S 87. *Radioactive Tracers in Antisera.*

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Methods hitherto used in studying the action and location of antisera include coupling with dyes or fluorescent materials. Radioactive tracers, however, possess obvious advantages, and it has been found possible to introduce radio-iodine into the globulin fraction of an antiserum without destroying its specific agglutinating properties, if the mean number of iodine atoms per globulin molecule is limited to about 3. With greater amounts of iodine there is a gradual falling off of antiserum agglutinating activity, owing to substitutions in the tyrosine residues, and oxidation of the globulin. The amount of iodine radioactivity carried on bacteria (*Proteus vulgaris*) when agglutinated by the specific antiserum can be used to measure the amount of agglutinin in the serum.

The active fractions of antisera, responsible for specific agglutination and other effects, can be separated by electrophoresis with the gamma-globulin. In mixtures of proteins, such as whole sera, an approximate estimate of the agglutinating activity can be made by the well-known technique of determining the agglutinating effect of the serum over a suitable range of dilutions. A qualitative tracer technique in which the antiserum is coupled with coloured or fluorescent molecules has been used by Marrack (*Nature*, 1934, **133**, 292), Coons *et al.* (*Proc. Soc. Exp. Biol. Med.*, 1941, **47**, 200; *J. Immunol.*, 1942, **45**, 159), and by McClintock and Friedman (*Amer. J. Roentgenol.*, 1945, **54**, 704).

The use of a radioactive tracer would offer obvious advantages in ease of location and measurement, and it has been found possible to introduce radio-iodine into the globulin molecule of an antiserum without seriously impairing the agglutinating power. Measurements of the radioactivity in iodinated *Proteus vulgaris* rabbit antiserum globulin have been used to estimate the mean number, n, of iodine atoms substituted into the globulin molecule (assuming mol. wt. 156,000) and the amount of iodinated globulin deposited on the bacteria when agglutination occurs. The antisera used contained H(flagellar)- or O(somatic)-agglutinins.

Conclusions and Discussion.—(*a*) For values of n up to 10, there is little change in the time necessary for the agglutination of bacterial H-antigen into large floccules. For $n = 30$—100 the time is greatly increased and the floccules become smaller.

(*b*) The percentage of the total radioactivity which can be precipitated by using an excess of bacterial suspension remains approximately constant for values of n up to 3 and then begins to decrease with increasing n. Some typical results are given below, for an H-antigen.

n	1·0	2·3	3·1	7·7
Total activity precipitated, %	8·1	8·1	9·3	5·7

A control with normal rabbit globulin gave only about 1% of precipitable radioactivity, which may have been due to naturally occurring agglutinins in the serum.

The retention of agglutinating properties when n is small is not, in itself, proof that the agglutination is due to iodinated globulin, since, if iodine atoms are substituted at random into the globulin, a considerable proportion of the globulin molecules will escape iodination altogether. However, the removal of radioactivity by the precipitated bacteria indicates a specific reaction also with iodinated globulin. As n increases, the proportion of un-iodinated globulin becomes very small, so that agglutination can only be attributed to iodinated globulin.

The iodine substitutes into the tyrosine residues (Bauer and Strauss, *Biochem. Z.*, 1929, **211**, 163; Kleczkowski, *Brit. J. Exp. Path.*, 1940, **21**, 98).

If iodine atoms are substituted at random into the tyrosine residues, of which there are about 58 per globulin molecule, then if $n \ll 58$ the percentage of globulin molecules containing m iodine atoms will be $100n^m e^{-n}/\underline{m}$. This percentage is tabulated below for certain values of n and m, to illustrate how the amount of un-iodinated globulin decreases as n increases. On iodination of the globulin, the amount of iodine substituted was always about 20%, the remainder being converted into iodide. Since for each iodine atom substituted into a tyrosine residue there will be one atom converted into iodide, it follows that about 60% of the iodine must be reduced in some other reaction, *i.e.*, the number of iodine atoms acting as an oxidising agent is approximately $3n$. It appears a reasonable supposition that the percentage of activity precipitated when n approaches zero represents the total agglutinin in the globulin. It should therefore be possible to adapt the technique for the accurate standardisation of a considerable variety of antisera.

	m.						
n.	0.	1.	2.	3.	4.	5.	6.
1	36·8	36·8	18·4	6·1	1·5	0·3	0·05
2	13·5	27·1	27·1	18·0	9·0	3·6	1·2
3	5·0	14·9	22·4	22·4	16·8	10·1	5·0
8	0·03	0·27	1·1	2·9	5·7	9·2	12·2

(*c*) By estimating the amount of O-agglutinin required just to precipitate a known number of bacteria in an O-antigen, it is found that the quantity required corresponds roughly to the formation of a unimolecular layer of globulin on the bacteria, there being about one globulin molecule to each 9000 A.^{2} of bacterial surface. According to Neurath (*J. Amer. Chem. Soc.*, 1939, **61**, 1841) the rabbit antibody globulin molecule is elongated with minor axes 37 A., major axis 274 A.

Experimental.

Preparation of Antigens.—The bacteria were centrifuged out of a 24-hour *Proteus vulgaris* broth culture, washed several times, and finally suspended in M/15-phosphate buffer of pH 7. The H-antigen was prepared by treating part of the suspension with 1% v./v. of formalin, the O-antigen by heating for a few minutes to 100°. The number of bacteria per ml. was found by counting with a hæmocytometer.

Preparation of Globulins.—64 Ml. of rabbit *Proteus vulgaris* HO (mainly H) antiserum, prepared by the usual technique, were diluted to 400 ml. with M/15-potassium phosphate buffer of pH 7, and 88 g. of sodium sulphate added. The solution was kept at 30—40° for 30 minutes with constant stirring. The precipitated globulin was filtered out (suction), dissolved in 100 ml. of phosphate buffer, reprecipitated with 22 g. of sodium sulphate, filtered out, washed with a 22% solution of sodium sulphate in phosphate buffer, and finally dissolved in 50 ml. of phosphate buffer. An O-antiserum and normal serum were similarly treated.

The globulin contents of the solutions were found by determining total nitrogen by the Kjeldahl method and assuming this to be 16% of the weight of globulin.

Preparation of Radio-iodine.—About 10 microcuries of carrier-free 8-day ^{131}I solution was mixed with 1 mg. of potassium iodide and oxidised with a slight excess of potassium dichromate and sulphuric acid. The dichromate was then reduced with an excess of oxalic acid, and the iodine distilled over into a few ml. of water. The iodine content (about 0·1 mg./ml.) was determined colorimetrically, and the activity in counts per minute per ml. by treating an aliquot portion with very dilute sodium hydroxide solution and evaporating it to dryness on a standard sample holder for counting. The weight of all samples counted was small, obviating any correction for self-absorption of the β-particles.

Iodination of Globulins : Example.—5 Ml. of an HO-globulin solution containing 4·6 mg. of globulin per ml. were mixed with 7·5 ml. of a solution containing 0·104 mg./ml. of radio-iodine. The iodine colour disappeared in a few minutes. After 15 minutes the solution was dialysed through a collodion bag into phosphate buffer until, after 24 hours, radioactivity measurements indicated that 99·9% of the dialysable iodine had been removed. An aliquot portion of the iodinated globulin solution was evaporated to dryness for counting to determine the total iodine combined, which was 0·146 mg., or 18·8% of the amount added, equivalent to an average of 7·7 atoms per globulin molecule.

Agglutination tests were all carried out at 56°, with the usual technique.

(*a*) 2 Ml. of M/15-potassium phosphate buffer, pH 7, +1 ml. of H-antigen, +1 ml. iodinated HO-antiserum globulin containing 0·22 mg. of globulin.

Approximate *n.*	Time for agglutination, mins.	Appearance.	Approximate *n.*	Time for agglutination, mins.	Appearance.
1	5	Coarse floccules	30	30	Fine floccules
3	5	,, ,,	100	120	,, ,,
10	5	,, ,,			

(*b*) 1·8 Mg. of iodinated HO-antiserum globulin, or iodinated normal globulin as a control, was mixed with 1 ml. of normal rabbit serum (in order greatly to increase the amount of globulins present, and thus prevent non-specific adsorption on the bacteria, which otherwise occurs) and 50 ml. of H-*Proteus* antigen. After 2 hours for agglutination the bacteria were filtered out on a paper disc the size of a standard sample counting dish, washed with phosphate buffer, dried, and the radioactivity measured. The weight of dried bacteria was so small that no correction was made for self-absorption of the β-particles.

The control, which had not agglutinated, was agglutinated by the addition of non-iodinated antiserum, and similarly treated. The filtrates were found incapable of causing further agglutination.

(*c*) A solution of iodinated O-antiserum globulin containing 14·4 mg./ml. was found by the above method to contain 4·0% of agglutinins. Agglutination tests were carried out by adding a *Proteus* O-antigen containing $6·9 \times 10^8$ bacteria per ml., filtering after 24 hours, and testing the filtrate with further antigen to see if any agglutinin was still present.

O-Antiserum globulin, ml.	0·10	0·05	0·02	0·01	0·005	0·002
Phosphate buffer pH 7, ml.	5	5	5	5	5	5
Proteus antigen, ml.	1	1	1	1	1	1
Agglutination, 24 hours	+	+	+	+	—	—
Agglutination by filtrate, with further 1 ml. antigen	+	+	+	—	—	—

These results indicate that approximately 0·01 ml. of globulin solution is necessary just to agglutinate 1 ml. of *Proteus* antigen. A surface area of $3\mu^2$ being assumed for each *Proteus* bacterium, the total surface is $6{\cdot}9 \times 10^8 \times 3 \times 10^8$ A.^{2} $= 2{\cdot}1 \times 10^{17}$ A.^{2}.

The number of antigenic globulin molecules is $14{\cdot}4 \times 0{\cdot}01 \times 0{\cdot}04 \times 6{\cdot}06 \times 10^{23}/1000 \times 156{,}000 = 2{\cdot}2 \times 10^{13}$. Consequently, the area occupied by each globulin molecule is about 9000 A.^{2}.

Results recently published by Pressman and Keighley (*J. Immunol.*, 1948, **59**, 141) confirm the conclusions reached on the retention of agglutinating activity after iodination.

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