	22.06	5	4.41	5.541
3	2.88	7.20				
4a	8.68	21.66	15.93	5	3.19	5.641
4	2.29	5.73				
5a	4.80	19.96	15.51	5	3.10	5.750
5	1.78	4.45				
6a	7.60	18.95	15.27	5	3.05	5.831
6	1.47	3.68				

TUMOR ALBUMIN

Temp. 0° C.

pH. 6.8 LOT # I

TUBE: #	CONC. M/L (10^{-6})	CONC. M (10^{-8})	AMT. BOUND: M (10^{-8})	MG. PROTEIN:	r (10^{-8})	-LOG A
Ia	5.19	13.00	9.12	5	1.80	5.810
I	1.55	3.88				
2a	5.85	14.64	9.81	5	1.96	5.714
2	1.93	4.83				
3a	6.82	17.06	11.11	5	2.21	5.623
3	2.38	5.95				
4a	8.21	20.55	12.51	5	2.50	5.492
4	3.22	8.04				
5a	9.72	24.36	14.55	5	2.91	5.403
5	3.96	9.81				
6a	14.26	35.65	20.44	5	4.09	5.217
6	6.07	15.21				
			LOT # II			
Ia	4.92	12.28	8.16	5	1.63	5.781
I	1.65	4.12				
2a	6.84	17.07	10.52	5	2.10	5.582
2	2.62	6.55				
3a	8.88	22.17	13.09	5	2.62	5.440
3	3.63	9.08				
4a	11.56	28.86	17.04	5	3.41	5.325
4	4.73	11.82				
5a	13.28	33.23	19.53	5	3.91	5.261
5	5.48	13.70				

TUMOR ALBUMIN

Temp. 0° C.

pH. 6.8 LOT # III

TUBE: #	CONC. : M/L (10^{-6})	CONC. : M (10^{-8})	AMT. BOUND: M (10^{-8})	MG. : PROTEIN:	r (10^{-8})	: -LOG A
Ia	4.44	11.09	7.94	5	1.59	5.899
I	1.26	3.15				
2a	5.68	14.23	9.03	5	1.81	5.681
2	2.08	5.20				
3a	6.28	15.71	9.73	5	1.95	5.622
3	2.39	5.98				
4a	6.32	15.75	9.17	5	1.83	5.580
4	2.63	6.58				
5a	10.48	26.22	15.32	5	3.06	5.361
5	4.36	10.90				
6a	12.20	30.49	17.09	5	3.42	5.271
6	5.36	13.40				
		LOT # IV				
Ia	14.04	35.10	19.30	5	3.86	5.199
I	6.32	15.80				
2a	12.88	32.24	18.44	5	3.69	5.260
2	5.52	13.80				
3a	10.28	25.73	15.71	5	3.14	5.397
3	4.01	10.02				
4a	7.36	18.43	11.01	5	2.20	5.527
4	2.97	7.42				
5a	6.20	15.46	10.46	5	2.09	5.699
5	2.00	5.00				
6a	5.52	13.76	9.41	5	1.88	5.761
6	1.74	4.35				

NORMAL GLOBULIN

Temp. 0° C.

pH. 6.8 LOT # I

TUBE #	CONC. M/L (10^{-6})	CONC. M (10^{-8})	AMT. BOUND: M (10^{-8})	MG. PROTEIN:	r (10^{-8})	-LOG A
Ia	6.53	16.26	.51	5	.10	5.201
I	6.30	15.75				
2a	8.88	22.20	.62	5	.12	5.064
2	8.63	21.58				
3a	10.94	27.39	.76	5	.15	4.973
3	10.65	26.63				
4a	12.92	32.32	.84	5	.17	4.902
4	12.59	31.48				
5a	14.56	36.36	1.01	5	.20	4.850
5	14.14	35.35				
6a	25.08	62.71	2.86	5	.57	4.621
6	23.94	59.85				
			LOT # II			
Ia	25.48	63.68	3.56	5	.71	4.619
I	24.05	60.12				
2a	22.00	55.00	2.15	5	.43	4.675
2	21.14	52.85				
3a	19.28	48.22	1.72	5	.34	4.731
3	18.60	46.50				
4a	16.12	40.29	1.09	5	.22	4.805
4	15.68	39.20				
5a	7.60	19.04	.54	5	.11	5.131
5	7.40	18.50				

PRE-CANCEROUS GLOBULIN

Temp. 0° C.

pH. 6.8 LOT # I

TUBE: #	CONC. M/L (10^{-6})	CONC. M (10^{-8})	AMT. BOUND: M (10^{-8})	MG. PROTEIN:	r (10^{-8})	-LOG A
Ia	29.48	73.76	3.25	5	.65	4.550
I	28.21	70.51				
2a	24.56	61.41	2.21	5	.44	4.623
2	23.85	59.20				
3a	15.24	38.11	1.42	5	.28	4.834
3	14.67	36.69				
4a	11.44	28.57	1.16	5	.23	4.961
4	10.96	27.41				
5a	8.28	20.74	.97	5	.19	5.102
5	7.91	19.77				
6a	6.64	16.63	.86	5	.17	5.201
6	6.30	15.77				

CANCEROUS GLOBULIN

Temp. 0° C.

pH. 6.8 LOT # I

TUBE #	CONC. M/L (10^{-6})	CONC. M (10^{-8})	AMT. BOUND: M (10^{-8})	MG. PROTEIN	r (10^{-8})	-LOG A
Ia	28.20	71.81	4.61	5	.92	4.561
I	27.54	67.22				
2a	19.31	48.91	2.20	5	.44	4.728
2	18.71	46.73				
3a	15.83	39.61	1.62	5	.32	4.818
3	15.22	38.00				
4a	13.00	32.54	1.13	5	.22	4.903
4	12.51	31.42				
5a	4.20	10.59	.60	5	.12	5.399
5	3.99	9.99				
		LOT # II				
Ia	29.32	73.29	4.26	5	.85	4.559
I	27.61	69.03				
2a	25.40	63.47	4.17	5	.83	4.625
2	23.72	59.30				
3a	23.00	57.46	3.14	5	.63	4.663
3	21.73	54.32				
4a	17.12	42.73	2.21	5	.44	4.791
4	16.21	40.52				
5a	10.32	25.78	.96	5	.19	5.003
5	9.93	24.82				
6a	5.52	13.82	.62	5	.12	5.278
6	5.27	13.20				

CANCEROUS GLOBULIN

Temp. 0° C.

pH. 6.8

LOT # III

TUBE #	CONC. M/L (10^{-6})	CONC. M (10^{-8})	AMT. BOUND: M (10^{-8})	MG. PROTEIN:	r (10^{-8})	-LOG A
Ia	26.04	65.07	3.75	5	.75	4.611
I	24.51	61.32				
2a	21.96	54.96	2.55	5	.51	4.679
2	20.95	52.41				
3a	17.12	42.80	1.91	5	.38	4.787
3	16.36	40.89				
4a	14.72	36.81	1.36	5	.27	4.848
4	14.20	35.45				
5a	7.56	18.90	.77	5	.15	5.140
5	7.25	18.13				
			LOT # IV			
Ia	27.32	68.33	4.31	5	.86	4.592
I	25.61	64.02				
2a	26.44	66.07	3.32	5	.66	4.601
2	25.01	62.75				
3a	20.12	50.31	2.61	5	.52	4.721
3	19.08	47.70				
4a	11.92	29.82	.95	5	.19	4.938
4	11.55	28.87				
5a	8.88	22.24	.91	5	.18	5.069
5	8.53	21.33				
6a	5.20	13.04	.57	5	.11	5.302
6	4.99	12.47				

TUMOR GLOBULIN

Temp. 0° C.

pH. 6.8 LOT # I

TUBE: #	CONC. :M/L (10^{-6})	CONC. :M (10^{-8})	AMT. BOUND: M (10^{-8})	MG. PROTEIN:	r (10^{-8})	-LOG A
Ia	29.16	72.93	2.41	5	.48	4.550
I	28.21	70.52				
2a	27.56	68.89	1.67	5	.33	4.571
2	26.89	67.22				
3a	20.72	51.75	1.00	5	.20	4.693
3	20.30	50.75				
4a	15.76	39.37	.72	5	.14	4.812
4	15.46	38.65				
5a	12.72	31.83	.56	5	.11	4.903
5	12.51	31.27				
6a	6.48	16.17	.37	5	.07	5.201
6	6.30	15.80				
		LOT # II				
Ia	31.16	77.91	2.76	5	.55	4.522
I	30.06	75.15				
2a	26.56	66.44	2.41	5	.48	4.592
2	25.61	64.03				
3a	23.92	59.82	1.32	5	.26	4.631
3	23.40	58.50				
4a	18.80	46.97	.92	5	.18	4.735
4	18.42	46.05				
5a	10.32	25.77	.57	5	.11	4.959
5	10.08	25.20				
6a	8.84	22.14	.41	5	.08	5.061
6	8.69	21.73				

TUMOR GLOBULIN

Temp. 0° C.

pH. 6.8 LOT # III

TUBE #	CONC. M/L (10^{-6})	CONC. M (10^{-8})	AMT. BOUND: M (10^{-8})	MG. PROTEIN:	r (10^{-8})	-LOG A
Ia	31.20	78.01	2.06	5	.41	4.518
I	30.38	75.95				
2a	22.40	56.02	1.27	5	.25	4.659
2	21.90	54.75				
3a	14.32	35.83	.71	5	.14	4.852
3	14.05	35.12				
4a	10.24	25.59	.61	5	.12	5.001
4	9.99	24.98				
5a	8.36	2.09	.27	5	.05	5.138
5	7.28	1.82				
			LOT # IV			
Ia	35.00	87.47	2.45	5	.49	4.469
I	34.01	85.02				
2a	24.52	61.33	1.61	5	.32	4.622
2	23.90	59.72				
3a	17.56	43.96	1.04	5	.21	4.765
3	17.17	42.92				
4a	14.40	35.97	.49	5	.09	4.850
4	14.19	35.48				
5a	10.84	27.11	.31	5	.06	4.969
5	10.72	26.80				
6a	5.32	13.32	.22	5	.04	5.281
6	5.24	13.10				

NORMAL RIBONUCLEOPROTEIN (RNP)

Temp. 0° C.

pH. 6.8

TUBE: #	CONC. M/L (10^{-6})	CONC. M (10^{-8})	AMT. BOUND: M (10^{-8})	MG. PROTEIN:	r (10^{-8})	-LOG A
Ia	19.11	47.78	.05	5	.01	4.719
I	19.09	47.73				
2a	15.81	39.53	.00	5	.00	4.802
2	15.82	39.53				
3a	12.96	32.40	.20	5	.04	4.893
3	12.88	32.20				
4a	10.61	26.53	.00	5	.00	4.975
4	10.61	26.53				

CANCEROUS RIBONUCLEOPROTEIN (RNP)

Temp. 0° C.

pH. 6.8

TUBE #	CONC. M/L (10^{-6})	CONC. M (10^{-8})	AMT. BOUND: M (10^{-8})	MG. PROTEIN	r (10^{-8})	-LOG A
Ia	10.00	25.01	.05	5	.01	5.002
I	9.96	24.96				
2a	4.20	10.53	.14	5	.03	5.385
2	4.12	10.39				
3a	13.00	32.51	.22	5	.04	4.893

TUMOR RIBONUCLEOPROTEIN (RNP)

Temp. 0° C.

pH. 6.8

TUBE #	CONC. M/L (10^{-6})	CONC. M (10^{-8})	AMT. BOUND: M (10^{-8})	MG. PROTEIN	r (10^{-8})	-LOG A
Ia	28.36	70.91	.00	5	.00	4.547
I	28.36	70.91				
2a	19.28	48.21	.04	5	.01	4.717
2	19.24	48.17				
3a	13.00	32.50	.21	5	.04	4.893
3	12.88	32.29				

NORMAL DESOXYRIBONUCLEOPROTEIN (DNP)

Temp. 0° C.

pH. 6.8

TUBE: #	CONC. : M/L (10^{-6})	CONC. : M (10^{-8})	AMT. BOUND: M (10^{-8})	MG. : PROTEIN:	r (10^{-8})	-LOG A
Ia	28.36	70.91	.13	5	.03	4.549
I	28.28	70.78				
2a	19.28	48.22	.04	5	.01	4.717
2	19.24	48.17				
3a	15.84	39.63	.00	5	.00	4.802
3	15.84	39.63				
4a	10.00	25.00	.22	5	.04	4.005
4	9.88	24.78				

CANCEROUS DESOXYRIBONUCLEOPROTEIN (DNP)

Temp. 0° C.

pH. 6.8

TUBE #	CONC. M/L (10^{-6})	CONC. M (10^{-8})	AMT. BOUND: M (10^{-8})	MG. PROTEIN	r (10^{-8})	-LOG A
Ia	26.04	65.10	.05	5	.01	4.587
I	25.96	64.95				
2a	17.08	42.71	.00	5	.00	4.767
2	17.08	42.71				
3a	14.74	36.63	.24	5	.07	4.839
3	14.62					
4a	11.52	28.85	.16	5	.03	4.943
4	11.44	28.69				

TUMOR DESOXYRIBONUCLEOPROTEIN (DNP)

Temp. 0° C.

pH. 6.8

TUBE #	CONC. M/L (10^{-6})	CONC. M (10^{-8})	AMT. BOUND: M (10^{-8})	MG. PROTEIN	r (10^{-8})	-LOG A
Ia	28.40	71.02	.16	5	.03	4.549
I	28.28	70.86				
2a	19.16	47.90	.12	5	.02	4.719
2	19.08	47.78				
3a	12.96	32.40	.11	5	.02	4.893
3	12.88	32.29				

BINDING PROPERTIES OF NORMAL AND CANCEROUS LIVER PROTEINS

by

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Man and most animals are faced with an abnormal, independent type of growth which we designate as a tumor or cancer. An indispensible part of this growth process is protein and protein synthesis.

Available evidence speaks against a very great difference between "normal" protein and "cancerous" protein. The implication that structural changes exist between these two types of protein has repeatedly appeared in the literature but conclusive evidence has been lacking.

Workers have found the tumor and normal liver to be composed of the identical amino acids, both qualitatively and quantitatively. Researchers have indicated that malignant tissue protein differs from normal tissue protein, both immunologically and serologically.

Miller offers significant evidence that a qualitative difference in protein may exist between the liver and tumor. He showed that the carcinogen (an azo dye) was found bound to the protein of the liver, but not present in the tumor itself, nor elsewhere in the body.

Young, mature, albino Sprague-Dawley rats, 200-240 grams in weight were fed 0.06 per cent m'-Me DAB for a period of twelve weeks. The animals were kept in individual screen bottom cages and food and water were administered "ad libitum." The dye was incorporated in the basal ration by dissolving it with heat in

the corn oil of the diet. The average amount of ration consumed daily, per rat, was 14.8 grams.

At the end of twelve weeks, the animals were anesthetized with ether and the livers removed. The livers were stored at -15° C. for five to ten days before being fractionated into albumin, globulin, ribonucleoprotein and desoxyribonucleoprotein. The fractionation procedure was performed at 0° C.

Studies were conducted upon normal, precancerous and cancerous liver and also the tumor evolved from the cancerous liver. The liver from five to seven rats usually constituted one group for the fraction procedure. Four different lots of each type of protein were employed and their reproducibility appeared to be quite satisfactory.

The proteins were dialyzed overnight with distilled water and then lypholyzed by means of acetone-dry ice. A dry, pure preparation of protein resulted.

The various kinds (normal, precancerous . . . etc. . . .) of albumin, globulin and the nucleoproteins were investigated in regard to their absorption spectrum, the spectra of the dye-protein complex and their degree of binding employing the dialysis- equilibrium technique.

Vast differences in the absorption spectra of the dye complexes with normal, precancerous, cancerous and tumor albumin suggest possible structural differences among these types of albumin. Both cancerous and precancerous albumin display an

identical spectral shift towards a higher wavelength. The spectral complex of normal and tumor albumin are also quite similar. Quantitative binding data and the absorption spectra of the various albumin irrevocably substantiate the normal-tumor and cancerous-precancerous resemblance. The physical appearance of the liver and tumor also add credence to this implication.

The above results also seem to offer support to a theory of carcinogenesis by Miller.

The nucleoprotein do not bind due to their abundance of negatively charged phosphate groups. The absorption spectra and the dye-protein complex of the various types of ribo and desoxyribonucleoprotein are nearly identical.

The globulin-methyl orange complex illustrates the same trend as was evident in the case of albumin, that of the same spectra of normal and tumor globulin when complexed with methyl orange.

In the case of both albumin and globulin, the cancerous protein bound the greatest.

Albumin, regardless of whether it is normal, precancerous, cancerous or tumor, has a far greater affinity for methyl orange than any of the other protein considered.