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Isolation and Characterization of a Rabbit Adrenal Autoantigen

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Summary. The antigenic components of adrenal extract were fractionated by chromatography on DEAE-cellulose. Using a stepwise elution procedure, it was found that a componenet of major interest, termed Pk3b, was eluted by a buffer of pH 6·70 and 0·09 M in phosphate-chloride. This antigenic preparation reacted with several rabbit antisera that had been prepared by isoimmunization with rabbit adrenal homogenate, in contrast to several other fractions, which reacted with only one (or two) of the antisera. Pk3b also showed a high degree of homogeneity, as shown by immunodiffusion, paper electrophoresis, and analytical ultracentrifugation. By the latter technique, an extrapolated sedimentation coefficient of 4·45S was determined. The further comparison of this antigen in immunodiffusion, along with an antiserum and the adrenal extract from the antiserum-providing animal, confirmed again that this is an autoantigen, and that it is one of five autoantigens of the adrenal gland.

INTRODUCTION

Previous reports have shown that adrenal tissue contains substances which are capable of eliciting the production of autoantibodies when injected into the same species of animal under intensive conditions of immunization. These successful results were achieved by the use of intradermal inoculation with the incorporation of Freund's complete adjuvant, and both the rabbit and the guinea-pig were used (Witebsky and Milgrom, 1962). It has also been shown that there are as many as four autoantigens in the rabbit system and an equal number of corresponding autoantibodies, as demonstrated by gel diffusion using concentrated preparations of the antiserum (Centeno, Shulman, Milgrom and Witebsky, 1965), and some chemical properties of the autoantigens have been studied (Centeno, 1962; Shulman, Centeno, Milgrom and Witebsky, 1965). The various proteins and antigens of adrenal tissue have been reviewed as a general topic in regard to adrenal specificity and autoimmunity (Glynn and Holborow, 1965; Shulman, 1971).

Attempts were then made to isolate one or more of these antigens by means of protein fractionation and especially by utilization of chromatographic procedures, and the major product among the purified fractions has been partially characterized.

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MATERIALS AND METHODS

Preparation of extracts

Rabbit adrenal glands were obtained in the frozen state from Pel-Freez, Rogers, Arkansas, and kept in this condition until used. Before processing, the adrenal glands were thawed and then trimmed in the cold room in order to remove the fat. Each portion of glands was homogenized in a Sorvall Omni-mixer with ice cold saline (0·15 M NaCl); the volume of saline was twice the weight of the tissue. The chamber was dipped into a cold bath at −8°. The suspension was chopped at top speed for 1 minute, allowed to cool, and chopped again for a second minute. This was then centrifuged in a Sorvall refrigerated centrifuge at 16,000 rev/min for 1 hour. Three layers were obtained, a layer of fatty material on the top, a red, clear, supernatant liquid in the middle, and a residue on the bottom. The supernatant was stored and the residue was processed again with a volume of 4 M NaCl equal to that of the original volume of saline. This was intended to produce an exhaustive extraction of the soluble components of the adrenal glands. The second supernatant fraction was pooled with the first one, and with a third supernatant which was obtained in a similar manner. The final saline extract, thus prepared, was somewhat turbid. It was found most expeditious for clarifying this turbidity to incubate for 1 hour at 37° with continuous gentle stirring, then to centrifuge the extract for 2 hours at 16,000 rev/min. A large precipitate was obtained which was discarded; the liquid phase was red and almost completely clear.

The extract was placed in a dialysis bag and embedded in solid sucrose which provided an osmotic concentrating effect. When the volume had been reduced to a suitable amount, the extract was dialysed against distilled water in the cold room for a week with changes of the dialysis bath twice a day. The bath contained sodium azide at 0·1 per cent as a preservative. After several days of dialysis, some of the extract components became insoluble, and this precipitate was removed from the system by centrifugation for 1 hour at 16,000 rev/min. This divided the system into: (1) a supernatant, labelled RAF-sol, for rabbit adrenal fraction, soluble in water, which contained most of the antigens, and (2) a precipitate, RAF-insol, for rabbit adrenal fraction, insoluble in water. The RAF-sol was used in the chromatographic studies to be described below. The precipitate was almost completely redissolved in a solution of 1 M NaCl and preserved for other studies. This RAF-sol fraction was dialysed against phosphate buffer, pH 7·8, 0·02 M.

Preparation of chromatographic system

DEAE-cellulose was obtained from Brown Company, Berlin, N.H., and was type 20, reagent grade, capacity of 0·93 mEq/g. The anion exchanger was suspended in 0·5 N NaOH and stirred in the cold room for a week. It was then washed with several portions of distilled water until there was no reaction with phenolphthalein. The alkaline treatment and the washing were repeated until the cellulose was quite white and the wash liquid was colourless. The resin was resuspended in the starting buffer, pH 7·8, 0·02 M.

Columns of 2·5 cm internal diameter were used. The DEAE-cellulose was poured as a slurry into the column. The column was refilled with additional amounts of the resin until it could receive no more. A pressure of about 10 lb/in.^{2} was applied and new portions of slurry were then added until the column had a height of 60 cm and did not diminish because of the pressure. A column prepared in this way generally has a flow rate of 5–8 ml 30 minutes; this was regulated by the hydrostatic pressure of a bottle filled with buffer

placed a few cm above the top of the column. The starting buffer was then allowed to run through the column until the resin was equilibrated. After applying the sample, a polyethylene container of 2000 ml capacity fixed at 10 cm above the level of the column provided a continuous flow of eluting buffer.

Fractions were collected by stepwise elution, using a GME time-activated fraction collector. Buffers with decreasing pH and increasing ionic strength were used. Optical density readings were taken with a Beckman DU spectrophotometer at 280 mμ, and every tube was examined. Buffers were changed after the optical density showed a steady and reasonably low value. Various fractions were pooled according to the outline of elution peaks, recorded by graphing the optical density versus the number of the tube. Pools were concentrated by osmotic effects of dry sucrose.

The preparative procedure is summarized in the flow sheet that is presented.

Gel diffusion method, antisera, and γ-globulin preparations

These have been described in a previous paper (Centeno *et al.*, 1965).

Electrophoretic analysis

Electrophoresis was performed by the filter paper strip method, using a Spinco model R apparatus with the procedure B, as described by the manufacturer.

Ultracentrifugal analysis

Samples were analysed in a Spinco ultracentrifuge model E, using schlieren optics and a single-sector cell. Sedimentation coefficients were measured and calculated by standard methods, as previously described (Shulman *et al.*, 1965), and utilizing the RTIC unit for temperature measurement. Corrections were made in the usual way. A value of 0·75 was assumed for the partial specific volume.

RESULTS

In the earliest studies, small columns were used (1 cm internal diameter and 60 cm long). A group of phosphate buffers, the pH and molarity values of which varied from 7·8 to 4·4, and from 0·02 to 2·4, respectively, were selected for trial. When the experimental conditions were established, larger columns, with a 2·5 cm internal diameter and length of 60 cm, were used.

The immunological activities of the various chromatographic fractions were tested against antisera obtained from rabbits 1919, 2030, and 2035 by immunodiffusion in agar gel. It was found that the fractions eluted with the buffers of pH 7·8, 7·0, 6·5, and 6·0 were antigenically active, since several precipitin bands developed. One of these bands was common to all three antisera. The corresponding antigen was mainly eluted with the buffer of pH 6·5, even though some activity could be detected in the fractions obtained with buffers of pH 7·0 and 6·0. As this component seemed to be the most active of all these antigens, it was chosen for the goal of further isolation.

Physicochemical and immunological studies performed on this fraction showed that it was heterogeneous. Consequently, new buffers were interpolated with the purpose of obtaining a better separation of these components. Finally it was found that a buffer solution of pH 6·70, 0·09 M produced the desired effect. The peak formerly eluted with the buffer of pH 6·5 was now divided (by these two buffers) into four components which were

identified as Pk3a1, Pk3a2, Pk3b, and Pk3c. This resolution is illustrated in Fig. 1, which shows the elution pattern from such a run, and the composition of the various buffers used in this fractionation is presented in Table 1. The yields from such a chromatographic run, as well as the detailed quantities and percentages of protein recovered from the different

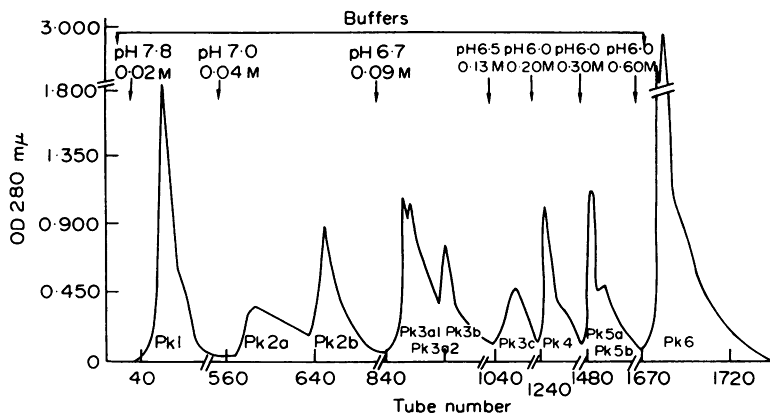


FIG. 1. Chromatogram of a rabbit adrenal saline extract on a 2·5 × 60 cm bed of DEAE-cellulose. The sample was 1204 mg of protein in a volume of 15 ml. Flow rate, 5 ml/30 minutes.

TABLE 1

BUFFERS USED IN THE CHROMATOGRAPHIC FRACTIONATION OF RABBIT ADRENAL AUTOANTIGEN

pH	Phosphate concentration (mol/l)	NaCl Concentration (mol/l)
7·80	0·02	—
7·00	0·04	—
6·70	0·06	0·03
6·50	0·08	0·05
6·00	0·10	0·10
6·00	0·10	0·20
6·00	0·10	0·50

TABLE 2

YIELDS OF THE FRACTIONS OBTAINED FROM A CHROMATOGRAPHIC COLUMN

Fraction	Mass (mg)	Recovery per cent	Buffer solutions used	
			pH	Molarity
Sample	1204			
Pk1	73·0	6·8	7·8	0·02
Pk2a	44·5	4·1	7·0	0·04
Pk2b	49·4	4·6	7·0	0·04
Pk3a1	71·1	6·7	6·7	0·09
Pk3a2	42·1	3·9	6·7	0·09
Pk3b	62·4	5·8	6·7	0·09
Pk3c	68·6	4·3	6·5	0·13
Pk4	68·9	6·4	6·0	0·20
Pk5a	40·4	2·9	6·0	0·30
Pk5b	37·8	3·6	6·0	0·30
Pk6	222·5	21·6	6·0	0·60
Final elution with HCl	300·2	28·5	2·0	0·50

fractions, are shown in Table 2. It is of interest that the number of adrenal glands used were approximately 2000, having an initial wet weight of 257 g. This value was reduced to a starting quantity of 1·20 g of protein after the processes of homogenization, clarification, and dialysis against distilled water. These new fractions (of the Pk3 group) were studied by ultracentrifugation, filter paper electrophoresis, and immunochemical procedures. All of them demonstrated that the four peaks shared a component whose greatest concentration and purity was located in the fraction Pk3b.

The analysis performed in paper electrophoresis indicated that the fraction Pk3b had a single component, as shown in Fig. 2. This is in agreement with the results furnished by the

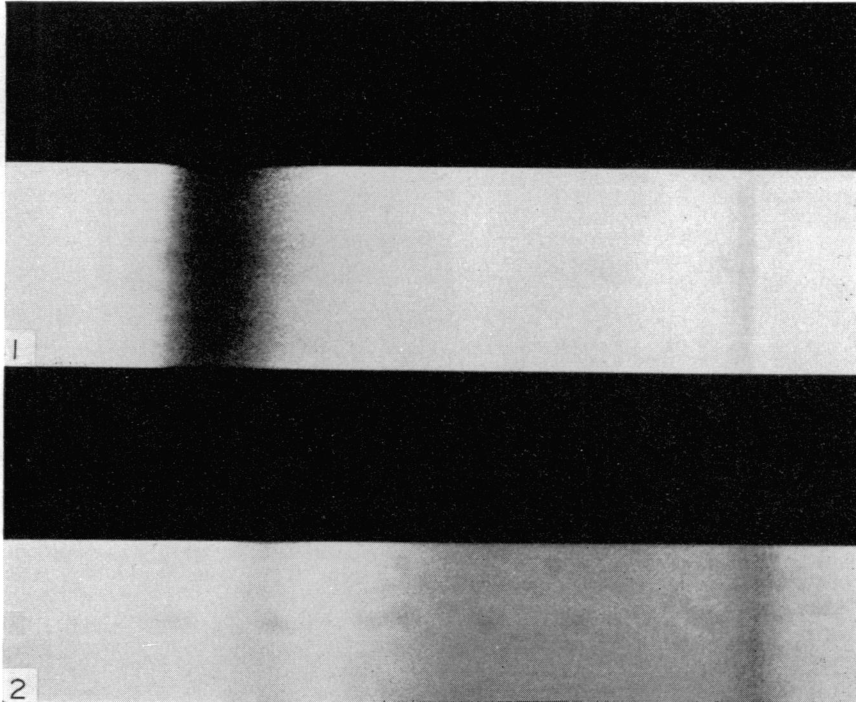


Fig. 2. Paper electropherogram of Pk3b fraction (strip 1) and the original rabbit adrenal saline extract (strip 2). The anode is on the left.

analytical ultracentrifuge (Fig. 3), where a single sharp peak is seen. Several samples were analysed in the ultracentrifuge over a range of concentrations. The values obtained, extrapolated to zero concentration and corrected to 20° in a medium of distilled water, gave a figure of 4·45S. The graph is shown in Fig. 4.

Antisera 1919, 2030, and 2035, whose preparation and diverse properties as anti-adrenal sera were described in a previous report (Centeno *et al.*, 1965), reacted with the isolated fraction in immunodiffusion plates, each giving a thick precipitin band. In addition, a minor component was found constantly with the antigen isolated in the described manner, as can be observed in Fig. 5, which shows the reaction, obtained in a system composed of the γ-globulin fraction isolated from serum 2030, reacting with a rabbit adrenal saline extract prepared from autologous tissue, and with the above-mentioned purified antigen.

To establish with which precipitin band of the total extract the antigen Pk3b gave a reaction of identity, the following experiment was performed. An immunodiffusion plate was made with (a) γ-globulin from rabbit 2030, incubated with the antigen Pk3b (four drops of γ-globulin solution and 1 drop of the antigen solution, which concentration was

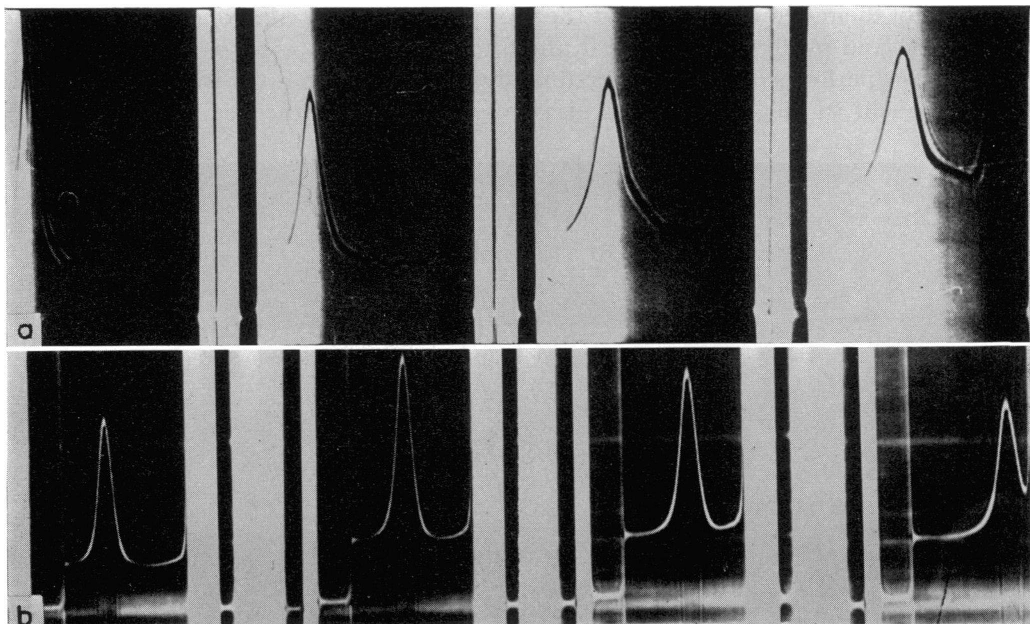


Fig. 3. Ultracentrifuge patterns. (a) Rabbit adrenal extract, protein concentration: 1·66 per cent. Photographs taken at 16, 24, 44, and 84 minutes after rotor reached speed of 59,800 rev/minute. Fraction Pk3b, protein concentration: 1·70 per cent. Photographs taken at 52, 68, 84, and 108 minutes after rotor reached speed of 59,800 rev/min. Direction of sedimentation is to the right.

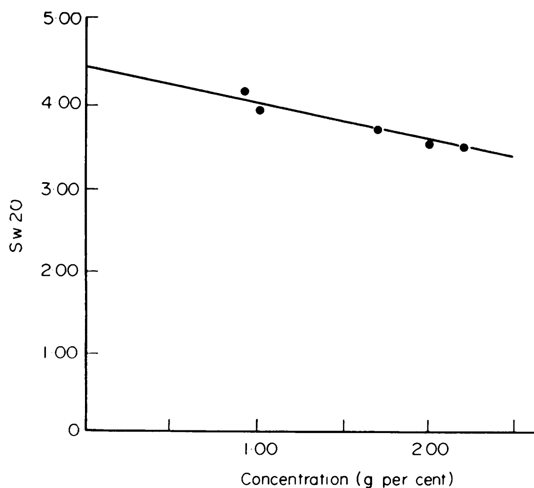


Fig. 4. Sedimentation coefficient of fraction Pk3b plotted against total protein concentration. Extrapolated value is 4·45S.

2 per cent) during 1 hour at room temperature, 12 hours at 4°, and finally centrifuged at 18,000 rev/min for 30 minutes; (b) the same γ-globulin without being absorbed, and (c) rabbit adrenal saline extract. The absorbed antibody preparation produced soluble complexes which formed a precipitin band when they met another portion of unabsorbed antibody. This band gave a reaction of identity with only one of those resulting from the reaction of saline extract and antiserum.

Other immunodiffusion studies carried out with this purified antigen, saline extracts made from other organs of rabbits, and medulla and cortex of bovine adrenals, tested

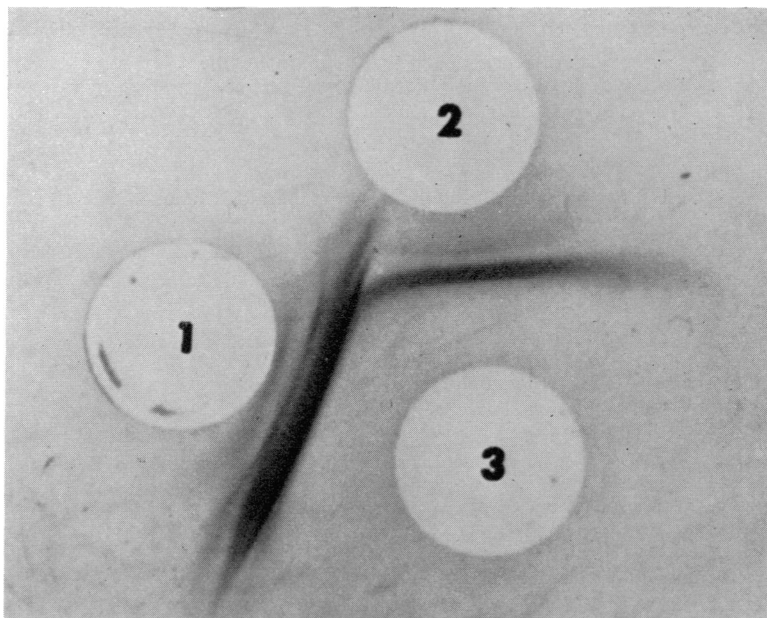


FIG. 5. Gel diffusion precipitation. Well 1: saline extract prepared with adrenals from rabbit 2030, protein concentration, 1·1 per cent. Well 2: fraction Pk3b, protein concentration, 2·1 per cent. Well 3: γ-globulin preparation from rabbit 2030.

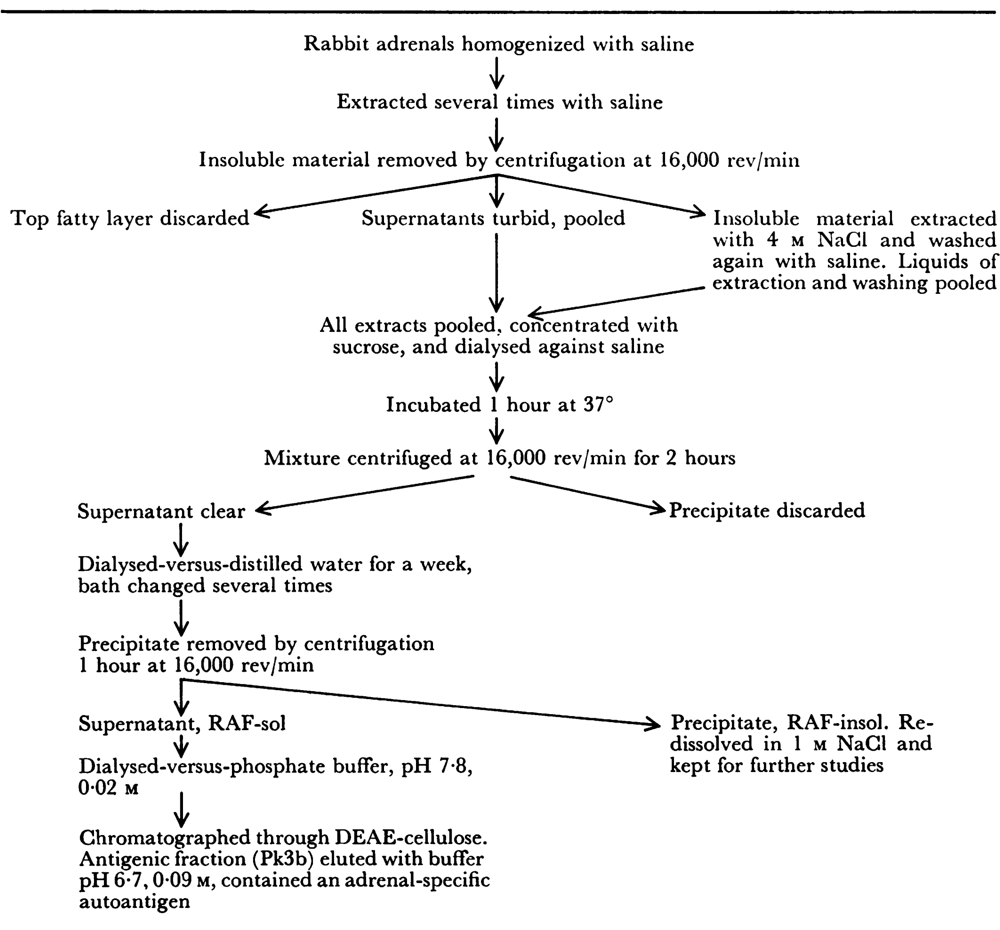
with the above-mentioned antiserum, showed that the antigen under study was shared only by the medulla of bovine adrenal. It was not possible to detect any cross reaction with saline extracts prepared with homologous kidney, liver, spleen, or lung.

The isolated antigen is highly unstable, being observed to give spontaneous precipitation in several weeks, even when stored at 4°, with or without sodium azide, or with a very small amount of toluene added to avoid the growth of bacteria.

The studies performed to obtain further purification encountered the inconvenience of the lability of the almost pure antigen. Nevertheless, some attempts were made for this goal. The sample identified as Pk3b was rechromatographed on small columns of DEAE-cellulose of 1 cm internal diameter and 30 cm height. Also, columns of Sephadex G-100 and G-200 were used with the same objective. None of these experiments yielded a purer substance or succeeded in resolving the sample into two or more components.

TABLE 3

FLOW CHART FOR PREPARATION OF ADRENAL AUTOANTIGEN



DISCUSSION

From a highly heterogeneous material, such as an extract of adrenal tissue, one component was finally isolated in a high grade of purity, according to the criteria of paper electrophoresis, analysis in the ultracentrifuge, and immunodiffusion in agar plates. Several methods have been used in the attempts to purify the antigen, termed Pk3b, including fractionation by salting out, zone electrophoresis on starch as inert support (Centeno, 1962; Shulman *et al.*, 1965), and chromatography on DEAE-cellulose. From all of these methods the latter was selected because it provided a product of high purity and also a reasonable yield.

From the distinct antigenic fractions obtained by chromatography on DEAE-cellulose, one was chosen because it reacted with several antisera. One can infer consequently, from these properties, that it was the most active autoantigen among all of those detected in the rabbit adrenal gland.

The high grade of purity of the isolated antigen is reflected in the results furnished by

the various analytical procedures employed in the present work. For example, Fig. 2 represents a comparative study, performed in paper electrophoresis, of a rabbit adrenal saline extract and the fraction Pk3b. It can be observed that the latter ran as a single fast component with the mobility of an albumin while the total extract gave a polydisperse pattern. It can also be noticed that the purified antigen has been concentrated many-fold as compared with its presence in the crude fraction.

A comparative study is also presented in Fig. 3a and b. The former is the analytical ultracentrifugal pattern of a rabbit adrenal total extract which is composed of a quite polydisperse system in contrast to the pattern presented in Fig. 3b which corresponds to the isolated antigen, the latter showing a very symmetrical boundary all along the experiment. This antigen possessed a sedimentation coefficient that extrapolated to zero concentration to give 4·45S. Therefore, this is not the antigen identified previously by Centeno (1962) and by Shulman, Centeno, Milgrom and Witebsky (1965), since the earlier one had a different sedimentation coefficient, which in fact was 6·6S. For this value of 4·45S, a mol. wt of the order of 60,000 could be estimated.

The autoantigenic nature of the fraction Pk3b is clearly demonstrated by the experiment shown in Fig. 5. The precipitin band formed by the antiserum from rabbit 2030 and the fraction Pk3b merges, giving a reaction of identity with one of the five precipitin bands formed by the serum 2030 and the saline extract prepared with the adrenal of the same animal. (This reaction thus occurs between two autologous reactants.)

The lability of the antigen Pk3b hindered all attempts to detect its inhibitory activity using techniques of passive haemagglutination such as that of Boyden (tannic acid) or BDB (bis-diazotized benzidine). Furthermore, by comparing the position of the precipitin bands obtained with the total extract and the isolated fraction, in Fig. 5, it can be observed that they were formed at an equal distance from the well where the specific γ-globulin was applied, in spite of the fact that the concentration of the purified fraction was twice that of the saline extract. This would suggest some loss in its antigenic properties. Nevertheless, the isolation of this antigen may be a step forward toward the knowledge of its structure and also to some possible elucidation of the phenomenon of autoimmunity.

Many tissue-specific antigens are mentioned in studies published in recent years. On the other hand, there are rather few references to purified autoantigens, and this is perhaps a demonstration of the many difficulties involved in the task of preparing them. However, outstanding progress has recently been made in the purification and chemical study of autoantigens of brain (Kies, Thompson and Alvord, 1965; Kibler, Shapira, McKneally, Jenkins, Seldon and Chou, 1969; Eylar and Hashim, 1968; Chao and Einstein, 1968) and testis (Voisin and Toullet, 1968); highly purified components have been described for thyroid (Ui, Tarutani, Kondo and Tamura, 1961; Shulman and Armenia, 1963; Salvatore, Salvatore Cahnmann and Robbins, 1964; Shulman, Mates and Bronson, 1967) and also certain cancer tissues (Karitzky and Burtin, 1967; Loisillier, Burtin and Grabar, 1968). The occurrence of distinctive autoantigenic determinant groups on the molecule of the major thyroid antigen has also been described (Shulman, 1968; Roitt, Torrigiani and Doniach, 1968; Ghayasuddin and Shulman, 1970).

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