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The Use of Radioactive Isotopes in Immunological Investigations

7. LABELLED PROTEINS AND PROTEIN ANTIGENS CONTAINING BOTH IODINE AND MUSTARD GAS SULPHONE GROUPS*

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In previous communications we have described the use of artificial protein antigens containing either iodine or mustard gas sulphone (di-2-chloroethyl sulphone; HO_2) in quantitative investigations on the precipitin reaction (Bournsell, Dewey, Francis & Wormal, 1947; Banks, Francis, Mulligan & Wormal, 1951a, b). In continuation of this work we wished to prepare antibodies to protein antigens containing two different introduced determinant groups, in order to study the reaction of these antibodies with antigens containing either or both of these groups.

Haurowitz & Schwerin (1943) have studied the properties of antibodies to iodinated azoproteins, but since, in these antigens, both determinant groups are mainly attached to tyrosine residues, and any one tyrosine residue can contain only one of these substituent groups, they are presumably well separated in the molecule. When proteins are treated with HO_2 , however, the reaction is mainly with the free amino groups, i.e. largely with the lysine residues, and the introduction of both iodine and HO_2 groups into the same protein molecule should thus yield an antigen containing two different determinant groups close together in some parts of the molecule and well separated in other parts, depending on the distribution of the tyrosine and lysine residues.

Natural protein antigens do not normally contain outstanding dominant groups comparable with those in chemically altered or conjugated protein antigens, and their serological specificity is ap-

parently related to characteristic spatial arrangements of groups of amino acids in the surface of the molecule. Thus it appears probable that their serological specificity is determined more by the configuration of a relatively large part of the molecule than by small isolated parts. Artificial antigens having different dominant groups attached to different amino acid residues might, therefore, more closely simulate the behaviour of natural protein antigens than do iodinated azoproteins.

In the experiments described here a study has been made of the conditions under which proteins can be labelled with both iodine and 'sulphone groups'. For some biological investigations it is advantageous to have two or more readily detectable labels attached to different parts of the protein molecule, with minimal chemical change in the protein, and we have, therefore, extended our experiments to include the preparation of protein derivatives containing small numbers only of iodine and sulphone groups, using reagents containing ^{131}I and ^{35}S respectively.

MATERIALS

Ovalbumin. Solutions of thrice-recrystallized hen ovalbumin in 0.9% (w/v) NaCl were used.

Serum globulins. The mixed horse serum globulins were prepared as described previously (Banks *et al.* 1951a).

Rabbit serum γ -globulins. These were prepared by the method of Kekwick (1940).

Iodine solution containing ^{131}I . Except where otherwise stated, this was prepared as described by Francis, Mulligan & Wormal (1951).

Di-2-chloroethyl sulphone containing ^{35}S . This was prepared essentially as described by Bournsell, Francis & Wormal (1946). In some preparations, however, the thio-

* Paper 6 of this series: Banks, Francis, Mulligan & Wormal (1951b).

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diglycol was chlorinated by shaking it in a sealed tube with excess conc. HCl and light petroleum (b.p. 50–60°) for 18 hr. at 60–70°, and the mustard gas was separated by evaporating the light petroleum layer. Oxidation to the sulphone was carried out in one stage by heating the mustard gas for 75 min. at 100° with an excess of 30% hydrogen peroxide and glacial acetic acid (Dr A. H. Ford-Moore, personal communication, 1940).

METHODS

For many experiments it is necessary to use an antigen which is as homogeneous as possible and of known molecular weight. Ovalbumin is a suitable protein antigen of this character, although, according to modern views, it is not homogeneous even after repeated recrystallization. Frequently, however, it is preferable to use proteins which are chemically more closely related to the blood and tissue proteins and which have undergone minimal treatment in their separation. For these reasons, and because they are more potent immunizing and precipitating antigens than is ovalbumin, we have used the mixed serum globulins in many of our experiments.

In the preparation of 'iodinated-sulphone-proteins', the methods used were essentially the same whether the material was required for immunization, in which case no radioactive isotope was introduced, or for use as an isotopically labelled test antigen in a precipitin reaction. Examples of both types of preparation are given below, but in every case where the non-radioactive material was used, a similar preparation of the desired degree of specific radioactivity may be obtained by using ^{35}S or ^{131}I .

Iodination of proteins. Full iodination of proteins was carried out by treating the solution with 0.1 N iodine in KI in the presence of NH_3 , as described by Banks *et al.* (1951*a*). When smaller amounts of iodine were to be introduced into the protein molecule less ammonia was used, in order to minimize any possible damage to protein due to the action of the ammonia.

Preparation of 'sulphone-proteins'. The reaction between HO_2 and proteins is apparently not simple, and the final products obtained from duplicate preparations often differ significantly in composition. It is, therefore, necessary to adhere closely to a standardized technique if repeatable results are required. In the preparations described below a suitable amount of HO_2 and one drop of phenol red solution were added to the protein solution, and the mixture was kept at 37° for several hours, with frequent shaking or stirring; drops of N-NaOH were added as required to maintain the pH at 7.5–8.0. With ovalbumin the mixtures were allowed to stand longer than were the mixtures of other proteins with HO_2 , otherwise the yield tends to be small. With serum globulins, however, appreciable amounts of derivatives insoluble at pH 7.5 are produced if the treatment is prolonged, particularly with the larger amounts of sulphone. The 'sulphone-proteins' were separated by adding 2N acetic acid to produce maximal precipitation, and were purified by dissolving them in water or 0.9% NaCl, with the addition of sufficient N-NaOH to bring to pH 7.5, followed by centrifuging and reprecipitation with acetic acid. This reprecipitation was repeated two or three times.

Preparation of rabbit γ -globulins trace-labelled with both ^{35}S and ^{131}I . Suitable small quantities of HO_2 (containing ^{35}S) were transferred to centrifuge tubes by evaporating (in a current of warm air) an ethanolic solution containing

1 mg. HO_2 /ml. The protein solution (about 3%, w/v) and indicator were then added, and the mixture was kept at 37° for 3 hr., with occasional shaking. Insoluble matter was removed by centrifuging and the reaction product was precipitated by adding solid Na_2SO_4 to give a concentration of 12% (w/v). The precipitate was centrifuged off and redissolved in 0.1 M phosphate buffer (pH 8) and again precipitated by Na_2SO_4 as before. The re-solution and reprecipitation were repeated a further twice. Iodination was then carried out by adding 0.1 ml. of 5N- NH_3 /ml. of protein solution and a saturated aqueous solution of iodine (without iodide) to which had been added carrier-free ^{131}I iodide. After 15 min. the solution was neutralized with N acetic acid and the double-labelled globulins were precipitated with Na_2SO_4 and redissolved and reprecipitated as described above, the final solution being filtered to remove any particulate matter.

Determinations of total nitrogen, iodine and sulphur. Total nitrogen determinations were made by a modified micro-Kjeldahl technique, as described previously (Banks *et al.* 1951*a*); sulphur was determined by the method of Boursnell *et al.* (1946), and iodine by the method of Shahrokh (1943).

Molecular weights and calculations. For the calculation of molecular ratios the values of 40 500 and 15.76% were adopted for the molecular weight and nitrogen content of ovalbumin (Chibnall, Rees & Williams, 1943), and 165 000 and 16% for the mixed horse serum globulins. The sulphone contents of derivatives prepared with non-radioactive HO_2 were calculated from the increase in sulphur contents.

In calculating the relative amounts of HO_2 and protein to be used in each preparation it was assumed that the reaction is confined to the free amino groups of the protein, and that ovalbumin and the serum globulins contain 1% free amino nitrogen.

Radioactivity determinations. Solutions for radioactivity determinations were evaporated, together with 2 or 3 drops of a 10% suspension of kaolin in 50% aqueous ethanol, on weighed nickel planchets (1.4 cm. diam.; The General Electric Co. Ltd., Kingsway, W.C. 2). The planchets were then reweighed in order that the appropriate self-absorption correction factor for the measured weight of the sample plus kaolin could subsequently be applied (for details, see Francis, Mulligan & Wormall, 1954). Radioactivity measurements were made with a bell-type Geiger-Müller counter (Twentieth Century Electronics, type B4 or EW3H, or G.E.C. type EHM2 or EHM2S, with 2 mg./cm.² mica window; or G.E.C. type GM4 with 7 mg./cm.² aluminium window for ^{131}I only) and a Panax E.H.T. and scaler unit type 44 or 100C. Determinations of ^{35}S and ^{131}I in the same sample were made by measuring the count rates with and without the superimposition of an aluminium filter of thickness 32 mg./cm.²; this filter completely absorbs the β -radiation from ^{35}S and reduces the count rate due to ^{131}I by a constant measurable factor.

RESULTS

In these investigations a study has been made of the amounts of iodine and mustard gas sulphone which react with ovalbumin and mixed serum globulins when (a) iodination of the protein precedes treatment with sulphone, and (b) the sulphone groups are introduced before the iodine atoms. Some typical results obtained with ovalbumin are given

in Tables 1 and 2, and with horse serum globulins in Tables 3 and 4.

Table 5 gives the results of experiments in which the γ -globulin fraction of an antiserum was 'trace-labelled' with both ^{35}S and ^{131}I , by sulphone treatment followed by iodination.

DISCUSSION

When proteins are treated with di-2-chloroethyl sulphone at 37° and pH 7-8, the main reaction is the formation of a derived protein with the free amino groups converted into 1:4-thiazan 1:1-dioxide

Table 1. *Iodination of 'sulphone-ovalbumin'*

Samples (10 ml.) of 6.5% ovalbumin solution were treated with varying amounts of HO_2 at 37° for 20 hr. The products, after three precipitations with 2N acetic acid, were made up to 10 ml. with 0.9% NaCl at pH 7.5 and centrifuged. Each solution was then treated with 5 ml. 5N- NH_3 , and fully iodinated with 0.1N- I_2 in KI. The products were precipitated with 2N acetic acid, reprecipitated twice and analysed for N, S and I.

HO_2 used		Composition of product			
Mol./free amino group	Mol./mol. protein	Additional S*		Iodine	
		%	Atoms/mol. protein	%	Atoms/mol. protein
0.5	14.5	†	†	6.24	19.9
2.0	58	1.87	23.7	5.79	18.5
5.0	145	2.96	37.5	4.60	14.7

* Calculated by subtracting the sulphur content of the ovalbumin (1.13%) from that of the product.

† The amount of product obtained in this case was too small to allow S determinations to be made.

Table 2. *Treatment of iodinated ovalbumin with HO_2*

Solutions of thrice-precipitated iodinated ovalbumin (3.9% protein in 0.9% NaCl at pH 7.5) were treated with varying amounts of HO_2 for 3 hr. at 37° and kept overnight at $0-4^\circ$. The products were purified by repeated reprecipitation as described in Table 1.

HO_2 used		Composition of product			
Mol./free amino group	Mol./mol. protein	Additional S*		Iodine	
		%	Atoms/mol. protein	%	Atoms/mol. protein
1.0	29	0.68	8.6	8.9	28.4
2.0	58	1.33	16.8	9.2	29.3
3.0	87	With these amounts of sulphone there was complete denaturation of the protein			
5.0	145				

* See footnote to Table 1.

Table 3. *Iodination of 'sulphone-globulins'*

Samples (10 ml.) of a 7.2% solution of horse serum globulins in 0.9% NaCl were treated with varying amounts of HO_2 for 4 hr. at 37° . The products, after three precipitations with 2N acetic acid, were made up to 10 ml. with 0.9% NaCl at pH 7.5, and centrifuged. Each solution was then treated with 5 ml. 5N- NH_3 and iodinated with 0.1N- I_2 in KI. The products were purified as described in Table 1.

HO_2 used		Composition of product			
Mol./free amino group	Mol./mol. protein	Additional S*		Iodine	
		%	Atoms/mol. protein	%	Atoms/mol. protein
1.0	118	1.42	73 (69)	10.1	131
2.0	236	1.42	73 (110)	10.2	133
3.0	354	2.32	119.5 (131)	8.8	114
5.0	590	Protein denatured by sulphone treatment			

* Calculated by subtracting the sulphur content of the globulins (0.88%) from that of the product.

Note. The figures in parentheses record the corresponding results of the direct determination of sulphone content in a similar experiment in which ^{35}S -containing HO_2 was used. From these results it is evident that the reaction of the protein with sulphone, when 2 mol. of non-radioactive sulphone/free amino group were used, was incomplete in this particular experiment.

Table 4. *Treatment of iodinated horse serum globulins with HO₂*

Solutions of 4 times precipitated iodinated globulin (1.06% protein in 0.9% NaCl at pH 7.5) were treated with varying amounts of ³⁵S-containing HO₂ at 37° for 4 hr. After precipitation and two reprecipitations with 2N acetic acid, the products were analysed for total N, I and ³⁵S.

<i>HO₂</i> used		Composition of product			
Mol./free amino group	Mol./mol. protein	Additional S*		Iodine	
		%	Atoms/mol. protein	%	Atoms/mol. protein
1.0	118	0.455	23.5	12.9	168
2.0	236	0.603	31.1	12.8	166

* Calculated from ³⁵S measurements.

Table 5. *Trace-labelling of proteins with ³⁵S and ¹³¹I*

Protein: separated γ -globulins from rabbit anti-ovalbumin serum. ³⁵S *label*: mustard gas sulphone containing ³⁵S (6.68 mc/m-mole). ¹³¹I *label*: aqueous iodine (0.24 mg./ml.) containing carrier-free ¹³¹I (20.32 mc/m-mole I₂).

Mustard gas sulphone (mol./mol. protein)		Iodine (atoms/mol. protein)		Isotope content (μ C/mg. protein N)	
Added	Combined	Added	Combined	³⁵ S	¹³¹ I
0	0	3.8	1.6	—	0.62
		9.5	4.5	—	1.73
		19.0	8.45	—	3.25
1.31	0.73	3.8	1.5	0.18	0.58
		9.5	4.6	0.18	1.77
		19.0	7.9	0.18	3.04
6.6	4.76	3.8	1.7	1.21	0.66
		9.5	4.45	1.21	1.71
		19.0	8.1	1.21	3.12

groups. The reaction is not stoicheiometric, since some of the sulphone is lost in side reactions, probably forming polymers of divinyl sulphone and some 1:4-oxathian 4:4-dioxide. Thus, with amounts of sulphone less than one molecule/free amino group, 70% or more of the sulphone reacts with the protein, but when larger amounts of sulphone are used, the percentage is less.

In order to obtain the maximal uptake of sulphone groupings, as much as a fivefold excess may have to be used, and the reaction may have to be allowed to continue for many hours. On the other hand, particularly when globulins or fully iodinated ovalbumin are being treated, these conditions frequently lead to extensive denaturation of the protein and loss of solubility at pH 7.5.

When both sulphone groupings and iodine groupings are introduced in significant quantities into the same protein there is always a decrease in the maximal amount of the second grouping which can be introduced (Tables 1-4). Thus treatment of 'sulphone-globulins' or 'sulphone-ovalbumin' with a small excess of iodine results in iodination to only 50-80% of that obtained with the native proteins, the extent of iodination decreasing with increase in the number of sulphone residues present. Sulphone treatment of fully iodinated ovalbumin results in reaction to the extent of only about 70% of that occurring with ovalbumin, but with serum globulins

the corresponding value is only 25-35%. It appears improbable that there are many groups in these protein molecules which are capable of reacting with both iodine and mustard gas sulphone, and this experimental finding is more probably attributable to steric hindrance. This could easily occur if the two groups in the protein molecule (e.g. tyrosine and lysine residues) which are reacting with the two reagents are close together, since the iodine atoms in diiodotyrosine, and the thiazan ring, are both large and fairly strongly polar groups.

We have observed in serological experiments with iodinated sulphone-treated proteins that the sulphone groupings have a greater effect than the iodine groupings in determining the immunological specificity of these antigens. It would seem desirable, therefore, when preparing 'double-labelled' antigenic protein derivatives of this type, to aim at securing the maximal uptake of iodine, if necessary at the expense of a somewhat diminished sulphone-content. This object is most conveniently achieved by fully iodinating the protein before treating it with sulphone. This procedure is particularly to be recommended with ovalbumin, since 'sulphone-ovalbumin', unlike iodinated ovalbumin and the double-labelled ovalbumin, is not well precipitated from solution when dilute acid is added. The iodinated protein should not be treated with

more than twice the theoretical amount of sulphone, since otherwise considerable denaturation may occur. If, however, the preparations are made in the reverse order, i.e. by treatment with sulphone before iodination, it is advisable to use a higher sulphone/protein ratio when preparing derivatives of ovalbumin, but not of globulins.

When 'trace-labelling' a protein with the two isotopes the above considerations do not arise, and it is probably immaterial which of the two groups is introduced first (Table 5). We have, however, invariably introduced the sulphone groups first, since it is during the sulphone treatment that there is the greater risk of damaging the proteins, and this is less likely to occur if these proteins have been submitted to only the minimum of preliminary chemical treatment.

SUMMARY

1. Methods are described for introducing into proteins both 1:4-thiazan 1:1-dioxide groups (by treatment with di-2-chloroethyl sulphone) and 3:5-diiodotyrosine groups.

2. The number of the two groups introduced into a protein is partly dependent on the order in which the iodination and treatment with sulphone are carried out.

3. The results are discussed with reference to the positions in the protein molecule taken up by the entering groups, and the efficacy of the method

for the preparation of double-labelled protein antigens.

4. A method of 'trace-labelling' proteins with both ^{131}I and ^{35}S is described.

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The Uncoupling of Oxidative Phosphorylation in Rat-Liver Mitochondria by Thyroxine, Triiodothyronine and Related Substances

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Martius & Hess (1951, 1952), Hoch & Lipmann (1953) and Maley & Lardy (1953) have shown that thyroxine, under certain conditions, can inhibit oxidative phosphorylation *in vitro*. This effect is taken to be related to the main physiological action of the thyroid hormone, as it can explain the rise in the basal metabolic rate caused by the thyroid hormone. Gross & Pitt-Rivers (1952) have suggested that triiodothyronine might be the active

form of the thyroid hormone, and if this is correct this substance is expected to be at least as effective in inhibiting oxidative phosphorylation as thyroxine. The inhibitory effects of thyroxine and triiodothyronine *in vitro* were therefore compared. The procedure of Krebs, Ruffo, Johnson, Eggleston & Hems (1953), in which the rate of incorporation of radioactive phosphate into adenosine phosphates is measured, was used. Rat-liver mitochondria served as a source of the phosphorylating enzyme system and succinate or β -hydroxybutyrate as substrate.

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