Biochemical Pharmacology, 1961, Vol. 7, pp. 109–122. Pergamon Press Ltd., Printed in Great Britain.

AN ABNORMAL SERUM COMPONENT IN EXPERIMENTAL POISONING BY CADMIUM AND OTHER METALS

D. J. LAWFORD*

Department for Research in Industrial Medicine
(Medical Research Council), The London Hospital, London, E.1

(*Received* 22 *December* 1960)

Abstract—An abnormal serum component was observed 24 hr after administration of a single dose of cadmium to albino rats by intraperitoneal injection, which gradually disappeared from the serum over a period of 9 days. The component was not observed on starch gel electrophoresis following very high or very low dosage, the optimum level being found to be 1 mg/kg of cadmium. The rate of migration of the material was not affected by the addition of CaEDTA to the system or the use of a more acid buffer.

Ammonium sulphate fractionation showed that the abnormal serum component was precipitated at between 35–40% saturation.

The *in vitro* addition of cadmium to samples of serum in varying concentrations revealed progressive alteration in the migration rate of the albumin band, but indicated no line comparable with the abnormal serum component.

The production of varying amounts of the component was observed following the injection of other metals, and mercury appeared to give a stronger line than cadmium, whereas lead gave no band at all.

The component was isolated from cadmium-poisoned rat serum in a reasonable state of purity, by means of neutral salt fractionation and zone electrophoresis.

An antiserum to the purified material was prepared in rabbits, and after suitable treatment, this antiserum was used to show the presence of the abnormal component in rat serum following very low intraperitoneal doses of cadmium.

INTRODUCTION

THE ingestion of small amounts of soluble cadmium salts has been shown to produce a rapid and severe anaemia[1–5] and an apparent considerable increase in the serum transferrin content of young albino rats,[6] whereas the injection of soluble cadmium salts can produce certain changes in the normal plasma protein relationship. Truhaut and Boudene,[7] in a study of the distribution of cadmium in rats following acute, subacute, and chronic administration by injection, found that following chronic administration, there appeared to be progressive hypoalbuminaemia and hyperglobulinaemia (an initial increase and a subsequent decrease in the α-globulins and a steady rise in the β-globulins levels). Barni *et al.*,[8] working along the same lines, found that the continuous injection of high doses of cadmium to rabbits produced an increase in the total blood proteins, a reduction in the albumin and α_1-globulin content being more than compensated by a progressive increase in the α_2, β and γ-globulins. As the poisoning progressed, it was observed that the α-lipoproteins decreased, while

Abbreviations: A.S.C., abnormal serum component. Part of work approved for Ph.D. thesis (London).

* Present address: Abbott Laboratories Ltd., 8 Baker Street, London, W.1.

the α_2, β and γ-glycoproteins increased. Odescalchi *et al.*[9] reported similar findings, and found in addition, an inverted albumin–globulin ratio, which suggested to them that the modifications in the concentrations of the serum proteins may have been related to certain liver changes that they had observed.

Stokinger and Wagner[10] have reported that the α-globulin fractions of the sera of dogs and rabbits were elevated, following exposure to small doses of cobalt.

The aim of the present work was to investigate in greater detail the serum protein changes produced in the albino rat, following the parenteral administration of cadmium salts.

MATERIALS AND METHODS

Animals

Adult Chester–Beatty strain rats were used.

Starch

Specially prepared Connaught Research Laboratories starch was used.

Buffer solutions

Borate buffer solutions. This solution was prepared in batches of 2 l. at a time and consisted of the following composition: 37·104 g of AnalaR boric acid, 4·8 g of AnalaR sodium hydroxide, made up to 2 l. with distilled water.

TRIS buffer solution. This solution was prepared in batches of 2 l. at a time consisting of the following composition: 18·413 g of tris-(hydroxymethyl)-aminomethane (melting point 170°C), 2·102 g of AnalaR citric acid, made up to 2 l. with distilled water.

Staining solution

A saturated solution of the dyestuff naphthalene black 10B 200 in the following solution: methanol, five parts; glacial acetic acid, one part; water, five parts.

Washing solution

The staining solution, minus the dyestuff.

Buffer systems

Continuous method. In this method, the Tris buffer solution was used to make up the gel and was also used in the buffer reservoir boxes. The system was run at 520 V, 10 mA and a voltage gradient of 2 V/cm for 16 hr.

Discontinuous method.[11] The gel was made up using Tris buffer solution, the reservoir boxes being filled with borate buffer solution. The system was run at 520V, 20 mA and a voltage gradient of 6 V/cm for 4 hr.

Starch gel electrophoresis

The method of Smithies[12] was used throughout these experiments.

Preparative electrophoresis

The apparatus designed by Porath[13] was used, in conjunction with the procedure of Gedin and Porath.[14]

Paper electrophoresis

The method of Kunkel and Tiselius[15] was used.

Apparatus for immune reactions

For the immune reactions in tubes, the method of Oakley and Fulthorpe[16] was used. In addition, the techniques of Öuchterlony,[17] and the micro-modification of Öuchterlony and Wadsworth[18] were employed.

Immunoelectrophoresis

It was found that starch was the most satisfactory medium for the separation of the abnormal serum component in rat serum, and the following method was used for the immunoelectrophoretic studies.

A plastic dish, 11·5 cm by 17·5 cm was filled to a depth of 2–3 mm with a solution of 1% agar (Ionagar no. 2 Oxoid) in sterile Tris buffer containing 0·5% sodium azide to prevent bacterial growth. The serum to be examined was subjected to starch gel electrophoresis in the usual manner, using the continuous buffer system, and slices were cut and placed face downwards on the surface of the agar. Strips of Whatman 3MM filter paper, 0·3 mm wide and of similar length to the starch gel slices were were dipped in antiserum and placed on the agar surface, parallel to the starch slices and at a distance of 3–4 mm from them (Fig. 1). The dish was carefully sealed and incubated at 37 °C for 7 days.

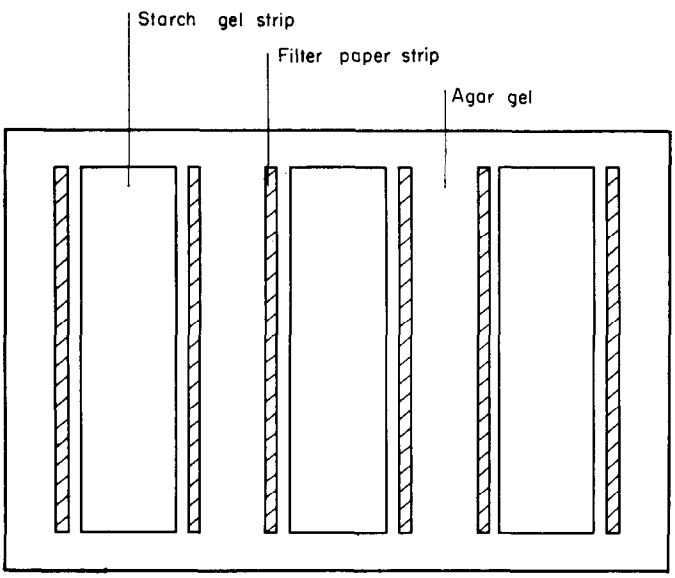

FIG. 1. Apparatus for immunoelectrophoresis. Actual size.

After marking the positions occupied by the starch gels and the filter paper strips, these were removed and the agar gel was suspended in trays of saline, with frequent changes for 3 days, in order to remove remaining soluble material. The agar gel was finally placed on a glass plate, covered with a fitted sheet of thick filter paper and allowed to dry. When dry, the surface was rinsed with water to remove the filter paper, and finally stained in methanol–water–acetic acid solution saturated with

naphthalene black, as described earlier. After suitable rinsing, the gel was dried and photographed.

Serum changes following parenteral administration of cadmium

1. *Appearance of an abnormal serum component.* Two groups of five male Chester–Beatty rats, aged 3 months, and weighing approximately 300 g were taken, one group serving as controls, the second group being given 1 mg/kg of inactive cadmium (as cadmium chloride) by intraperitoneal injection. Samples of blood were taken from each animal by cardiopuncture after 24 hr. The blood was allowed to clot and the sera were submitted to starch gel electrophoresis using both the continuous and the discontinuous buffer systems.

Although several changes of a minor character were observed between the control and experimental animals, the most outstanding feature was the appearance of a new band in the sera of the cadmium rats, which was not apparent in the sera of the control rats (Fig. 2). This band could be readily observed when the continuous buffer system was used, but was slightly less obvious using the discontinuous buffer system.

2. *Effect of varying doses of cadmium.* Five male adult rats were given 0, 0·025, 0·1, 0·2 and 1 mg/kg body weight of inactive cadmium as cadmium chloride, daily for 4 days. Starch gel analyses were made on sera from these animals taken 1, 2, 3 and 4 days after the commencement of dosing. The A.S.C. band appeared after 24 hr and increased to a maximum up to 3 days, but appeared to be slightly weaker after a further day (Fig. 3). Other changes were seen to occur at the 1 mg/kg level, notably a reduction of the albumin band, and a visible decrease in the globulin level similar to those observed when cadmium was administered orally.

Properties of the abnormal serum component

1. *Effect of pH on electrophoretic mobility.* Serum containing the A.S.C. was subjected to starch gel electrophoresis using a continuous Tris buffer system adjusted to pH 7·0. No alteration in the speed of migration of the A.S.C. was seen, although other serum components migrated a smaller distance in a measured time.

2. *Effect of incorporating EDTA.* The addition of a small amount of disodium dihydrogen ethylenediaminetetra-acetic acid to the serum, made no difference to the speed of migration of the A.S.C., suggesting that it was not cadmium-bearing, a finding which was subsequently confirmed.

3. *Rate of appearance and disappearance.* Three male rats were given a single dose of 1 mg/kg of cadmium, as inactive cadmium chloride, intraperitoneally. Blood samples were collected from the tail at 0, 6 and 24 hr after the injections, and thereafter daily for 2 weeks. Starch gel electrophoresis using the continuous buffer system showed that the A.S.C. band first appeared after 24 hr, reached a maximum after 2 days, and gradually disappeared with time, becoming invisible 9 days after the injection.

4. *Optimum dose of cadmium to produce the A.S.C.* In order to see if the production of A.S.C. was proportional to the size of dose following a single intraperitoneal injection, six pairs of 3-month-old male rats were injected with the dose range,

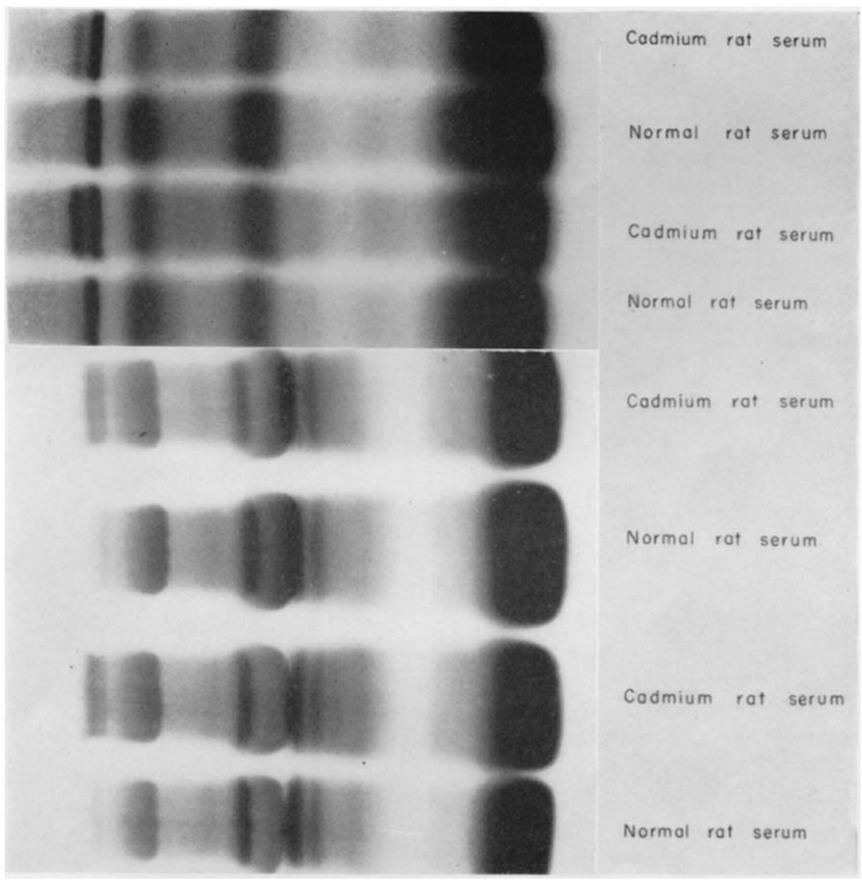

FIG. 2. Appearance of abnormal component in the sera of rats 24 hr after the intraperitoneal injection of 1 mg/kg of cadmium as cadmium chloride. (1) Continuous buffer system. (2) Discontinuous buffer system.

facing page 112

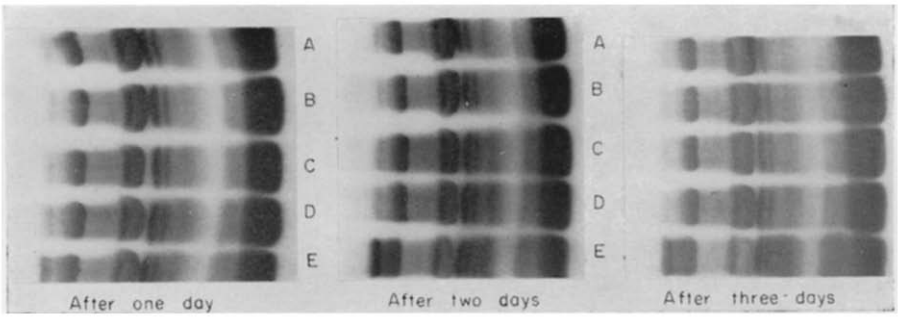

FIG. 3. Starch gel electrophoresis patterns of sera of rats given various daily doses of cadmium a cadmium chloride by intraperitoneal injection. A, normal rat serum. B, 0·025 mg cadmium/kg daily C, 0·100 mg cadmium daily. D, 0·200 mg cadmium daily. E, 1·000 mg cadmium daily.

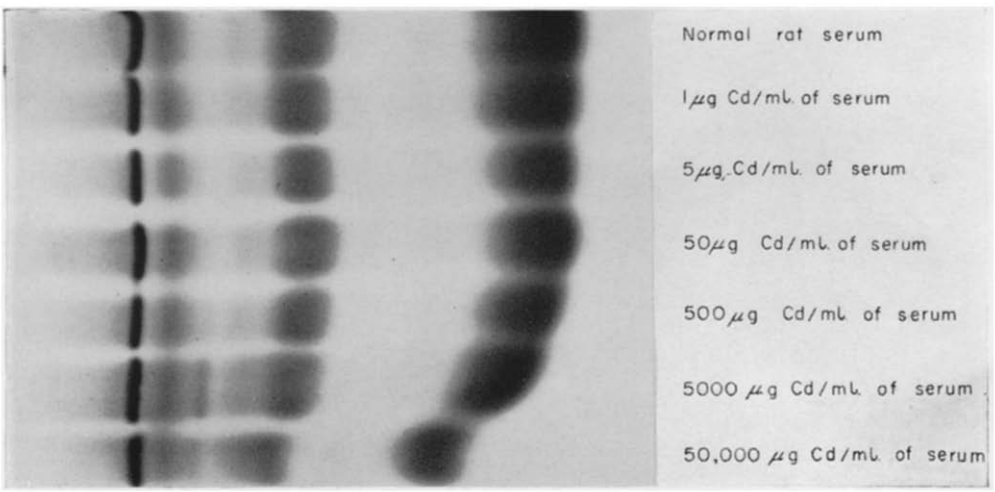

FIG. 4. *In vitro* addition of cadmium to rat serum. Starch gel electrophoresis patterns of normal rat serum containing the indicated concentrations of cadmium (continuous buffer system).

0·5, 1·0, 1·5, 2·0, 2·5 and 3·0 mg/kg of inactive cadmium as cadmium chloride in saline. Blood was taken after 24 and 48 hr and the sera were subjected to starch gel electrophoresis, using the continuous buffer system.

After 24 and 48 hr, the animals given 2, 2·5 and 3 mg/kg of cadmium showed no A.S.C. line. The animals that received 0·5 and 1·5 mg/kg showed faint lines at 24 hr and slightly stronger ones at 48 hr. The animals that were given 1 mg/kg exhibited the strongest A.S.C. lines at 24 hr and even stronger ones at 48 hr. The optimum conditions for production of the A.S.C. line are therefore the administration of 1 mg/kg of cadmium and removal of the serum 2 days afterwards.

5. *Precipitation by ammonium sulphate.* Five samples of serum containing the A.S.C. were treated with saturated ammonium sulphate solution to give a range of saturation of 30–35–40–45–50 per cent. The precipitated sera were left at 0 °C for 1 hr and then centrifuged at 3000 rev/min for 20 min. The supernatant fluids were removed, dialysed free of the ammonium sulphate, and dialysed against normal saline prior to the electrophoresis. The precipitates were dissolved in 0·5 ml of distilled water and dialysed against water and finally saline. Starch gel electrophoresis using the continuous buffer system showed that at 35 per cent saturation of the serum, virtually all of the A.S.C. was precipitated, and at 40 per cent saturation, all of the A.S.C. was out of solution, within the limits of visibility of this technique.

In vitro addition of cadmium to normal serum

The delay of 24 hr following cadmium administration before the A.S.C. appeared in the serum, made it improbable that the A.S.C. was a result of the direct interaction between cadmium and an existing serum protein. This possibility was examined, however, by the following experiment. Inactive cadmium, as cadmium chloride, was added to normal rat serum so as to give the following concentrations: 1, 5, 50, 500, 5000 and 50,000 μg of cadmium per ml of serum. The mixtures were left for 1 hr in an incubator at 37 °C, then run on a starch gel, using the continuous buffer system. The only serum protein to be seriously affected was the albumin fraction, which migrated more slowly as the concentration of cadmium in the serum increased. The other bands in the serum appeared unaffected, and no line corresponding to the A.S.C. was observed (Fig. 4).

Formation of abnormal serum component by other metals

In order to determine whether the production of the A.S.C. was a specific symptom of cadmium absorption, four male rats were taken and given the following treatments:

Treatment 1. Saline daily for 3 days by intraperitoneal injection.
Treatment 2. Cadmium (1 mg/kg) daily for 3 days by intraperitoneal injection.
Treatment 3. Zinc (1 mg/kg) daily for 3 days by intraperitoneal injection.
Treatment 4. Mercury (1 mg/kg) daily for 3 days by intraperitoneal injection.

Blood was taken 1, 2 and 3 days from the commencement of dosing and the results revealed that the mercury administrations produced the A.S.C. in the blood rather more abundantly than cadmium, whereas zinc did not produce the band at the given dose rate. Much larger doses of zinc did, however, produce the A.S.C. A rat was given 20 mg/kg of zinc (as zinc acetate) daily for 2 days by intraperitoneal injection and 24 hr after the second dose, the animal was killed and samples of blood and peritoneal

fluid were collected. The injections had produced considerable fluid in the peritoneum and it was possible that the A.S.C. had originated from this site, with subsequent removal by the blood stream. Starch gel electrophoresis of the sera and peritoneal fluid using the continuous buffer system showed, however, that although the peritoneal fluid contained all the normal serum proteins in comparable amounts to the serum, its content of A.S.C. was very much smaller.

An extension of these experiments was made to observe the A.S.C.-producing capabilities of a range of metals. Pairs of adult male rats were given a single intraperitoneal injection of 1 mg/kg of the following metals: beryllium, cadmium, chromium, cobalt, copper, iron, lead, manganese, mercury and nickel. A further pair of rats served as controls and received a single injection of saline. The metals were administered as the acetate, and were neutralized before injection, with the exception of the beryllium salt, which was slightly acid. The animals were bled after 24 hr, the sera from each pair was pooled and examined by starch gel electrophoresis, using the continuous buffer system. The results showed (Fig. 5) that the intensity of the A.S.C. line produced varied considerably with the metal administered, in the following sequence:

$$\text{mercury} > \text{cadmium, beryllium, copper} > \text{manganese}$$

Faint bands were produced with chromium and nickel, barely perceptible bands with iron and cobalt and no visible band at all with lead.

Isolation of the abnormal serum component

The initial observation of the appearance of the A.S.C. posed many questions, and as a first step to answering some of them, a separation and partial purification of the material was attempted.

Apparatus. The electrophoretic separation described in this section was carried out using the apparatus described by Porath[13] and the procedure of Gedin and Porath.[14] The separation column consisted of a glass tube 150 cm in length and 2 cm internal diameter, surrounded by a 5 cm internal diameter cooling jacket. Purified cellulose (250 g) was well mixed with 2·2 l. of buffer and the column was carefully poured as directed. The homogeneity of the column was checked by pouring 2 ml of saturated DNP-aspartic acid, in buffer solution, in at the top and eluting down the column. The DNP-aspartic acid eluted out in 14 ml of solution, thereby indicating a satisfactorily packed column, which was then ready for use.

Buffers used. A borate–phosphate buffer, pH 8·4, and ionic strength 0·05, of the following composition was used in the experiments: 2·34 g of sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$), 4·29 g of sodium tetraborate ($Na_2B_4O_7.10H_2O$), made up to 1 l. and degassed on a water pump 20 min before use.

Method. The column was used to effect an electrophoretic separation of the whole serum of rats injected with radioactive cadmium. Absorptiometer measurements and subsequent starch gel electrophoresis of the fractions showed that the abnormal serum component migrated at a slightly slower rate than the albumin fraction (Fig. 6). With this information, a sample of the material was isolated as follows.

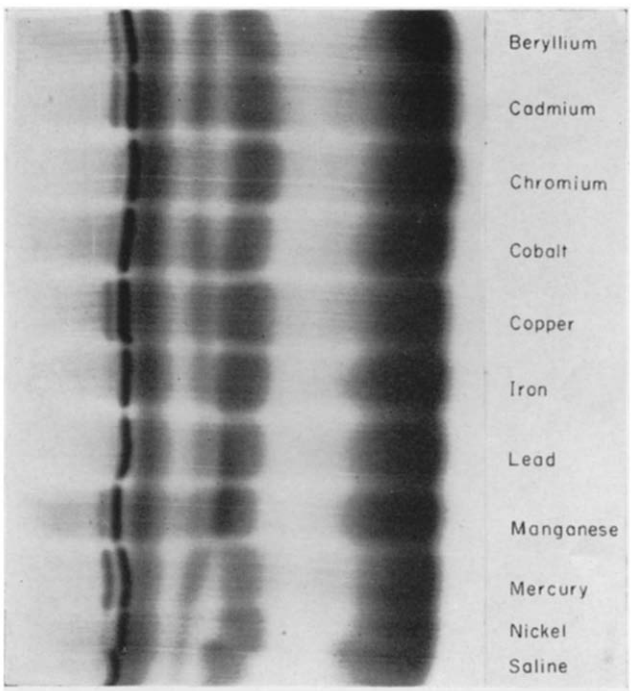

FIG. 5. Starch gel electrophoresis patterns of the sera of rats taken 24 hr after the intraperitoneal administration of 1 mg/kg of the metals indicated (continuous buffer system).

facing page 114

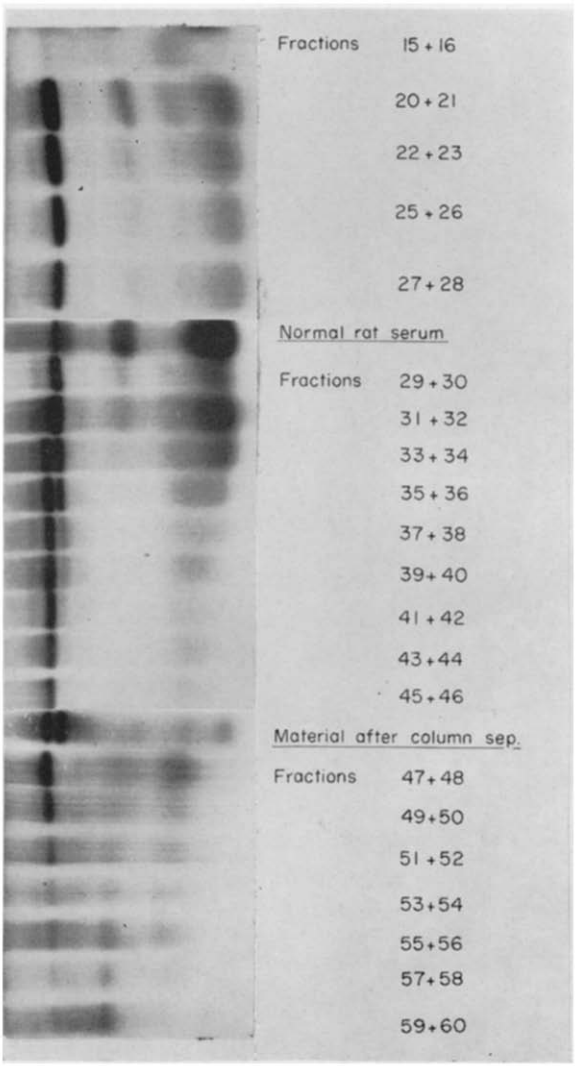

FIG. 7. Starch gel electrophoresis patterns of the freeze-dried fractions from the preparative electrophoresis column (continuous buffer system).

Thirty-six male adult rats, aged 3 months and weighing 320–350 g were given two doses of 1 mg/kg of radioactive cadmium on successive days, and were completely bled by cardiopuncture on the third day. The blood was left to clot for 1 hr at room temperature, put in the refrigerator at 0 °C for 2 hr and finally centrifuged at 3000 rev/min for 30 min. The serum was removed and twice its volume of distilled water was added, the resultant solution being left for 30 min, for haemolysis of the remaining red cells to take place. Saturated ammonium sulphate solution was then added to give a level of 40 per cent saturation. The mixture was left at 0 °C for 1 hr, and then spun at 3000 rev/min for 30 min. The precipitate was dissolved in saline, and dialysed against frequent changes of distilled water during the following day. Dialysis was continued until the water gave no reaction with barium chloride solution. The material was finally freeze-dried, and a sample reserved for future analysis. The freeze-dried material weighed 1·95 g.

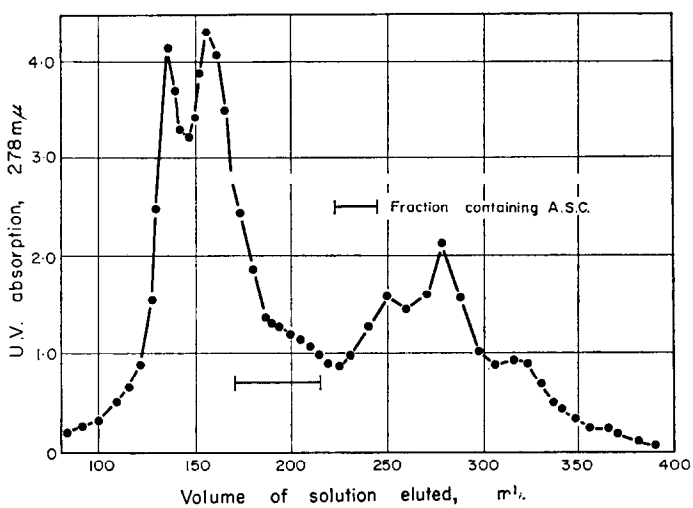

FIG. 6. Ultra-violet absorption curve. Fractions of cadmium rat serum from preparative zone electrophoretic column.

Some (1·9 g) of the freeze-dried material was dissolved in 22 ml of the borate–phosphate buffer, and centrifuged for 30 min before application to the column. The band of liquid 9·3 cm in depth, was drawn carefully into the cellulose medium, and the electrophoretic separation was allowed to run for 28 hr, at a voltage of 1200 V, and a current of 25 mA. At the end of the experiment, the column was eluted with the degassed borate–phosphate buffer solution and collected by a mechanical fraction cutter, in 4–5 ml fractions, at a rate of four per hr, until 400 ml had been eluted. This volume represented the hold-up volume of the column. The volumes of the samples were carefully measured and the u.v. absorption curve at 278 mμ was plotted. The samples were finally dialysed separately against distilled water for 3 days (three changes) at 0 °C, combined in pairs in the range fraction 15–86, freeze-dried and submitted to starch gel electrophoresis using the continuous buffer system. By examination of these gels (Fig. 7) it could be seen that the A.S.C. appeared at fraction 33 and

was almost absent after fraction 60. Fractions 35–52 (inclusive) were bulked together, dissolved in distilled water and freeze-dried. The final weight of the freeze-dried material was 620 mg and radioactivity measurements on the sample were negative.

The degree of purification affected by this method can be seen from Fig. 8, in which the material at stages after ammonium sulphate precipitation, and after the electrophoretic separation, were compared with a normal serum sample, by both continuous and discontinuous buffer systems. The two or three impurities observed in the starch gel analyses of the final material are, of course, serum proteins which migrate with the A.S.C. in the immediate post-albumin position in free electrophoresis.

Experiments with the isolated material

Electrophoresis on other media. Samples of the isolated A.S.C. were subjected to paper electrophoresis according to the method of Kunkel and Tiselius.[15] The material appeared to migrate at the same speed as in the column, that is, slightly slower than the albumin band, although serious trailing was observed. Attempts to use cellulose acetate strips in place of paper met with singularly little success, the material moving very slowly as an haphazard, very diffuse band, in both borate and barbitone buffers.

Sensitivity of starch gel electrophoresis. The sole criterion of the presence of A.S.C. in specimens of serum had, until this point, been its visibility as a stained band after starch gel electrophoresis. The following experiment was designed to determine the limit of sensitivity of this technique.

Five solutions of A.S.C. in saline were prepared, to give 1, 2, 3, 4 and 5 mg/ml of solution. These solutions, together with a normal serum, were subjected to starch gel electrophoresis using the continuous buffer system, and the stained gels were thoroughly washed for 3 days, with several changes of solution. The limit of visibility of the stained A.S.C. line was reached with the 2 mg/ml solution and therefore, the limit of sensitivity by this method was between 1 and 2 mg/ml of material.

Abnormal serum component—immunological studies

Preparation of antiserum to abnormal serum component. Although no A.S.C. has been observed in normal rat serum by the stained gel method, there was a distinct possibility that the substance was present in amounts too small to be detected. Obviously a more sensitive technique would be required before it could be definitely stated that this material was an abnormal component and not merely one which was already present in very small amounts.

Under suitable conditions the technique of immunological precipitation between antigen and antibody has been shown by many workers in other fields to be extremely sensitive. With this object in view, experiments were performed to determine whether the A.S.C. was antigenic, and if so, whether the antiserum so produced could be used as a sensitive method for the determination of small amounts of A.S.C. in serum.

Five male lop-eared rabbits, aged 7 months, were used for this experiment and were given the following injections:

Injection 1. 3 ml of oil (Bayol F), 1 ml of Arlacel A, 1 ml of sterile buffered saline, containing 12·5 mg of A.S.C., 12·5 mg of dried Canetti strain T.B. bacillus. The dried bacillus was ground up in the Bayol F oil, the remainder of the constituents added

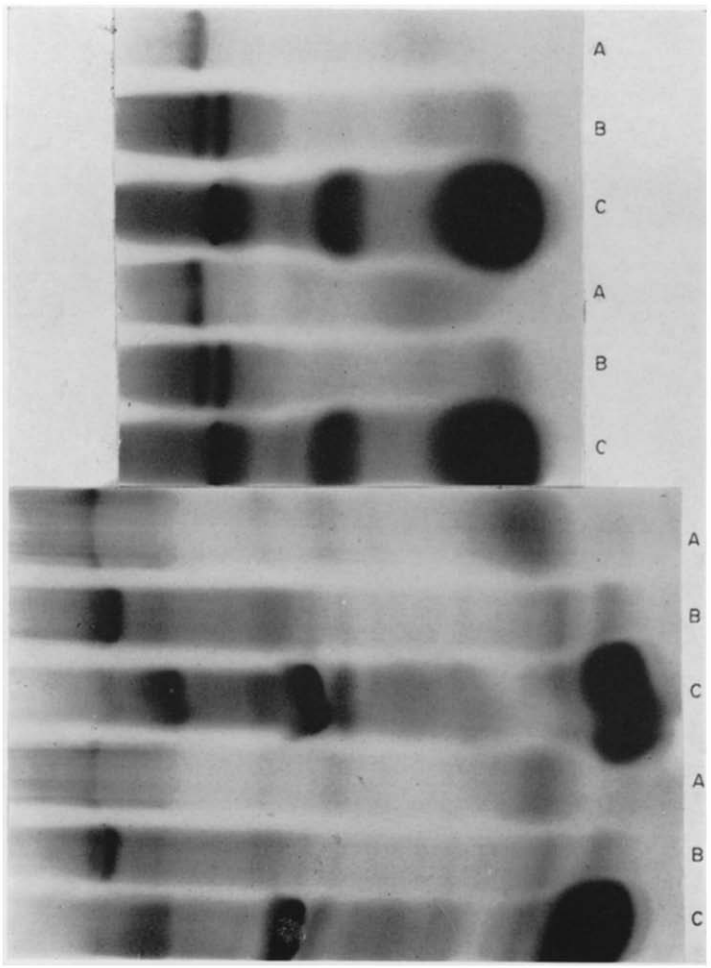

FIG. 8. Starch gel electrophoresis patterns of the material containing the abnormal serum component, at various stages of purification. (1) Continuous buffer system. (2) Discontinuous buffer system. A, final isolated material. B, cadmium serum after salt fractionation. C, normal rat serum.

facing page 116

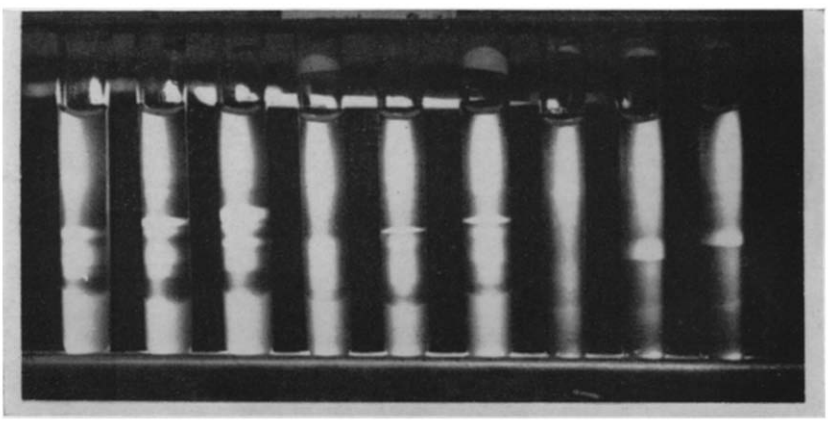

FIG. 9. Precipitin lines between antiserum and normal and cadmium rat serum. Top layer: 1, 4 and 7, normal rat serum; 2, 5 and 8, cadmium rat serum (*A*); 3, 6 and 9, cadmium rat serum (*B*). Bottom layer: 1, 2 and 3, 100 per cent rabbit antiserum (treated); 4, 5 and 6, 50 per cent rabbit antiserum (treated); 7, 8 and 9, 10 per cent rabbit antiserum (treated).

and the whole mixture thoroughly emulsified. A small volume (0·4 ml) of this solution was injected intramuscularly into each hind leg and the animals were left for 4 weeks.

Injection 2. 12·5 mg of A.S.C. in 0·3 ml of buffered sterile saline, 2·2 ml of aluminium phosphate gel. A small volume (0·4 ml) of this solution (equivalent to 2 mg of A.S.C.) was injected intravenously into each animal, two injections being given at an interval of 3 days. The animals were bled from the ear vein 1 and 2 weeks after the second injection and the serum obtained from these two bleedings was pooled and frozen in a deep freeze refrigerator until required. A volume of 170 ml of rabbit antiserum was obtained.

Precipitin reactions using the antiserum. The technique used in these experiments was that described by Oakley and Fulthorpe[16] with slight modifications. Ionagar no. 2 (Oxoid) and sterile buffered saline were used to prepare solutions containing 0·25% and 0·5% agar, incorporating 0·3% sodium azide as bacteriocide. Precipitin tubes of a suitable size were prepared with an inner film of agar and set up with three 1-cm layers of agar solution as follows: top layer, 0·1 ml of 0·5% agar solution, 0·1 ml of antigen solution; middle layer, 0·2 ml of 0·25% agar solution; bottom layer, 0·1 ml of 0·5% agar solution, 0·1 ml of antiserum. The layers were allowed to set, then stoppered, incubated at 37 °C and examined after 1, 3, 5, 7 and 14 days.

Precipitation of impurities. Starch gel electrophoresis of the isolated A.S.C. had already shown that two or three other serum components were visible. These proteins would, in all probability stimulate the production of antibodies when injected with the A.S.C. It was necessary, therefore, to precipitate the undesirable antibodies by the addition of suitable amounts of normal rat serum.

Varying amounts of normal rat serum were added to constant volumes of rabbit antiserum and the resultant solutions were tested for precipitin lines when matched against a solution of the A.S.C. in saline. It was found that the addition of 5% normal rat serum was sufficient to remove from the antiserum all the extraneous antibodies but one, whereas ten times this amount was necessary to afford complete removal of the remaining impurity. The addition of one part of normal rat serum to twenty parts of rabbit antiserum was considered sufficient for the purpose, allowance being made where necessary for the additional weak impurity.

Limit of detectibility of the A.S.C. An experiment to determine the optimum ratio of the A.S.C.–antibody system using the Oakley and Fulthorpe technique, showed that 1 ml of the rabbit antiserum reacted with 5 mg of A.S.C. at the optimum ratio. With this information, the limit of detectibility of the A.S.C., using the precipitin reaction, was estimated by the following procedure: Rabbit antiserum, to which 5 per cent of normal rat serum had been added, was matched with a 5 mg/ml solution of A.S.C. in order that optimum conditions should be obtained. Dilutions of A.S.C., using saline in one series and normal rat serum in the other, were matched against corresponding dilutions of the antiserum in saline (Table 1).

The limit of visibility in both series of tubes was the reaction occurring between 1 : 300 antiserum and the equivalent dilution of A.S.C. The limit of detectibility by this method then, is of the order of 16 μg/ml of A.S.C.

(The lines in tubes 16–20 were observed to be slightly lower than the corresponding

lines in tubes 6–10, and appeared to indicate the possibility that normal rat serum contained finite amounts of the A.S.C., thus causing depression of the band. This matter was examined in later experiments.)

Precipitin lines in cadmium rat serum. Two male rats *A* and *B* were given daily injections of 0·6 mg/kg of non-active cadmium by intraperitoneal injection for 3 weeks and at the end of this time the animals were bled by cardiopuncture.

Rabbit antiserum was treated with 5 per cent normal rat serum in the usual way and dilutions of this material giving 10 per cent, 50 per cent and 100 per cent of the original mixture were matched against serum obtained from a normal rat and also from rats *A* and *B* (Fig. 9).

The additional lines due to the presence of A.S.C. in the sera of rats *A* and *B* can readily be seen.

TABLE 1

Tube no.	Antiserum dilution (saline)	A.S.C. in saline (μg/ml)	Tube no.	Antiserum dilution (saline)	A.S.C. in rat serum (μg/ml)
1	1 : 1	5000	11	1 : 1	5000
2	1 : 20	250	12	1 : 20	250
3	1 : 40	125	13	1 : 40	125
4	1 : 60	83	14	1 : 60	83
5	1 : 80	62·5	15	1 : 80	62·5
6	1 : 100	50	16	1 : 100	50
7	1 : 160	31·3	17	1 : 160	31·3
8	1 : 200	25	18	1 : 200	25
9	1 : 300	16·7	19	1 : 300	16·7
10	1 : 500	10	20	1 : 500	10

The possibility that the A.S.C. had generated an auto-immune antibody to itself was examined by matching sera from rats *A* and *B* and a normal rat, against solutions containing 5, 1 and 0·1 mg/ml of A.S.C. in saline. A diffuse band was observed in all the tubes after 3 days, but the reactions between *A* and *B* and the A.S.C. were seen to be much stronger than those observed between the normal rat serum and the A.S.C. After 7 days, all the bands had disappeared.

The experiment was extended to examine the reactions of normal rat serum and combined sera from *A* and *B*, with dilutions of A.S.C. in saline to give 50, 10, 5, 1 and 0·1 μg/ml of A.S.C.

Two weaker bands were visible in those tubes containing normal rat serum, but two much stronger bands in a similar position were observed in the tubes containing serum from rats treated with cadmium. Gradual disappearance of the bands with time was observed as in the previous experiment. No explanation of this phenomenon is offered.

Immunoelectrophoresis

The isolated A.S.C. fraction contained several trace amounts of normal rat serum protein and by means of the technique of immunoelectrophoresis, the position of the various antigen–antibody reactions resulting from these impurities could be seen in relation to the main A.S.C.–A.S.C. antibody precipitin line.

Immunoelectrophoresis between A.S.C. and the rabbit antiserum. A solution of 10 mg/ml of the isolated A.S.C. in saline was subjected to starch gel electrophoresis using the continuous buffer system. The resultant gel was sectioned, one part being stained with naphthalene black solution, and the other being laid face downwards on a suitably prepared agar gel. Strips of filter paper were dipped in the rabbit antiserum, blotted damp dry and applied to the agar gel as previously described. After 7 days the following pattern was observed (Fig. 10). Accurate measurements of the gel showed that the A.S.C.–A.S.C. antibody reaction occurred at point *A*, other precipitin lines being due, presumably, to reactions between impurities in the A.S.C. material, and corresponding antibodies in the rabbit antiserum.

Precipitin reactions using the A.S.C. antiserum. Since Öuchterlony[17] introduced a method for the observation of precipitin lines by their formation in an agar gel contained in a glass Petri dish, this principle has found wide application in bacteriology and immunochemistry. A micro-modification of this method by Öuchterlony and Wadsworth,[18] using agar-coated glass microscope slides, has the advantage of conserving material and giving a result in a shorter time.

Effect of adding normal rat serum to rabbit antiserum. The Öuchterlony plate technique was used to examine the effect of addition of increasing volumes of normal

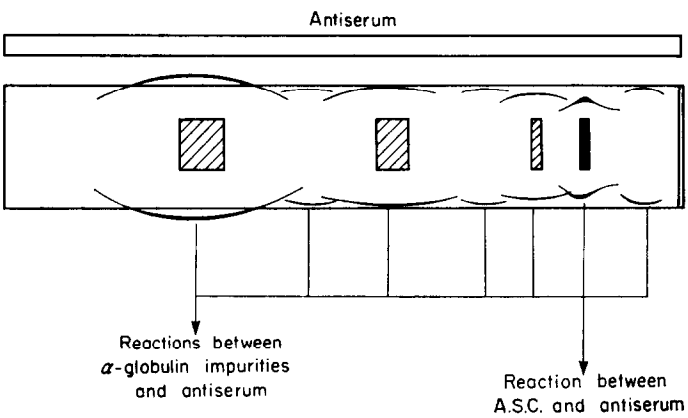

FIG. 10. Immunoelectrophoresis: precipitin bands between the isolated abnormal serum component and its rabbit antiserum.

rat serum to the rabbit antiserum, when the latter was matched against a solution of A.S.C. A solution of 3 mg/ml of A.S.C. was placed in the centre hole, and in the surrounding six holes, appropriate volumes of the following mixtures (Fig. 11): (1) Rabbit antiserum. (2) Rabbit antiserum (1 vol.) plus normal rat serum (0·05 vol.). (3) Rabbit antiserum (1 vol.) plus normal rat serum (0·5 vol.). (4) Rabbit antiserum (1 vol.) plus normal rat serum (1 vol.). (5) Rabbit antiserum (1 vol.) plus normal rat serum (3 vols.). (6) Normal rat serum.

It can be seen that there are multiple lines between the A.S.C. in the centre and the untreated antiserum. The addition of 0·05 vol. of normal rat serum to 1 vol. of rabbit antiserum left only two lines, and the addition of 0·5 vol. of normal rat serum to 1 vol. of rabbit antiserum gave a single line which did not appear to diminish in

intensity with increasing concentrations of normal rat serum. No line was formed between the rat normal serum and the A.S.C. solution.

Precipitin lines following varying intradermal doses of cadmium. Three rats were given single injections of 1, 2 and 3 mg/kg of inactive cadmium, as cadmium chloride, intradermally, blood specimens being taken after 24 hr. The sera from these rats, together with normal rat serum and a 0·5 mg/ml. solution of A.S.C. were matched on a micro-Öuchterlony plate against a 1 : 30 dilution of rabbit antiserum to which 5 per cent normal rat serum had been added. All the specimens gave precipitin lines except the normal rat serum.

Precipitin lines following low intraperitoneal doses of cadmium. Three rats were given single injections of 10, 50 and 100 μg/kg of inactive cadmium, as cadmium chloride, by intraperitoneal injection, saline being administered to a control rat. On examination of the sera after 24 hr, using the micro-Öuchterlony technique, easily visible lines were observed between the antiserum and the sera of the rats receiving 50 and 100 μg/kg. An almost imperceptible line was visible at the junction between the antiserum and the serum of the rat given a dose of 10 μg/kg. Evidence of the production of the A.S.C. can therefore be demonstrated after the injection of between 10 and 50 μg/kg of cadmium.

DISCUSSION

The introduction of the technique of electrophoresis of proteins has stimulated a great deal of research in experimental and clinical medicine, notably in attempts to correlate possible deviations from the normal body proteins with the progress of the particular disease or disorder under study. In addition, the modern techniques of ultracentrifugation and immunochemistry have been applied to this problem.

The investigation of possible serum protein changes in the rat following the administration of soluble cadmium salts, was initially conducted by the author using the method of filter paper electrophoresis. It soon became obvious, however, that although gross changes in the serum protein pattern could be identified in this way, a more sensitive method would be required to observe smaller deviations from the normal. It was decided to make use of the horizontal starch gel technique devised by Smithies,[12] and this method was used almost exclusively in the investigations reported in this paper.

An abnormal serum component was observed 24 hr after the intraperitoneal administration of a single dose of cadmium. Previous experiments[19] had shown that the level of cadmium in the blood following a single intraperitoneal injection, dropped to a negligible value after 5 hr, but, nevertheless, the possibility that the abnormal component was the result of a direct cadmium–serum combination was examined by the addition of cadmium to normal rat serum *in vitro*. The results indicated a progressive lowering of the migration rate of the albumin fraction, from which cadmium–albumin binding may be inferred, but no band comparable with the abnormal serum component was seen on starch gel electrophoresis of the sera from this experiment. At the time of production of the abnormal serum component, that is, 20–24 hr after the injection, the administered cadmium had already been laid down in the tissues of the body, and 50 per cent of this cadmium could be found in the liver. It seems possible

that the abnormal serum component is the result of aberrated biosynthesis of the serum proteins, resulting from the action of cadmium on one or more of the liver constituents.

Further evidence for this theory is afforded by the difficulty experienced in separating a sample of the abnormal serum component from the serum protein migrating immediately in front of the abnormal component in starch gel electrophoresis, using the continuous buffer system. Earlier experiments using British Drug Houses Ltd. Soluble Starch, appeared to indicate a complimentary role for these two components, i.e. as the abnormal component increased, the adjacent protein decreased. This phenomenon was not so apparent in the present experiments, but would probably repay further investigation.

Another interesting aspect of this work was the observation that at relatively high doses of, for example, 2 mg/kg of cadmium, the abnormal serum component was not produced. If the action of low doses of cadmium results in an abnormal biosynthesis, then higher levels of cadmium may well prove completely cytotoxic to the cells concerned in the production of the abnormal component.

In addition to cadmium, several other metals, when injected intraperitoneally, caused the production of the abnormal serum component. Mercury, indeed, appeared more effective, than cadmium, whereas beryllium and copper were equally effective, manganese, chromium and nickel somewhat less effective, lead giving no comparable reaction at all. Stokinger and Wagner[10] have reported that the α-globulin fractions of the sera of dogs and rabbits were elevated following exposure to small doses of cobalt. This finding may possibly be related to the faint band found in the rats receiving cobalt, although the serum neuraminic acid levels were normal, as opposed to the elevated levels found by Stokinger and Wagner. This point obviously requires further investigation.

Electrophoretic analysis has shown that a large range of different diseases, having a common syndrome of malnutrition and wasting, tend to give a similar overall serum pattern.[20] Such diseases as nephrosis, cirrhosis, hepatitis, multiple myeloma, syphilis, tuberculosis, sarcoid, leprosy, malaria, lobar pneumonia, rheumatic fever, rheumatoid arthritis and lupus erythrematosis disseminatus, have all been shown to produce a lowered albumin content and an increased globulin content of the plasma. General observations of altered plasma protein distribution with the occurrence of various diseases, are of somewhat limited value, unless extended to include a more detailed study of specific changes. The production of an abnormal serum component by several metals, and cadmium in particular, can be considered rather different from the serum changes apparent in these diseases, especially in view of the very small doses required to produce it, and the comparatively short time before its appearance in the serum.

Acknowledgements—The author is indebted to Dr. Donald Hunter and Dr. A. I. G. McLaughlin for interest and encouragement with the work. Thanks are also gratefully extended to Professor C. J. O. R. Morris and Dr. R. G. White for advice and assistance with the techniques of electrophoresis and immunology, respectively, and to Miss Kathleen Ballard and Mr. B. J. Biles for valuable technical help.

REFERENCES

1. E. W. Schwartze and C. L. Alsberg, *J. Pharmacol.* **21**, 1 (1923).
2. C. O. Johns, A. J. Finks and C. L. Alsberg, *J. Pharmacol.* **21**, 59 (1923).
3. O. G. Fitzhugh and F. H. Meiller, *J. Pharmacol.* **72**, 15 (1941).
4. R. H. Wilson and F. DeEds, *Arch. Industr. Hyg.* **1**, 73 (1950).

5. L. E. DECKER, R. U. BYERRUM, C. F. DECKER, C. A. HOPPERT and R. F. LANGHAM, *Arch. Industr. Hlth.* **18**, 228 (1958).
6. D. J. LAWFORD, *Nature, Lond.* In press.
7. R. TRUHAUT and C. BOUDENE, *Arh. Hig. Rada* **5**, 19 (1954).
8. M. BARNI, M. DE FELICE and L. REALE, *Folia Med.* **41**, 168 (1958).
9. C. P. ODESCALCHI, P. ANDREUZZI and A. T. VILLA, *Folia Med.* **41**, 1179 (1958).
10. N. E. STOKINGER and W. C. WAGNER, *Arch. Industr. Hlth.* **17**, 273 (1958).
11. M. D. POULIK, *Nature, Lond.* **180**, 1477 (1957).
12. O. SMITHIES, *Biochem. J.* **61**, 629 (1955).
13. J. PORATH, *Biochim. Biophys. Acta* **22**, 151 (1956).
14. H. I. GEDIN and J. PORATH, *Biochim. Biophys. Acta* **26**, 157 (1957).
15. G. H. KUNKEL and A. TISELIUS, *J. Gen. Physiol.* **35**, 89 (1951).
16. C. L. OAKLEY and A. J. FULTHORPE, *J. Path. Bact.* **65**, 49 (1953).
17. Ö. ÖUCHTERLONY, *Acta Path. Microbiol. Scand.* **32**, 231 (1953).
18. Ö. ÖUCHTERLONY and C. WADSWORTH. Unpublished, referred to in *Progress in Allergy* Vol. 5. Karger, Basle, Switzerland.
19. D. J. LAWFORD, *J. Lab. Clin. Med.* In preparation.
20. A. B. GUTMAN, *Advanc. Protein Chem.* **4**, 155 (1948).