Thermal Coagulation of Serum Proteins

II. Deficient Coagulation in Cancer and the Iodoacetate Index*

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Frequently in human cancer the serum proteins are deficient in quantity and abnormal in kind. The qualitative abnormalties have not been precisely defined but are recognizable *inter alia* by defective coagulation when the serum proteins are subjected to heat as will be demonstrated in this paper. The simple determination of the lowest concentration of proteins which can coagulate provides useful information in recognition of the presence of cancer in man. The iodoacetate index is another quantitative device for recognition of the abnormality of serum protein permitting the over-all coagulative defects to be expressed in mathematical form.

In his valuable review of the status of proteins in cancer Toennies (13) has recently assembled much of the published data concerning the serum proteins in cancer. Numerous investigations have shown that on a statistical basis the serum of man and animals with malignant tumors contains a lowered concentration of total proteins and albumin and a low albumin/globulin ratio; the fibrinogen level is usually increased in the blood plasma in cancer.

Only a few studies have been made of the coagulation of serum proteins by heat. Ehrentheil and Weis-Ostborn (6) and these authors with Luger (9) studied the coagulative effects of heat on serum and found pronounced differences between cancer and pernicious anemia; with respect to normal serum, less flocculation occurred in the serum of cancer patients while greater turbidity developed in the sera in pernicious anemia. The serum of patients with cancer has been investigated by means of the Weltmann reaction (15) which consists of heating serum in the presence of progressively decreasing concentrations of calcium chloride, the serum proteins being very dilute in final concentration. The Weltmann reaction apparently does not depend on the concentrations of protein present. In this test serum flocculates over a rather constant range of concentration of the electrolyte; the range is more restricted in a variety of debilitative states such as hepatic and cardiac diseases and fibrous pulmonary tuberculosis, where flocculation occurs only in the stronger concentrations of calcium ions. Roeloffs (11) observed a decreased "range" of flocculation in malignant states but Teufl (12) related this defect to necrotic or regressive changes in tumor tissue or to inflammatory complications in the neoplasm.

In contrast to this evidence as well as to the data reported in the present paper, but reconcilable with them, are the observations of Black, Kleiner and Bolker (2) who demonstrated that *plasma* from cancer patients tended to undergo heat coagulation more rapidly than plasma from normal individuals. It was evident from their studies that an increased level of blood fibrinogen was of importance in this effect although they state that no absolute stoichiometric relationship existed between the increased coagulation reaction and the fibrinogen content of plasma.

Griffin and Baumann (7) found that homogenates of livers from rats fed *m'*-methyl-*p*-dimethylaminoazobenzene failed to coagulate when heated in a bath of boiling water whereas similar preparations of normal rat liver coagulated completely after being boiled for only a few minutes.

The coagulation of protein by heat is a complex physico-chemical change which is affected by many factors (8) including pH, total ionic strength and charge density, the concentration of non-electrolytes as well as by the presence of certain

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promoting and inhibiting agents. The known inhibitors which act in low concentration are fatty acids and alkyl sulfonates (1), (5), thymus nucleate (4) and iodoacetate[1] (8).

At pH 7.4, halogenated acetates in varying effectiveness have a peculiar property (8) of irreversibly inhibiting the thermal coagulation of proteins; it is of interest that chemical substances closely related to iodoacetate such as the methyl ester of iodoacetate, iodoacetamide, iodoacetone, etc., promote rather than inhibit coagulation. Although the mechanism of the inhibition of protein coagulation by iodoacetate is unknown, clearly this compound reacts with those molecular linkages essential to clot formation.

The iodoacetate index is an expression relating the inhibition of clotting by iodoacetate to the quantity of protein present in serum and hence to the total availability of linkages essential to coagulation. In this paper conditions are described under which the relation between serum proteins and iodoacetate is linear.

METHODS

Reagents.—

1. *M/15 phosphate buffer, pH 7.4.* Dissolve 1.743 gm. of potassium di-hydrogen phosphate, KH_2PO_4, and 7.652 gm. of anhydrous di-sodium hydrogen phosphate, Na_2HPO_4, in water and dilute to 1 liter.
2. *Normal sodium hydroxide.*
3. *0.1% phenolsulfonephthalein.* A few drops of N sodium hydroxide are added to 0.1 gm. of phenolsulfonephthalein and the solution is diluted to 100 ml.
4. *0.06 M iodoacetate.* Iodoacetic acid was recrystallized twice from a mixture of benzene and petroleum ether. 0.279 gm. of iodoacetic acid is placed in a 25 ml. volumetric flask and dissolved in 10 ml. of phosphate buffer; add 2 drops of phenosulfonephthalein. As a pH control, similar amounts of buffer and indicator are added to another flask containing no iodoacetate. N sodium hydroxide is added drop-wise to the flask containing iodoacetic acid until the color matches that of the control solution of buffer and indicator. The iodoacetate solution is then diluted to the mark with phosphate buffer. Iodoacetate solutions were prepared freshly each day.
5. *3 M sodium chloride.* Dissolve 17.56 gm. of sodium chloride in water and dilute to 100 ml.

Equipment.—We used 1 ml. pipettes graduated in 0.01 ml. divisions, with the tips drawn out to a fine point. Pyrex glass tubes (10×75 mm.) were employed in the tests which were conducted in a bath of vigorously boiling water. The water bath was large so that boiling was not interrupted upon introduction of a metallic rack containing the tubes.

Determination of Protein and Albumin Content.—Total proteins of serum were determined by the micro-Kjeldahl technique of Ma and Zuazaga (10) after digestion with sulfuric acid in the presence of copper and selinite (3). The proteins were estimated by multiplying by 6.25 the total nitrogen corrected for non-protein nitrogen.

Serum albumin was determined by the same techniques after precipitation of the globulins with 23 per cent sodium sulfate and ethyl ether (14).

Clinical material.—The coagulative characteristics of the sera of 300 individuals were studied; these people were classified in three categories, each of 100, respectively, of apparently healthy persons, patients with cancer and patients with non-malignant pathology; not all of the tests were run on all of the sera. The entire group was assembled from a hospital population, either patients or attendants; each of the individuals was seen, at least, by one of the authors, while in most cases the clinical and pathologic studies were rather more extensive.

EXPERIMENTAL

Iodoacetate index.—For each serum 10 tubes were set up containing 0.25 ml. of serum and 0.12 ml. of 3 M sodium chloride. Increasing amounts of the 0.06 M iodoacetate solution were placed in the tubes with correspondingly decreasing quantities of buffer (Table 1), the total amount of these two solutions measuring 0.38 ml. Thus the series consisted of a step-wise titration of 0.25 ml. of serum in 1:3 dilution against 9 to 22.8 μM of iodoacetate. The tubes were agitated to mix the contents thoroughly and then were placed in a bath of vigorously boiling water for 30 minutes.

After boiling, each tube was inverted and shaken very gently several times to disrupt any surface coagulation at the air-water interface. In this test, by our definition, serum is coagulated when it is thoroughly solid and will not pour. The end-point is the largest amount of iodoacetate, expressed in micromoles, in which serum coagulates.

The iodoacetate index is derived from the end-point extrapolated to 1 ml. divided by grams of total protein or albumin in 100 ml. of serum:

$$\text{Iodoacetate index} = \frac{\mu\text{M iodoacetate} \times 4}{\text{protein in gm. per 100 ml. of serum}}$$

[1] In this paper, iodoacetic acid brought to pH 7.4 is designated as iodoacetate.

Least concentration of coagulable protein.—Eight 10×75 mm. tubes were set up for each test so as to contain a final concentration of 15 to 33 per cent of serum. To the tubes were added progressively increasing amounts of serum—0.15, 0.18, 0.2 ml. . . . 0.33 ml. of serum, and the volume was made up to 1 ml. with M/15 phosphate buffer 0.85, 0.82, 0.8 ml. . . . 0.67 ml. The contents were thoroughly mixed and the tubes were placed in a bath of boiling water for thirty minutes. Each tube was inverted without shaking, and if the contents did not pour, it was read as coagulation. The lowest percentage of serum which coagulated was the end point. The data were related to the protein content in 100 ml. of serum. As an example of the calculation, the final volume is 1.0 ml.; if the end point is 0.2 ml., and the total protein content is 6 gm. per 100 ml., the least concentration of coagulable protein is:

$$\frac{0.2}{1.0} \times 6 = 1.2 \text{ gm. per } 100 \text{ ml.}$$

TABLE 1

ARRANGEMENT OF TUBES FOR DETERMINATION OF IODOACETATE INDEX

Tube no.	1	2	3	4	5	6	7	8	9	10
Iodoacetate—ml.	0.15	0.18	0.20	0.23	0.25	0.28	0.30	0.33	0.35	0.38
Iodoacetate—μM	9.0	10.8	12.0	13.8	15.0	16.8	18.0	19.8	21.0	22.8
Buffer—ml.	.23	.20	.18	.15	.13	.10	.08	.05	.03	0.0
Serum—ml.	.25	.25	.25	.25	.25	.25	.25	.25	.25	.25
Sodium chloride, 3 M—ml.	.12	.12	.12	.12	.12	.12	.12	.12	.12	.12

Quantitative relationship between iodoacetate and protein concentration.—To determine the relationship between these components, thermal coagulation titrations were carried out between increasing amounts of serum 0.5 to 1 ml. and varying amounts of iodoacetate, 1.5 to 54 micromoles dissolved in phosphate buffer, at each serum level; the total volume in each tube was 1.5 ml. In graphic form, the results appear as a paraboloid curve (Fig. 1). In one experiment, the iodoacetate end point for 0.5 ml. of serum was 4.5 μM while for 1 ml. the end point was 42 μM.

Repeating this experiment in the presence of concentrated sodium chloride, a rectilinear relationship was observed between the concentrations of iodoacetate and protein. In argeement with earlier findings, increased electrolyte concentration promotes protein coagulation; we found further that larger amounts of iodoacetate are required to block coagulation than in the absence of added salt. Despite this straight-line relationship, proportionality does not exist between the iodoacetate end point and the protein content; thus, in this experiment (Fig. 1) coagulation of 28 mg. of protein was blocked by 27 μM of iodoacetate while 56 mg. of protein required 75 μM to inhibit clotting.

Coagulative characteristics of serum versus plasma.—In a series of 20 normal individuals and 10 patients with cancer, coagulation tests were made simultaneously on oxalated and heparinized plasma and blood serum. With respect to the tests described in this paper, plasma did not differ essentially from serum in its quantitative characteristics of coagulation. The plasma of normal persons like the serum has a greater capacity for thermal coagulation than that of patients with cancer. The remainder of this paper is concerned exclusively with observations on serum.

RESULTS

Least concentration of coagulable protein.—The greatest dilution of serum to clot in phosphate buffer was 16 ml. per 100 ml.; all sera in this series coagulated when the concentration of serum was 42 per cent or greater.

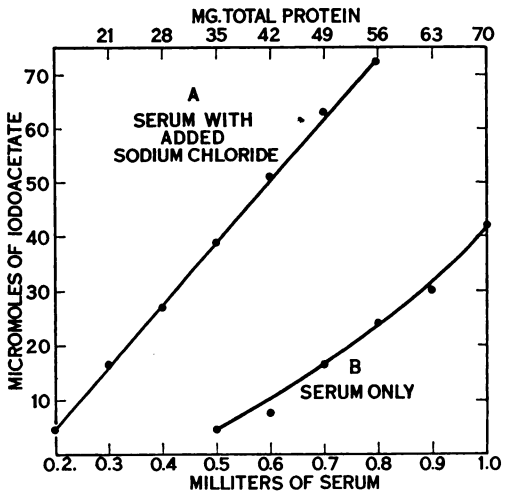

FIG. 1.—Inhibition of thermal coagulation of varying amounts of serum by iodoacetate: A, in the presence of 3 M sodium chloride; B, without added salt. The final volume in each case is 1.5 ml.

The lowest concentration of serum to undergo coagulation under the conditions defined was expressed in terms of grams of total protein per 100 ml. of serum. All of the sera from 84 normal individuals clotted at levels of 1.476 gm. per cent, or

less; the sera of 7 patients with cancer coagulated at levels of less than 1.5 gm. per cent. These consisted of cancer of the prostate, well controlled, 2, uncontrolled, 1; cancer of colon, 1; polyposis of rectum, 1; lymphosarcoma, 1; excised "solitary" metastasis, 1.

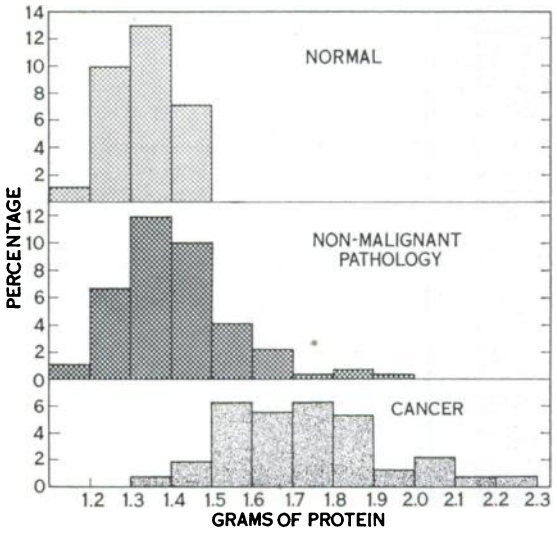

FIG. 2.—Histogram of least concentrations of total serum protein to undergo thermal coagulation. The percentage is on the basis of the entire group of 268 individuals.

With reference to the group of individuals with least coagulable protein concentrations of 1.5 gm. per cent or more, there were no normal individuals; 76 patients with cancer; and 21 patients with non-malignant disease. Hence, 76/83 cases or 92 per cent of patients with cancer and 21/101 cases or 21 per cent of persons with non-malignant pathology including tuberculosis fell in this arbitrary group which was free from healthy individuals.

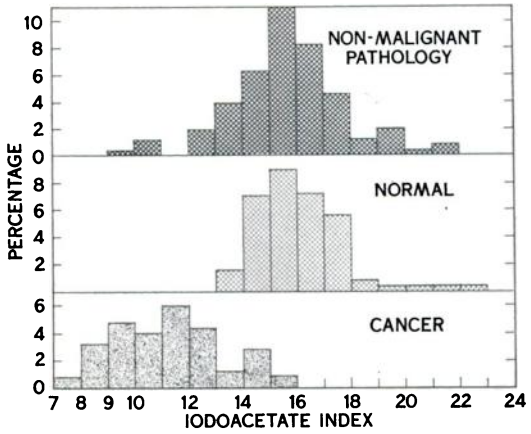

FIG. 3.—Histogram of iodoacetate indices on the basis of albumin content of serum. The percentage is on the basis of the entire group of 244 individuals.

The iodoacetate index in malignant disease.—

(a) *On the basis of serum albumin.* 244 individuals were available in this group. With reference to an iodoacetate index of 12.99 or less, there were 68 cases in all: no normals; 10 patients with non-malignant pathology including tuberculosis: and 58 patients with malignant disease. The 10 patients with non-malignant pathology included: pulmonary tuberculosis, 6; chronic nephritis, 2; lobar pneumonia, 1; infected hydronephrosis, 1.

With reference to an iodoacetate index of 13 or more, the group wherein all of the normals fell, there were 176 cases available. There were 12 cases

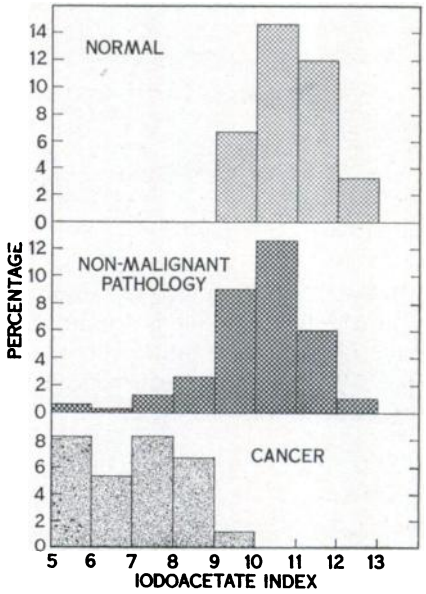

FIG. 4.—Histogram of iodoacetate indices on the basis of total protein concentration of serum. The percentage is on the basis of the entire group of 283 individuals.

with cancer in this group, namely: controlled cancer of the prostate, 2; uncontrolled cancer of the prostate, 2; cancer of breast, 2; cancer of lung, 2; cancer of cervix, 1; testis, 1; penis, 1; and rectum, 1.

Considering an iodoacetate index of 12.99 or less on the basis of albumin content, 58/70 cases or 83 per cent of patients with malignant disease fell in this group; also in this category were 10/100 cases of non-malignant pathology including tuberculosis or 10 per cent of the cases.

(b) *On the basis of total serum protein.*—Determinations were made on the serum of 283 individuals classified in three groups: normal, non-malignant pathology including pulmonary tuberculosis, and cancer. With reference to the group of individuals with an iodoacetate index of 8.99 or less, there were: no normal persons; 16 patients with non-malignant pathology; and 85 patients with cancer. The 16 individuals with non-malig-

nant pathology consisted of: all patients with pulmonary tuberculosis, 7; chronic nephritis, 3; benign prostatic hypertrophy, 2; one patient, respectively, with lobar pneumonia, infected hydronephrosis, aortic aneurysm, and pregnancy.

In the group with an iodoacetate number of 9 or larger (Fig. 4), there were 100 normals, 79 patients with non-malignant pathology, and 3 cases of malignant disease. The 3 patients with cancer consisted of 1 patient in whom an apparently solitary metastatic lesion had been excised surgically two weeks previous, and 2 patients with cancer of the prostate believed to be well controlled by antiandrogenic measures.

The iodoacetate index of 8.99 or less on the basis of total protein content of serum was chosen because in this series it excluded all apparently healthy persons. In the group of patients whose serum was found to have an iodoacetate index of less than 9, 85/88 cases or 96.6 per cent of patients with cancer fell in this group. The three exceptions of cancer not recognized under this system, consisted of patients in whom the carcinoma was apparently well controlled. There were in this group, however, 16/95 cases of non-malignant pathology or 16.8 per cent of non-cancer false positives. All of the patients with non-malignant pathology whose values fell in this category had serious disease.

DISCUSSION

These experiments revealed a defect in the coagulative capacity of the serum proteins with respect to the linkages available for thermal coagulation. In all cases the data are referred to protein concentration. In measuring the least concentration of protein which can undergo coagulation in phosphate buffer, the larger the value the greater is the deviation from normal; with the iodoacetate indices the lower values imply greater abnormalities.

Clearly, patients with cancer seldom have as high an iodoacetate index (Table 2) as normal subjects. It is impossible to attribute this striking discrepancy to chance alone. Precautions were taken to protect the data against statistical artifact. Thus tests on the various types of subjects were run in a haphazard order which we believe to be effectively random, and the laboratory work was generally, though not always, done in ignorance of the status of the subject. The conventional statistical résumé in terms of series, size, sample mean and standard deviation are given in Table 2. The striking appearance of Figures 2 to 4 makes a formal test of statistical significance virtually superfluous but the conventional t-tests were made and, as was to be expected, the results were incompatible with a hypothesis that the difference between the coagulability of our normal and cancerous subjects is due to chance alone.

The coagulation reaction is not specific as a diagnostic instrument although we have found it to be rather useful. From a practical standpoint, the difficulty is not in the failure to recognize active malignant disease, but in the false positive

TABLE 2

STATISTICAL ANALYSIS OF THERMAL COAGULATION OF SERUM

	No. of patients in series	Range	Median	Mean	Standard deviation
Iodoacetate Index $\frac{\mu M \text{ Iodoacetate}}{\text{Gm. Protein}}$					
(1) Normals	100	9.20–12.60	10.82	10.81	0.7538
(2) Non-malignant Pathology	88	8.36–13.00	10.28	10.39	0.9104
(3) Tuberculosis (Pulmonary)	7	5.24– 7.64	7.60	7.23	1.1773
(4) Cancer	88	5.0 – 9.96	7.12	6.43	2.3560
Iodoacetate Index $\frac{\mu M \text{ Iodoacetate}}{\text{Gm. Albumin}}$					
(1) Normals	74	13.44–22.5	15.79	16.10	1.6660
(2) Non-malignant Pathology	94	13.13–21.11	15.94	16.18	1.6960
(3) Tuberculosis (Pulmonary)	6	10.26–12.74	11.50	11.51	1.0940
(4) Cancer	70	7.93–16.48	10.76	10.15	3.8560
Lowest Coagulable Protein Concentration Protein, Gm./100 ml.					
(1) Normals	84	1.147–1.476	1.320	1.334	0.1646
(2) Non-malignant Pathology	94	1.164–1.720	1.390	1.391	0.1670
(3) Tuberculosis (Pulmonary)	7	1.578–1.924	1.718	1.734	0.1360
(4) Cancer	83	1.355–2.268	1.718	1.723	0.1979

$$\text{Standard deviation} = \sqrt{\frac{\Sigma xi^2 - x\,\Sigma xi}{n-1}}, \quad \text{where} \quad \begin{array}{l} \Sigma xi = \text{sum of all values in the series} \\ \bar{x} = \text{sample mean} \\ n = \text{no. of patients in the series} \end{array}$$

reactions. For example, in all respects the serum of patients with pulmonary tuberculosis resembled cancerous serum in its coagulative aspects. The serum of patients with benign tumors coagulated like that of normal individuals.

Relating the iodoacetate index to the total protein content of serum gave the most useful information. With an arbitrary index of 9 as the minimum for normal serum, all active cancers were correctly classified as far as confirmation of the clinical diagnosis is available. The patients suffering from non-malignant pathology who fell in this group with a defective coagulation reaction had serious diseases and were far from normal.

In the iodoacetate index (total protein content) the blood of 6 newborn infants as obtained from the umbilical cord just after birth coagulated like that of normal adults. In the case of 1 pregnant woman, the index was slightly lowered (8.72) while in 5 cases the iodoacetate index was in the normal range.

SUMMARY

In most cases of human cancer there is a qualitative defect in the proteins of serum which may be identified by the thermal coagulation tests. The defect is not specific and reactions similar to that in cancer are obtained in the presence of pulmonary tuberculosis and some acute massive inflammatory processes as well. The defect was not observed in normal pregnancy, in new-born infants, or in non-pulmonary tuberculosis.

By determining the iodoacetate index as related to the total protein content of serum, it was found that all of 85 consecutive clinically active cancers fell in a group with a low index (less than 9). However, 16 of 95 patients with non-malignant pathology fell in this same range.

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