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Biochem. J. (1960) **77**, 12

Immunochemically-Active Cross-Linked Polystyrene Preparations

By L. H. KENT AND J. H. R. SLADE

Microbiological Research Establishment, Porton, Wilts

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In recent years the immune reaction has been used, directly or indirectly, for the fractionation of antibody proteins. Application of such methods has been reviewed by Isliker (1957). For example, Campbell, Luescher & Lerman (1951) have chemically conjugated antigen protein to a polymer such as cellulose and have used the product in a column to fractionate antibody. In the course of a more general attempt (Tozer, Cammack & Smith, 1958) to separate antigens by means of their immunochemical specificities, G. H. Whitham, B. T. Tozer & H. Smith (unpublished work) attempted to invert the method used by Campbell *et al.* (1951), and, by conjugating antibody to cellulose, use the product to fractionate antigens. The amount of antibody protein which can be conjugated in this manner is not large and the stoichiometry of the immune reaction is such that the ratio of antigen combining with antibody is usually low. The result is that the capacity of such conjugated material is also low. It occurred to us that greater capacity could be achieved by using antibody protein to cross-link linear polymers such as a styrene, suitably substituted with a group capable of reacting with reactive centres or groups in the protein molecule e.g. tyrosyl and sulphydryl groups. While this work was in progress Gyenes, Rose & Sehon (1958) reported a similar approach to the fractionation of antibody proteins. The present paper describes the preparation of two types of such cross-linked polymers from styrene and antibody protein, in which the chemical conjugation is achieved in one by means of a diazo group and in the other by means of a mercury atom. A preliminary account of some of this work has already been published (Kent & Slade, 1959).

MATERIALS AND METHODS

Antigens. *Pasteurella pestis* toxin was prepared by $(\text{NH}_4)_2\text{SO}_4$ precipitation from the protein fraction of *Pasteurella pestis* grown on a casamino acid-glucose medium at 28° (Englesberg & Levy, 1954). Anthrax protective antigen was prepared as a culture filtrate prepared by the method of Thorne & Belton (1957). Ovalbumin was prepared by Na_2SO_4 precipitation according to the method of Kekwick & Cannan (1936).

Antibodies. Ovalbumin antiserum was prepared in rabbits and supplied by Dr H. Smith and Dr B. T. Tozer. Plague antiserum was prepared in rabbits by Dr A. P. MacLennan and was active against the toxin and several other, but not all, known antigens of *Pasteurella pestis*; 1 ml. of antiserum neutralized 4 mg. of toxin. A hyper-immune anthrax antiserum was prepared in horses by Mr F. C. Belton using a spore vaccine (Sterne Strain).

γ -Globulins. These were prepared from both normal and immune sera by Na_2SO_4 precipitation by the method of Kekwick & Record (1941) and stored as freeze-dried powders.

Polyaminostyrene. In the initial experiments linear polystyrene, Distrene 80 (British Resin Products Ltd. Hayes, Sully, Penarth, Glamorgan, Wales) was nitrated with a mixture of HNO_3 and H_2SO_4 to the extent of approximately one nitro group per aromatic ring; the nitro compound was reduced with SnCl_2 and HCl by the method of U.S. Pat. 2,366,008. The polyaminostyrene so prepared, though fully soluble in dil. HCl , gave, surprisingly, a sparingly soluble diazonium salt. This was traced to the presence in the product of a small percentage of sulphonic acid groups, derived from the H_2SO_4 used during the nitration. When HNO_3 alone was used in this stage, a soluble diazonium salt was formed. The method finally adopted was as follows.

Granular Distrene (20 g.) was mixed with 100 ml. of fuming HNO_3 (95%, w/w; sp.gr. 1.5) and warmed on a water bath until dissolved. Heating at 100° was then continued on the steam bath (usually for about 30 min.) until

a test sample, obtained by precipitation with water, washed well and dried, showed a nitrogen content of 9–10% by the Kjeldahl method modified for nitro compounds (Pregl, 1945). The product was then precipitated with water, washed well, drained and transferred to a flask fitted with a reflux condenser; SnCl_2 (200 g.) and conc. HCl (300 ml.) were added and the mixture was slowly heated to reflux on the hot-plate for 4 hr. We are indebted to Dr P. Jackson for informing us that this reaction may become violent, even in the later stages after the initial vigorous reaction has subsided. Almost all the solid dissolved to give a light-brown solution of the amine hydrochloride. The solution was filtered through a large sintered-glass filter (porosity 2 or 3) and the small residue was washed with a little water.

A solution of NaOH (300 g./300 ml. of water) was added slowly to the cooled filtrate with stirring. The polyaminostyrene was precipitated as a dirty white solid, whereas the tin hydroxides at first precipitated redissolved in the excess of alkali. The polyaminostyrene was collected by filtration through a thick layer of cellulose pulp on a large sintered-glass funnel (porosity 2) and washed well with N-NaOH solution until it was largely free of tin salts. The precipitate was protected from strong light and from the air by keeping it covered with liquid as much as possible to prevent its darkening. It was drained, and the cake of polyaminostyrene separated from the filter-paper bed as completely as possible and redissolved in warm 2N-HCl containing 10% of NaCl . The solution was adjusted to pH 1.0–1.5 and the tin was precipitated as sulphide by a stream of H_2S and removed by filtration through a layer of filter-paper pulp in a sintered-glass funnel. The solution was freed from H_2S by heating gently on a hot-plate in a stream of N_2 . The yield could be calculated by evaporating a known volume to dryness, drying at 105–110° to give total solids and subjecting the residue subsequently to ignition, which gave the NaCl content. If the NaCl ash contains any Sn , the precipitation of free polyaminostyrene with excess of NaOH should be repeated, followed by dissolving again in 2N-HCl . A final concentration of 2% of polyaminostyrene was convenient.

Polystyrylmercuric acetate. Mercuric oxide (25 g.) was dissolved in 22 ml. of 72% perchloric acid (sp.gr. 1.7) and the solution was cooled and diluted slowly, with cooling and stirring, with 200 ml. of amyl acetate. Distrene (10 g.) was dissolved in 400 ml. of amyl acetate by gently warming on a water bath and the solution of mercuric perchlorate was added dropwise, with vigorous stirring, until any sticky precipitate that formed had redissolved. The pale-yellow solution was allowed to stand at 20°; a cream-coloured gelatinous precipitate formed and the reaction appeared to reach completion in 2 hr. The precipitate was filtered off and washed with amyl acetate until a test portion remained clear on dilution with 90% ethanol; it was then washed with 90% ethanol until it was free of amyl acetate and wetted by successive washing with 50% ethanol, 20% ethanol and, finally, with water.

The resulting polystyrylmercuric perchlorate was converted into the acetate by stirring with successive portions of aqueous 25–30% sodium acetate solution, to which a few drops of acetic acid had been added, until the filtrate after treatment was free of perchlorate. The solid was washed free of sodium acetate, drained well on the filter pump and stored in the damp state.

The mercury content of the product varied somewhat

from batch to batch but was normally between 50 and 60%, based on a dried sample.

Conjugation with antibody protein

By means of polyaminostyrene. In general it is advantageous to use a weight of polymer approximately equal to that of the protein used.

A 2% solution of polyaminostyrene in 2N-HCl (10 ml.) was cooled to between 0 and -5° , diazotized by dropwise addition of 1.5 ml. of 14% NaNO_2 solution and kept at about -5° for 20 min. The excess of nitrous acid was destroyed by adding 2 ml. of 6% urea solution (previously cooled to 0°) and keeping the mixture between 0 and -5° , with occasional stirring, for about 1 hr. until a test drop failed to turn a moistened starch-iodide paper blue. The solution was cooled in ice- NaCl to about -10° , and 12 ml. of N-NaOH (also previously cooled to -10°) was added slowly with stirring. The stirring must be thorough and the pH must not rise at this stage above 4 or cross-coupling, with resulting precipitation, will occur. The protein solution (0.20 g. in 1% NaCl solution) and crystalline sodium acetate (1 g.) were quickly added, with stirring, when the pH rose to about 6 and a brown precipitate of polystyryl-azoprotein began to form. The mixture was stirred gently overnight at 0° ; the solution was adjusted to pH 8 with a few drops of dilute Na_2CO_3 solution and again stirred gently for 24 hr. at 0° . Normally all the protein will have been removed from the supernatant at this stage, as shown by a negative phosphotungstic acid test. The precipitate was then filtered off through a sintered-glass filter (porosity 3 or 4), well washed with 0.9% NaCl , resuspended in 0.9% NaCl and stirred successively at room temperature with further small amounts (5–10 mg.) of protein until the latter was no longer absorbed from solution. Any unchanged diazonium groups were then inactivated by stirring the solution with 0.2 g. of resorcinol in 5% sodium acetate solution for 24 hr.; the filtered product was washed with 0.9% NaCl until the filtrate was free from resorcinol. Preparations required to show the highest degree of specificity of adsorption were then allowed to stand for a further 24 hr. in 0.9% NaCl containing a few milligrams of the protein used and finally washed with 0.9% NaCl and stored in the same solvent.

For brevity, such preparations are referred to below as diazo 'resins', qualified by the word 'immune' if they contain antibody protein.

By means of polystyrylmercuric acetate. Damp polystyrylmercuric acetate, equivalent to about 10 times (dry weight) as much polymer as protein to be treated, was transferred to a metal or plastic container and shaken vigorously for $\frac{1}{2}$ hr. with a glass bead and sufficient 5% sodium acetate solution to give a thin creamy suspension. The γ -globulin or other protein solution, containing 10–40 mg. of protein/ml. in 5% sodium acetate solution, was added and the mixture was stirred gently at $0-5^\circ$ for 48 hr. If all the protein had reacted, as shown by a negative phosphotungstic acid test on the supernatant, the mixture was stirred with successive small quantities (5–10 mg.) of protein until some remained in the supernatant. The precipitate was filtered off. Any unchanged mercury groups were inactivated by stirring for 24 hr. at $0-5^\circ$ with a M -solution of monothioethylene glycol in 5% sodium acetate, 2 ml./g. (dry wt.) of 'resin' being used. At the end of this

treatment the pH should be between 5.5 and 6.5 and the supernatant should contain excess of thiol, as shown by its ability to decolorize 0.01 N-iodine solution; but the 'resin' itself, after thorough washing, should fail to give a blue colour when a sample is treated on a white tile with a drop of diphenylcarbazone reagent (0.1 % in ethanol). The 'resin' was then washed with 0.9 % NaCl, stirred again for 24 hr. with 10 mg. of protein in 0.9 % NaCl, the mixture was filtered, the residue was washed with 0.9 % NaCl and stored at 0–5° in 0.9 % NaCl.

Such preparations are referred to below as mercury 'resins', qualified by the word 'immune' if they contain antibody protein.

Analytical methods

Total nitrogen. This was determined by the Kjeldahl method.

Protein. This was determined from the total nitrogen, estimation by a modified Sakaguchi-Weber (Weber, 1930) technique (Scanes & Tozer, 1956), or quantitative ninhydrin (Cocking & Yemm, 1954) on 6N-HCl hydrolysates (100°; 18 hr.) by using the appropriate protein as standard. The last-mentioned method was usually used by way of confirmation in view of the possibility of producing traces of NH_3 , but the results obtained always agreed well with those from the other two methods.

Mercury. This was determined by Kjeldahl decomposition and colorimetric estimation by (1) diphenylcarbazone (Snell & Snell, 1949) and (2) H_2S in the presence of gum (Snell & Snell, 1949).

Quantitative precipitin test. Antigen was determined by total N determinations on the specific precipitate according to the method of Heidelberger, Sia & Kendall (1930).

Antigen assays. Anti-anthrax protective antigen was assayed both by a neutralization technique against a standard antiserum on agar-diffusion plates by the method of Thorne & Belton (1957) and by a rabbit-skin test devised by Belton & Henderson (1956).

Pasteurella pestis toxin was determined by assay for toxicity in white mice, the LD_{50} being between 15 and 20 μg .

RESULTS AND DISCUSSION

Composition of the 'resins'

The polyaminostyrenes from which the diazo 'resins' were prepared had nitrogen contents between 10.0 and 11.2 % ($[\text{-CH}(\text{C}_6\text{H}_4\cdot\text{NH}_2)\cdot\text{CH}_2\text{-}]_n$ requires N, 11.7 %). The polydiazonium salts derived from the nitration with a mixture of nitric acid and sulphuric acid were capable of combining with 20–30 % by weight of protein. When nitric acid alone was used the capacity for protein was increased to 60–70 % by weight of the final 'resin'. The product, though softer than the normal ion-exchange resin is nevertheless easily handled. When drained on a sintered-glass funnel, the moist 'resins' contain as much as 80–90 % by weight of water. In order to minimize the risk of protein denaturation, these 'resins' were always stored and handled in the moist state, usually suspended in dilute electrolyte.

The introduction of mercury, by the method given, readily yielded products containing 55 % of mercury ($[\text{-CH}(\text{C}_6\text{H}_4\cdot\text{HgOAc})\cdot\text{CH}_2\text{-}]_n$ requires Hg, 55.1 %). The solubility characteristics of the perchlorate formed initially suggest that some degree of cross-linkage occurs during the process. This is probably why the capacity of the modified polystyrene for protein is considerably lower than that of polydiazostyrene. The protein content varied between 12 and 20 %, calculated on the dry weight of 'resin', with a mean of about 15 %. These 'resins' also held considerable amounts of moisture. The moist product, after draining on a sintered-glass filter, usually contained 50–70 % by weight of water. The 'resins' were stored as moist powders.

Adsorption characteristics of 'resins'

From the physical constitution of these 'resins' some degree of non-specific adsorption can be expected, owing partly to physical factors and to non-specific protein-protein interaction. In an attempt to assess the capacity of these 'resins' for specific adsorption, tests were made with proportions of antibody and antigen in the region of the equivalence zone, determined from the precipitin curve (ovalbumin), neutralization tests (*P. pestis* toxin) or by direct absorption tests with diffusion plates (anthrax antigen). Except where a pure antigen is used, it is difficult to assign numerical values to the specific-adsorption efficiency of these resins, owing to the inherent inaccuracies of both bioassay and immunochemical assay of specific protein. Nevertheless it is not difficult to demonstrate a semi-quantitative selectivity of an immune 'resin' for its homologous antigen. It is much more difficult to demonstrate complete specificity (i.e. that the 'resin' takes up the appropriate antigen and that alone, from a mixture of proteins). To obtain a measure of non-specific adsorption we compared the behaviour of the immune 'resins' with that of exactly similar 'resins' prepared with γ -globulin from a normal animal.

To test these 'resins' we chose three systems of increasing complexity, using as antigen (a) ovalbumin (a pure antigen) in buffer solution, (b) the protective antigen against anthrax in a culture filtrate (small quantities of a well-characterized antigen in the presence of much other protein) and (c) a protein fraction from *P. pestis*, containing a toxin active against mice (LD_{50} 15–20 μg .) but also significant though minor amounts of other antigens.

Specificity. A sample of a diazo 'resin', made from anti-ovalbumin serum and containing 120 mg. of γ -globulin (1.5 times the theoretical quantity), when treated with 2 mg. of ovalbumin in 2 % potassium bicarbonate solution, removed 82 % of protein as shown by the quantitative precipitin

test. With double the quantity of 'resin' only traces of protein were left in solution. Under comparable conditions, the same 'resin' removed only 9.2 and 10.0 % of bovine-serum albumin from solution, in duplicate experiments. In another experiment a mercury 'resin', containing 48 mg. of anti-ovalbumin γ -globulin, when treated with a solution containing 0.75 mg. of ovalbumin, removed 93 % of the latter. Double amounts of 'resin' removed all but traces of ovalbumin. This 'resin' also removed only 10 % of serum albumin under comparable conditions.

The protective antigen against anthrax behaves abnormally in the precipitin test, and from neutralization data it is difficult to assess the zone of equivalence. In consequence, the equivalent proportions of antibody to antigen were determined by successive additions of immune 'resin' to a standard volume of culture filtrate until no antigen was detectable in the supernatant by the agar-diffusion-plate technique. An amount of immune diazo 'resin' containing 3.5 mg. of γ -globulin was required to neutralize 1 ml. of culture filtrate, whereas with an immune mercury 'resin' an amount containing 1.5 mg. of γ -globulin was equivalent to 1 ml. of filtrate. This difference suggests that the diazo type of coupling reduces the capacity of antibody for antigen. In approximately these proportions both the diazo and mercury immune 'resins' remove the protective antigen completely from culture filtrate without significant decrease in the total protein content (quantitative Sakaguchi estimation).

Amounts of immune 'resin' containing 8 mg. of γ -globulin would remove 1 mg. of *P. pestis* toxin from solution, a figure in approximate agreement with the neutralization data. But somewhat larger quantities of cellulose pulp, kieselguhr or even powdered azobenzene would do the same. Our earlier work with this toxin was largely vitiated by its adsorption on sintered-glass filters. Even millipore filters have a small but significant capacity for adsorbing the toxin. Evidently non-specific adsorption is of great importance and our efforts were concentrated on decreasing it to the minimum (see below).

In order to assess the magnitude of the effect of non-specific adsorption, the adsorptive capacity of 'resins' containing γ -globulin from normal animals has been compared with that of the immune 'resins' under identical conditions. After adsorption, the 'resin' is washed well with buffer (1 % sodium chloride-1 % phosphate buffer, pH 7.8) and 5 % potassium bicarbonate solution and the total filtrate assayed.

An amount of diazo 'resin', containing 400 mg. of normal γ -globulin (twice the equivalent amount of immune 'resin') removed only 10 % of oval-

bumin from a 0.9 % sodium chloride solution containing 3 mg. of ovalbumin and only a trace from a similar solution in 5 % potassium bicarbonate. In a similar experiment an amount of mercury 'resin' containing 120 mg. of normal bovine-serum γ -globulin removed no significant amounts of ovalbumin from a solution containing 2.5 mg. in potassium bicarbonate solution (total nitrogen and quantitative precipitin test), whereas a mercury 'resin' containing the same quantity of anti-ovalbumin γ -globulin removed 85 % (quantitative precipitin), or 80 % (total nitrogen) from a similar solution.

Similarly, amounts of both 'resins' containing 10 mg. of normal, horse γ -globulin (three times the equivalent for an immune diazo 'resin', six times the equivalent for an immune mercury 'resin') removed no detectable amount of anthrax protective antigen from 1 ml. of culture filtrate, as judged by diffusion-plate assays. Although this assay method might not detect even as much as a 20 % decrease in antigen, the use of considerably greater amounts of normal 'resin' than of immune 'resin' emphasizes that non-specific adsorption is small.

The position was markedly different with *P. pestis* toxin. This was chosen as an antigenically complex material, about which much was known. The antiserum used was active against other components in addition to toxin but reacted weakly with antigens 3 and 4 of Crumpton & Davies (1956). The experiments with immune 'resins' reported above showed also that much protein was adsorbed with the toxin. The amounts were such as to suggest strongly that non-antigenic components were being removed. This was confirmed by experiments with 'resins' containing normal γ -globulin. With amounts of both types of 'resin' equal to the amounts of immune 'resin' required to remove all antigen from solution, non-specific adsorption to the extent of 20-30 % of the toxin occurred regularly, accompanied by at least an equivalent proportion of total protein. Potassium bicarbonate (5 % solution) appeared to be the best suspending and washing medium, and with this solution a non-specific adsorption as low as 15 % was recorded in one experiment.

Attempts have been made to decrease the magnitude of the non-specific adsorption. It is not due solely to protein-protein interactions, since 'resins' containing only 20 % of γ -globulin show a similar effect to those containing 60 %. Methods other than the action of resorcinol were tried for removing unchanged diazonium and mercury groups. These included the action of diethylamine and of cuprous chloride for diazo groups, and methanethiol and thioglycolic acid for the mercury 'resins', but the non-specific adsorption was not

decreased. It seems therefore that this ready adsorbability is a property of the antigenic preparation itself.

Biological activity of adsorbed antigen

Antigen adsorbed on immune 'resins' is firmly held. No systematic attempts have been made to release it, but it is not removed by prolonged washing with 0.9% sodium chloride or dilute buffers, including 5% potassium bicarbonate. It seemed important to attempt to determine whether the antigen had retained its biological activity after adsorption in this fashion. In view of the many data on the immunization levels for anthrax in experimental animals available in this Establishment, the anthrax protective antigen was chosen as the test case. Rabbits and guinea pigs were injected subcutaneously with suspensions of both types of immune 'resins' on which the protective antigen had been adsorbed. In all experiments an amount of antigen equal to that contained in 1 ml. of the culture filtrate (Thorne & Belton, 1957) was administered and the degrees of immunity produced were compared with those produced by the alum preparation of Belton & Strange (1954). This preparation, at 1 ml. of culture filtrate level, gives a high degree of protection in rabbits and a moderate degree of protection in guinea pigs if given in two doses 10 days apart, but a low degree of protection if given in one dose. The same total dose of antigen (half adsorbed on 'resin' and half on alum) was also administered in one dose. The animals were challenged with 200 times the median lethal dose (LD_{50}) of anthrax spores 21 days after inoculation. The results are shown in Table 1.

Table 1. *Comparison of 'resin' preparations containing anthrax antigen with an alum preparation (Belton & Strange, 1954)*

The numbers of animals surviving injection of the preparations shown in the first column and subsequent challenge with 200 lethal doses of anthrax spores are given in columns 3 and 4.

Form of antigen	No. of doses	Animals surviving challenge	
		Rabbits	Guinea pigs
Alum	2	4/4	6/10
Alum	1	1/4	0/10
Diazo 'resin'	1	3/4	4/10
Hg 'resin'	1	3/4	1/4*
Diazo 'resin' + alum	1	3/4	5/10
Hg 'resin' + alum	1	4/4	6/10
Controls			
0.9% NaCl	1	0/4	0/10
Diazo 'resin' without antigen	1	0/4	0/10
Hg 'resin' without antigen	1	0/4	0/4*

* Six guinea pigs died before challenge.

The immune 'resins' without antigen were included as controls to eliminate the remote possibility that the small amount of bound antibody administered was establishing a degree of passive immunity. The mercury preparations proved somewhat toxic for guinea pigs, killing six out of ten when administered with antigen or without it as a control (Table 1). Half the quantity of mercury 'resin' administered with alum produced no deaths. Dr M. C. Lancaster kindly carried out autopsies on the guinea pigs that died before challenge; their condition was typical of heavy-metal intoxication. None of the rabbits showed any such symptoms. All deaths after challenge were confirmed as due to anthrax infection.

A few experiments have also been carried out to determine the antibody response in sheep to anthrax antigen adsorbed on these 'resins'. An amount of antigen equal to that in 5 ml. of culture filtrate (Thorne & Belton, 1957) was administered intramuscularly: (a) adsorbed on an immune mercury 'resin'; (b) adsorbed on an immune diazo 'resin'; (c) as an alum precipitate both in one and in two doses. The antibody levels in the sera after 3 and 6 weeks were determined by their abilities to neutralize a standard amount of anthrax toxin in the rabbit-skin test of Belton & Henderson (1956) and of standard amounts of antigen in the agar-diffusion-plate test of Thorne & Belton (1957). As expected, single doses of the alum preparation produced no antibody response detectable by either method of assay, whereas in two doses satisfactory levels were attained. The mercury 'resin' with adsorbed antigen in a single dose gave levels comparable with the alum preparation in two doses. The diazo 'resin' in one dose gave a positive response in only one animal out of six. This suggests not only that diazo coupling decreases the capacity of the antibody for antigen but also that the latter may be less tenaciously held. We have also found (unpublished observation) that diazo coupling tends to decrease the combining power of antibody for its homologous antigen.

SUMMARY

1. Highly swollen linear chains of polystyrene have been cross-linked by means of antibody protein, by using diazo coupling and coupling via the sulphhydryl group of the protein by means of a mercury atom.

2. Preparations containing 15% to over 60% of protein, by weight, are described.

3. Such products are able selectively to adsorb their appropriate antigen from solution.

4. With two antigens out of the three investigated, namely ovalbumin and anthrax protective antigen, the action is markedly specific, only small

quantities of heterologous protein being adsorbed. With the third antigen, *Pasteurella pestis* toxin, non-specific adsorption is substantial.

5. With the anthrax preparation, the antigen, when adsorbed on the polymer, retains its biological competence as judged by its ability to stimulate antibody production and to produce immunity to challenge in animals after injection.

We wish to thank our colleagues, Mr F. C. Belton, for supplies of anthrax antiserum and anthrax antigen and for assays of the latter, Dr A. L. MacLennan for anti-plague serum and *Pasteurella pestis* toxin and for mouse-toxicity tests, Dr H. Smith and Dr B. T. Tozer for anti-ovalbumin serum and also Sir Harry Melville, F.R.S., Dr H. T. Hookway and Mr D. K. Hale of the Department of Scientific and Industrial Research for helpful discussions.

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Biochem. J. (1960) **77**, 17

The Chemical Nature of the Toxic Compounds Containing Fluorine in the Seeds of *Dichapetalum toxicarium*

BY R. A. PETERS, R. J. HALL AND P. F. V. WARD

A.R.C. Institute of Animal Physiology (Biochemistry Department), Babraham, Cambridge

AND N. SHEPPARD

The University Chemical Laboratory, Cambridge

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A fluoro-octadecenoic acid has been isolated in small amounts from the seeds of *Dichapetalum toxicarium* (Peters & Hall, 1959). It was obtained by fractionation of the oil soluble in acetone at -20° on silane-treated Hyflo Supercel columns with liquid paraffin and aqueous acetone as the stationary and mobile phases (Howard & Martin, 1950; Crombie, Comber & Boatman, 1955). In this Laboratory, the fraction from the columns eluted by aqueous 55% (v/v) acetone was purified by precipitation as the calcium salt. However, analysis showed that, with one exception, all our small samples were contaminated with 5–10% of an impurity. Reversed-phase paper chromatography showed that this fraction contained up to three components and it was clear that the structure of the fluoro-octadecenoic acid could not be

determined finally by this method. The present paper describes an alternative method of preparing gram quantities of the pure fluoro fatty acid, and the determination of its structure as fluoro-oleic acid (ω -fluoro-*cis*- Δ^9 -octadecenoic acid). During the final stages of the purification another fluorine-containing component was found in small amounts. Unlike fluoro-oleic acid this is a solid at room temperature and its chemical structure has not yet been determined.

MATERIALS AND METHODS

Special chemicals. The activated charcoal was from Hopkin and Williams Ltd. and was previously washed with light petroleum (b.p. $40-60^{\circ}$). Linolenic acid, linoleic acid and oleic acid were from Nutritional Chemicals Ltd. and

Bioch. 1960, 77