A Comparison of the Serum Proteins of Normal Rats with those of Rats bearing Liver Tumours

By P. N. CAMPBELL, BARBARA A. KERNOT AND I. M. ROITT

Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1

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The persistent demand for a reliable method for the diagnosis of the presence of malignant tumours has led to numerous investigations on the composition of the serum proteins of animals bearing such tumours. So far, with the exception of myeloma in man, the serum proteins of the tumour-bearing animal have not been shown to differ from those of animals under other pathological conditions in any characteristic way. (Studies on the plasma proteins of patients with cancer were reviewed by Winzler in 1953.) In spite of this discouraging situation it was felt that yet another effort to investigate the problem by recently introduced techniques and discriminating immunological methods might be of value. The proteins in the serum of rats bearing well-characterized primary liver tumours induced by feeding 4-dimethylaminoazobenzene have therefore been compared with the serum proteins of the normal rat. While this work was in progress Darcy (1957) published a paper in which the composition of the serum proteins of normal and tumour-bearing rats was compared by immunological methods. The results now presented confirm and extend these studies.

A preliminary report of some aspects of this work has appeared (Campbell, Kernot & Roitt, 1957).

MATERIALS AND METHODS

Animals. Rats were male Wistar albinos highly inbred in this Institute. Liver tumours were induced by feeding the rats on a diet containing 0.06% of 4-dimethylaminoazobenzene as described by Campbell (1955). Partial hepatectomy in rats was performed by the method of Higgins & Anderson (1931).

Rabbit antisera. An alum-precipitate of freshly obtained rat serum was prepared by the method of Proom (1943). Rabbits were given this suspension by intravenous injection as previously described (Campbell, 1955). The antiserum obtained was not fractionated before use, but was stored at -20° .

Precipitation in agar gel. This was carried out by the method of Ouchterlony (1953). Sodium azide (1%) was incorporated into the medium as a bactericide. The wells were not replenished. The plates were photographed by the method of Lawson (1957). When it was necessary to compare the antigenic composition of the different rat sera, some normal serum was included in the agar used for the plate. This follows the method of Björklund (1952, 1953).

Immuno-electrophoresis. This was carried out in agar by a slight modification of the method of Grabar & Williams (1955).

Fractionation of plasma proteins

Salt. When $(\text{NH}_4)_2\text{SO}_4$ was used an equal volume of a saturated solution was added to rat serum obtained by heart puncture. The precipitate was removed by centrifuging and was washed with a solution of half-saturated $(\text{NH}_4)_2\text{SO}_4$. The precipitate was dissolved in water, dialysed and freeze-dried. The protein in the supernatant from the $(\text{NH}_4)_2\text{SO}_4$ fractionation was recovered in a similar manner.

When Na_2SO_4 was used for fractionation 2 vol. of 27% (w/v) Na_2SO_4 dissolved in potassium phosphate buffer (pH 7.8, I 0.1) was added to 1 vol. of rat serum, to give a final concentration of 18% (w/v) Na_2SO_4 . The precipitate and supernatant were treated as described for $(\text{NH}_4)_2\text{SO}_4$ fractionation.

Paper electrophoresis. The fractionation was followed by electrophoresis on Whatman no. 3 MM paper, with veronal buffer (pH 8.6, I 0.05) as described by Flynn & de Mayo (1951). In general, this buffer gave much better results than did that containing acetate described by Roberts & Kelley (1956). More recently, satisfactory results have been obtained with the 2-amino-2-hydroxymethylpropane-1:3-diol (tris)-ethylenediaminetetra-acetic acid (EDTA) buffer of Aronsson & Grönwall (1957). The method of Kohn (1957), which involves the use of cellulose acetate paper with veronal buffer, was also employed.

Zone electrophoresis. The method of Porath (1956) in which electrophoresis is carried out in a column was used. The columns were packed with cellulose prepared as described by Flodin & Kupke (1956) in a borate-phosphate buffer (0.0115 M- $\text{Na}_2\text{B}_4\text{O}_7$ -0.0155 M- NaH_2PO_4 , pH 8.6, I 0.05).

RESULTS AND DISCUSSION

Paper electrophoresis

The serum proteins of the rat do not give discrete bands when subjected to paper electrophoresis in veronal buffer as do those of man and rabbit. The results obtained in the present experiments were, in general, similar to those obtained by Roberts & Kelley (1956), although the buffer used by these authors was not found satisfactory. No system of paper electrophoresis has so far been found which gives completely satisfactory results for rat serum. Thus the best overall picture is obtained on Whatman no. 3 MM paper with veronal buffer. Under these conditions the α_1 -globulin is only

just separated from the albumin (Fig. 1a), as may be more clearly shown when the proteins precipitated after half-saturation of the serum with ammonium sulphate are subjected to electrophoresis (Fig. 1b). If a clearer separation between these two components is required the method of Kohn (1957) with cellulose acetate paper and veronal buffer is to be recommended (Fig. 1d). An excellent separation of the α_1 -, α_2 - and β -globulins may be obtained by the use of the tris-EDTA buffer of Aronsson & Grönwall (1957). However, in this case the front of the albumin band is rather diffuse (Fig. 1c).

The paper-electrophoretic method of Aronsson & Grönwall (1957) revealed a difference between the proteins in the α_1 -, α_2 -globulin band of the normal and tumour-bearing rat serum. The latter appeared to contain additional components compared with that of the normal serum.

Salt fractionation

Paper electrophoresis of the two fractions obtained by half-saturation with ammonium sulphate showed that both contained albumin, although the amount in the precipitate was very small compared with that in the supernatant. The fractionation of the γ -globulin was complete in that it was present only in the precipitate. There was no satisfactory fractionation of the α_1 -, α_2 - and β -globulins by this procedure.

The use of sodium sulphate to fractionate the sera has the advantage that there is a complete separation of both albumin and γ -globulin. Thus

Fig. 1. Paper electrophoresis of rat serum proteins under various conditions. (a) Whole serum, Whatman no. 3MM paper, veronal buffer. (b) Precipitate obtained by half-saturating the serum with $(\text{NH}_4)_2\text{SO}_4$. Whatman no. 3MM paper, veronal buffer. (c) Whole serum, Whatman no. 3MM paper, tris-EDTA buffer. (d) Impure albumin obtained by zone electrophoresis on cellulose columns (Campbell & Stone, 1957). Cellulose acetate paper, veronal buffer.

the albumin was confined to the supernatant and the γ -globulin to the precipitate. The other components were distributed between the two fractions.

Zone electrophoresis

The result of submitting normal serum to zone electrophoresis after removal of the precipitate obtained by half-saturation with ammonium sulphate is shown in Fig. 2. No consistent difference between the results obtained with this serum fraction from normal and tumour-bearing animals has been obtained. However, when the precipitate obtained by half-saturation of normal serum with ammonium sulphate was submitted to zone electrophoresis the pattern shown in Fig. 3 was obtained, whereas electrophoresis of a similar fraction from tumour-bearing animals gave the somewhat different pattern shown in Fig. 4. The difference between the central parts of these two patterns has been a consistent finding and suggests

Fig. 2. Zone electrophoresis of normal-rat serum proteins after removal of the precipitate formed by half-saturation with $(\text{NH}_4)_2\text{SO}_4$. Protein (50 mg.) was added to the column (46 cm. $\times$ 1.5 cm.) packed with 'cellulose'. A current of 10 mA (500 v) was applied for 24 hr. An indication of the composition of the peaks is given diagrammatically. The diagrams represent the results obtained by paper electrophoresis in veronal buffer.

Fig. 3. Zone electrophoresis of normal-rat serum proteins precipitated from whole serum by half-saturation with $(\text{NH}_4)_2\text{SO}_4$. Conditions were as described for Fig. 2.

Fig. 4. Zone electrophoresis of serum proteins of tumour-bearing rat precipitated from whole serum by half-saturation with $(\text{NH}_4)_2\text{SO}_4$. Conditions were as described for Fig. 2.

an increase in some component in the tumour serum compared with the normal having the properties of an α -globulin.

Differences between the sera revealed by immunological techniques

When the Ouchterlony technique of diffusion of antibody and antigen in an agar gel was applied to serum from normal and tumour-bearing rats and their corresponding antisera, more lines were revealed in the serum from tumour-bearing rats than from the normal serum. For this reason in all subsequent experiments antiserum to the tumour serum was used. A typical result is shown in

Plate 3 (a). The difference may be more clearly defined by absorbing the antibodies to normal serum according to the Björklund (1953) technique. If a similar plate is set up differing only in that the agar contains some normal serum, then, as shown in Plate 3 (b), the tumour serum shows an additional line compared with the normal. It may be observed that the pattern of the precipitated proteins around the centre well is distorted by the advancing antigen.

If instead of mixing the normal serum with the agar the normal serum is placed in the centre well together with the antiserum the result shown in Plate 3 (c) is obtained. It will be observed that here three extra lines are present in the tumour serum. When the amount of normal serum in the centre well is reduced a line does appear with normal serum and this line is confluent with one of the lines in the tumour serum. This suggests that at least one of the components is merely present in the tumour serum at an increased concentration compared with the normal. Further evidence on this point will be presented later.

Owing to the simplicity of the Björklund technique further characterization of the additional antigens in the tumour serum was made by this method. When instead of fresh serum the serum used was freeze-dried and then dissolved in 0.9 % sodium chloride (1.5 mg. of protein/0.2 ml. of saline), the tumour serum again gave the additional line, showing that the responsible antigen withstood freeze-drying and was probably not a lipoprotein. Ammonium sulphate appears to effect a fractionation of the component, for the supernatant failed to give a line whereas the precipitate gave a line when tested at a concentration of 1.5 mg. of protein/0.2 ml. of saline. On the other hand, the use of sodium sulphate did not effect a fractionation of the component, both the precipitate and the supernatant giving the additional line.

To obtain further information about the nature of the additional components in tumour serum the fractions obtained after zone electrophoresis of the ammonium sulphate precipitate were tested. All three fractions (see Fig. 4) gave a line, but, as judged by the distance of the line from the centre well, time of appearance and bulk of precipitate, fraction B contained more of the antigen than did either fraction A or fraction C. This experiment suggested that additional components in the tumour-serum proteins had the mobility of an α -globulin. Further evidence on this point was obtained by immunoelectrophoresis. The results of this experiment are shown in Fig. 5. It can be seen that the tumour serum contained two additional components having the mobility of α_1 - and α_2 -globulins respectively in the buffer of Aronsson

& Grönwall (1957). A control experiment in which the normal serum replaced the tumour serum failed to reveal any lines.

The specificity of the unknown components with respect to tumour serum was studied as follows. Tests were made on the fractions obtained after zone electrophoresis of the proteins of normal serum precipitated by half-saturation with ammonium sulphate (fractions *A* and *C*, Fig. 3). These fractions both showed the presence of the component. Thus it is clear that the component(s) is present in normal serum and is merely increased in tumour serum. Serum obtained from a rat 40 hr. after a partial hepatectomy was then tested, together with that from a pregnant rat. In each a line appeared at about the same time as with the tumour serum and all three lines merged. Thus not only is there an increase in the concentration of the component in tumour serum but also in other conditions involving rapid growth.

An attempt was made to determine whether all or any of the components were glycoproteins, by staining the Ouchterlony agar plate with the periodic acid-Schiff's reagent according to the method of Uriel & Grabar (1956). The results obtained were consistent with the view that at least one of the components was a glycoprotein.

The increase in the concentration of the proteins comprising the α -globulin fraction of the plasma in the rats bearing liver tumours compared with normal rats is in agreement with the work of Bernfeld & Homburger (1955), who studied the influence of tumour growth on the plasma proteins in mice and later (Nisselbaum & Bernfeld, 1956) isolated glycoproteins from the plasma of both normal and tumour-bearing mice. The results of the present experiments are in close accord with those of Darcy (1957) already mentioned. The

Björklund technique used with Ouchterlony agar plates has, however, greatly simplified the detection of the proteins, the concentration of which is increased in the tumour-bearing animal. Zone electrophoresis has provided confirmatory evidence of the nature of the substances and immunoelectrophoresis has shown that the concentration of at least two plasma proteins having different electrophoretic mobilities is increased in the tumour-bearing rats.

Although these experiments do not provide any evidence for a difference between normal serum and the serum from tumour-bearing animals which is confined to such animals, they do indicate a change in the serum proteins which arises as a result of growth. Bucher, Scott & Aub (1951) have shown that substances in the blood can initiate mitosis. Thus when one partner of parabioc twins was subjected to partial hepatectomy the rate of mitosis of the unoperated animal rose. Whether the components studied in the present investigation are responsible for controlling the growth of tissues or whether they are produced as a result of growth is not known.

SUMMARY

1. The composition of the serum proteins of normal rats and those bearing liver tumours has been compared by paper electrophoresis, zone electrophoresis and immunological methods.

2. The electrophoretic methods, particularly zone electrophoresis, showed that the serum protein of the tumour-bearing animals contained more protein of the mobility of the α -globulins than did normal serum.

3. Immunological methods, particularly the Ouchterlony plate method as modified by Björklund, showed that one or more components of the normal serum proteins were present in increased concentration in the tumour serum. Similar results were obtained with serum from either pregnant rats or rats after partial hepatectomy.

4. Immunoelectrophoretic methods showed that the proteins present in an increased concentration in the tumour serum had mobilities corresponding to α_1 - and α_2 -globulins respectively.

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Fig. 5. Immunoelectrophoresis of serum proteins from a tumour-bearing rat. The proteins were first separated by electrophoresis on Whatman no. 3MM paper with tris-EDTA buffer. The paper was cut longitudinally, one half being stained and the other placed on a sheet of agar as indicated. The separation of the proteins into bands as revealed by the stained strip is also indicated. A channel was cut in the agar and this was filled with antiserum to the serum proteins of the tumour-bearing rat absorbed with one-fifth of its volume of serum from a normal rat (cf. Plate 3c). The two lines on the diagram are tracings of a photograph taken after 10 days.

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EXPLANATION OF PLATE 3

- (a) Ouchterlony plate in which an antiserum to the serum proteins of a tumour-bearing rat was placed in the centre well. The other wells contained either whole serum from normal rats or serum from tumour-bearing rats diluted with saline as indicated. Photographed after 12 days.
- (b) Ouchterlony plate set up exactly as described in (a) except that the agar contained 1% (v/v) of normal-rat serum.
- (c) Ouchterlony plate in which the centre well contained antiserum to tumour serum absorbed with one-fifth of its volume of normal-rat serum. The side wells contained either tumour serum or normal serum diluted with saline as indicated. Photographed after 10 days.

Mitochondria from Embryonic Tissues of the Chick

1. PREPARATION, CHARACTERIZATION AND SOME ENZYMIC PROPERTIES

BY N. H. CAREY* AND G. D. GREVILLE

Department of Biochemistry, University of Cambridge

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Whereas the isolation and properties of subcellular particles from normal adult tissues (review: Hogeboom & Schneider, 1955), tumour tissue (Schneider, 1946), plant tissues (review: Hackett, 1955) and yeast (Linnane & Still, 1955) have been extensively studied, no comparable work seems to have been done with embryonic tissues. Claude (1938, 1940) centrifuged particles from a homogenate of previously frozen chick-embryo pulp in phosphate buffer; they contained some ribonucleic but no deoxyribonucleic acid. Steinbach & Moog (1945) and Moog & Steinbach (1946) obtained granules from homogenates of chick embryos in water by centrifuging at room temperature, and studied their phosphatase and adenosine triphosphatase activities.

Recknagel (1950) homogenized eggs of the frog *Rana pipiens* in phosphate buffer and separated a granular fraction which contained about 70% of the cytochrome oxidase activity of the homogenate, with a much higher specific activity, and which stained with Janus green B. A similar mitochondrial fraction, in which the cytochrome and succinate oxidases were localized, was obtained by

Boell & Weber (1955) from homogenates in sucrose solution of embryos of the toad *Xenopus laevis*.

Nuclear, mitochondrial and microsomal fractions have been obtained by differential centrifuging of homogenates in sodium chloride-phosphate medium of embryos of the grasshopper *Melanoplus differentialis* (Bodine & Lu, 1950, 1952); all the succinate oxidase activity was in the mitochondrial fraction.

Hutchens, Kopac & Krahl (1942) separated from homogenates of eggs of the sea urchin *Arbacia punctulata* a 'mitochondrial' fraction which, however, contained only 1% of the cytochrome oxidase activity. On the other hand, Weber is reported by Boell & Weber (1955) to have found that homogenates of eggs of *Paracentrotus lividus* yield large granules which sediment between 600 and 12 000 g, have the characteristics of mitochondria and contain 90% of the cytochrome oxidase activity of the homogenate.

Clearly, cell-fractionation studies with embryonic tissue have so far been scanty and incomplete. Attempts have been made to separate a fraction containing enzymes that are associated with mitochondria in adult mammalian tissues, but in few cases have the workers carried out the fractionation in sucrose medium which, like certain special salt

* Present address: Department of Pharmacology, George Washington University School of Medicine, Washington, D.C., U.S.A.