Formation of Specific Antibodies and γ-Globulin *in vitro*. A Study of the Synthetic Ability of Various Tissues from Rabbits Immunized by Different Methods

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Although there is abundant evidence that specific antibodies are formed in lymphoid tissues (e.g. spleen and lymph nodes) of immunized animals, relatively little is known about the extent to which different tissues contribute to overall synthesis of specific antibody or of other γ-globulin in the whole animal.

[^{14}C]Glycine has been shown to be incorporated into antibody by slices of spleen and liver taken from animals immunized intravenously (Ranney & London, 1951; Keston & Dreyfus, 1951), and by granulomatous tissue developed at the site of administration of antigen in an adjuvant mixture (Askonas & Humphrey, 1955). When the relative rates of incorporation of labelled amino acid into antibody by tissue slices taken from different organs of the guinea pig were compared with the populations of antibody-containing cells in those organs, Askonas & White (1956) found a generally good correlation between the two measurements. There is therefore some justification for considering that the ability of tissues to incorporate amino acids into specific antibody or other γ-globulin *in vitro*, under suitable experimental conditions, is a measure of the relative ability of the tissues to synthesize these proteins. Because of its simplicity we have used this method to study antibody synthesis by different tissues in rabbits immunized with one or more antigens and by different routes. Our results show that the pattern of antibody synthesis varies widely under different conditions, but that it is always accompanied by increased synthesis of other γ-globulin. The reasons for this and the mechanism of formation of these proteins by the tissue slices are discussed.

METHODS

Immunization of animals

Rabbits (albino or sandylop strains) were bred at the National Institute for Medical Research and were fed on pelleted Diet no. 18 (Bruce & Parkes, 1940). They weighed 2·0–2·8 kg. at the time of use.

Intravenous immunization. Pneumococci type 3 killed with formalin (Kauffmann, Björneboe & Vammen, 1938) were injected intravenously as a suspension containing 4×10^9 organisms/ml. Increasing amounts (0·1–1 ml.) were given thrice weekly for 3–4 weeks. In some rabbits the courses of injection were repeated after a rest period of 2 months.

Ovalbumin [four times recrystallized, and precipitated with alum (Porter, 1955)] was administered according to a schedule similar to that used for pneumococci. The amounts injected on each occasion were increased gradually from 0·5 to 5 mg.

Immunization with Freund's adjuvant. Ovalbumin was emulsified in a modification of Freund's adjuvant mixture (Freund & McDermott, 1942) by using Unguentum Alcohol Lanis B.P. and Light Liquid Paraffin B.P. The final concentration of ovalbumin was 10 mg./ml. and of heat-killed tubercle bacilli 1 mg./ml. Two injections of 1 ml. were given either intramuscularly or intraperitoneally at intervals of 1 week. The precipitating antibody content of the serum rose gradually for 6–8 weeks after the last injection, and then remained fairly constant for several weeks. Large cystic granulomatous masses (weighing 30–100 g.) developed at the injection sites.

A few animals received one or two intravenous injections of alum-precipitated ovalbumin 4 weeks after intramuscular administration of ovalbumin in adjuvant.

Serum-antibody levels were measured by quantitative precipitation in the presence of slight excess of antigen. After standing overnight at 2° the precipitate was washed three times at 0° and dissolved in 0·1 N-NaOH, and the amount of protein was estimated by measuring the absorption at 280 mμ.

Tissue-slice technique

^{14}C-*Labelled amino acids.* Biosynthetic [^{14}C]glycine and L-[^{14}C]valine were obtained from the Radiochemical Centre, Amersham, Bucks, with specific activities of 56 and 53 μc/mg. respectively.

Preparation of tissue slices. The immunized rabbits were bled from the ear exhaustively and then killed by injecting 120 mg. of pentobarbital intravenously. The organs to be tested were removed immediately, rinsed in cold 0·9% NaCl solution and chilled on ice. After each tissue had been weighed, appropriate amounts were chopped finely (to about 0·3 mm.) with an automatic chopper (McIlwain & Buddle, 1953) and with small scissors by hand. Portions of chopped tissue (0·3–1 g.) were weighed and incubated as described below.

Incubation of tissues. The tissues were incubated in Hanks's medium (Hanks, 1948), containing 0·3 g. of glucose, a complete amino acid mixture (5 mg. of N), prepared from acid-hydrolysed rabbit plasma protein with

added tryptophan, and 5000 units of penicillin in 100 ml. Each portion of tissue was suspended in 0·5 ml. of the animal's own serum and 4·5 ml. of medium containing 2–3 μc of [^{14}C]glycine or L-[^{14}C]valine in silicone-treated flasks. The flasks were gently shaken at 37°, and gassed continuously with $O_2 + CO_2$ (95:5, v/v) mixture. At the end of the incubation Merthiolate (Eli Lilly and Co.) was added to a concentration of 10^{-4} M, together with several milligrams of inactive amino acid to dilute ^{14}C-labelled amino acid in the medium and so to diminish adsorption of the label on protein precipitates later. In order to break up cells the incubation mixture was frozen in an ethanol–solid CO_2 mixture at −70° and then allowed to thaw. Carrier antibody was provided for each flask by addition of antiserum or purified γ-globulin containing antibody (sufficient to yield a total of 5–10 mg. of antigen–antibody precipitate). The contents of each flask were homogenized for 1 min. in a Potter-type homogenizer, and the total volumes adjusted to 10–12 ml. with M/15 phosphate buffer, pH 7·2. Sediment was removed by centrifuging for 20 min. at 18 000 *g* at 3°, and the supernatant was freed of fat by centrifuging for 20 min. at room temperature at 1500 *g*; the clear extract was used for isolation of antibody and other γ-globulin. In certain cases the sediment was treated as described below under 'Preparation of tissue residue'.

Isolation of specific antibody and other γ-globulin

Precipitable specific antibody was isolated by addition of a slight excess of antigen (one-tenth of the weight of antibody for ovalbumin and one-twentieth for pneumococcal capsular polysaccharide), followed by incubation of the tubes for 30 min. at 37° and 18 hr. at 1°. The specific precipitates were centrifuged, washed three times with 0·9% NaCl solution containing inactive amino acids, then with 50% aqueous ethanol, with hot ethanol and finally with ether. They were dried at room temperature.

The remaining γ-globulin was precipitated with 18% (w/v) Na_2SO_4; after being redissolved in a smaller volume it was reprecipitated with 16% and finally with 12% (w/v) Na_2SO_4, according to the method described for human γ-globulin by Kekwick (1940). The product was dialysed against distilled water, dried from the frozen state, and redissolved in 0·9% NaCl solution. Insoluble material was removed by centrifuging and to the supernatant was added 10 vol. of ethanol at 0°. The precipitated protein was washed with 50% ethanol, ethanol and ether and dried. The freeze-dried material was analysed electrophoretically in veronal buffer, pH 8·6, *I* 0·1. It corresponded to the components of rabbit serum moving slowest during electrophoresis at pH 8·6, and was not evidently contaminated with any other globulins or with albumin.

Preparation of tissue residue

The insoluble material, which was sedimented after homogenization, was washed with 0·9% NaCl solution, and protein was then precipitated by addition of trichloroacetic acid to a concentration of 5% (w/v). The precipitate was washed twice with boiling trichloroacetic acid solution, followed by hot and cold ethanol, and finally dried with ether.

Determination of radioactivity

Dried protein was plated at infinite thickness on 0·3 or 1 cm.^{2} disks, and the radioactivity determined by counting on an automatic recording Geiger counter with thin end-window, to an accuracy of at least ±5%. When necessary, counts were corrected to infinite thickness.

Calculation of relative capacity of tissues to form antibody

The specific radioactivity of the isolated antibody was found to be proportional to the amount of tissue incubated (as was to be expected, since the weight of newly formed radioactive antibody was negligible compared with the weight of inactive carrier-antibody added). Hence the relative synthetic abilities of the tissues could be compared by calculating the radioactivity of the isolated antibody/unit wt. (0·1 g.) of tissue incubated. As a standard of comparison for all the experiments, spleen was chosen arbitrarily to represent 100% activity, and the percentage activity of other tissues was determined relative to it.

In one experiment tissue slices were incubated separately with [^{14}C]glycine and L-[^{14}C]valine, and the relative synthetic activities of the tissues were compared, on the basis of their abilities to incorporate either of these amino acids into antibody. Although the absolute levels of radioactivity were not the same for each amino acid, the relative synthetic abilities were the same within experimental error.

RESULTS

Time-course of incorporation of [^{14}C]*amino acids into protein*

Fig. 1 shows the course of incorporation of [^{14}C]-amino acid into specific antibody, other γ-globulin and tissue-debris fractions when slices of lymph glands from an animal immunized against ovalbumin were incubated for varying periods of time in the presence of ^{14}C-labelled amino acids. It may be seen that the specific radioactivities of the antibody and γ-globulin follow an S-shaped curve. During a preliminary period of 10–15 min. no radioactivity appears, whereas after 30 min. radioactivity increases steeply for 2 hr., after which the rate of increase falls off. Incorporation of radioactivity into insoluble tissue protein, however, follows a straight line throughout the period of the experiment. This suggests that the slices lose the capacity to form antibody and γ-globulin earlier than the capacity to incorporate [^{14}C]amino acids into insoluble particles and structural components. The length of time during which incorporation of [^{14}C]amino acids into antibody protein continued varied with different tissues; chopped granuloma tissue (from the site of injection of the adjuvant mixture) continued to incorporate [^{14}C]glycine into antibody for up to 6 hr. and spleen slices for more than 4 hr. (see Fig. 2). In experiments designed to compare the antibody-forming capacity of different tissues an incubation time of 3 hr. was therefore chosen.

It must be emphasized that the absolute values for specific radioactivities depend greatly upon the

amount of carrier protein added. Since there was added two to three times more 'normal' γ-globulin than antiovalbumin, the absolute rates of incorporation into these two proteins in Fig. 1 were in fact quite similar.

The tissue protein not extracted with saline represents a complex mixture of structural proteins and proteins associated with subcellular particles. No carrier protein was added to this fraction, hence the specific antibody and γ-globulin secreted by the cell had a much higher specific radioactivity than the tissue protein.

Effects of cell damage on incorporation

Rapid freezing and thawing of the tissues before slicing reduced the uptake of radioactivity into antibody on subsequent incubation by 90–95 %.

On several occasions the effect was examined of homogenizing spleen, bone marrow and lymph glands for 15–30 sec. in isotonic salt media, or in 0·25 or 0·29 M-sucrose, or in the sucrose medium containing inorganic salts described by Zamecnik & Keller (1954). After nuclei and whole cells had been centrifuged at 1500 *g* for 10 min. the supernatant containing mitochondria and microsomes was incubated aerobically, or anaerobically in the medium of Zamecnik & Keller (1954) with 3 μc of added [^{14}C]glycine or L-[^{14}C] valine. In no case was incorporation of ^{14}C into specific antibody or other γ-globulin observed.

Even slight damage to cells appeared greatly to diminish uptake of radioactivity into antibody. For example, when a suspension of cells was obtained by teasing out cells from a lymph gland by means of a needle, the cells looked somewhat damaged under the microscope; upon subsequent incubation under standard conditions very little ^{14}C was incorporated into antiovalbumin or into other γ-globulin, whereas considerable uptake took place into insoluble tissue protein (Table 1). This was in contrast with experiments with sliced tissues, and suggests either that antibody-producing cells were not teased out from the gland or, more probably, that under our conditions whole undamaged cells are necessary for formation of extractable antibodies and other γ-globulin.

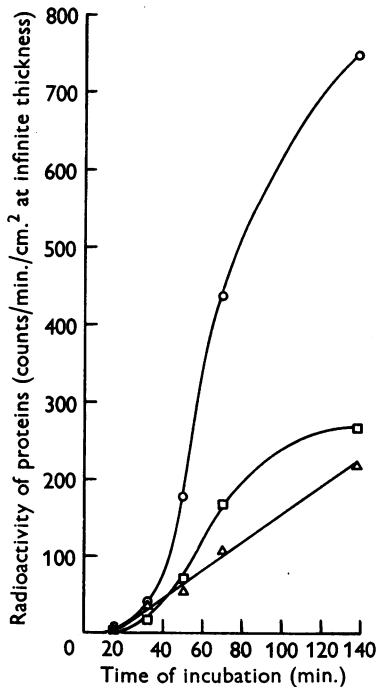


Fig. 1. Time-course of incorporation of [^{14}C]glycine into antiovalbumin, γ-globulin and insoluble tissue protein by sliced lymph glands. Portions (0·35 g.) of sliced lymph glands (from a guinea pig immunized with ovalbumin in adjuvant) were incubated in the usual medium (see Methods) containing 2·5 μc of [^{14}C]glycine. ○, Antiovalbumin; □, other γ-globulin fraction; △, tissue protein not extractable by isotonic saline–phosphate.

Specificity of incorporation of [^{14}C]*amino acids into antibody*

To test the extent of contamination of the antigen–antibody precipitates obtained by the technique described, antibody against the capsular polysaccharide of pneumococci type 3 was added to tissue slices from animals immunized against ovalbumin; after incubation for 3 hr. in the usual way, each antibody was isolated by precipitation with the appropriate antigen. The pneumococcal antibody was precipitated first, and had less than one-tenth of the radioactivity of the antiovalbumin.

Table 1. *Effect of damage to cells on incorporation of* [^{14}C]*amino acids into antibody and γ-globulin*

Lymph glands (pooled) from a rabbit immunized subcutaneously with ovalbumin in adjuvant. Incubations were carried out in the presence of 2·5 μc of [^{14}C]glycine in the usual medium except where otherwise indicated.

	Radioactivity, counts/min./cm.² (at infinite thickness)		
Lymph gland	Anti-ovalbumin	γ-Globulin	Residual tissue protein
Slices (0·35 g.)	762	320	220
Damaged cells*	8	25	435
Homogenate†	0	2	53

* Cells were teased from lymph glands (1 g.) by means of a needle.

† Homogenate was prepared from lymph glands (1 g.) and incubated in 0·29 M-sucrose–salt medium (Zamecnik & Keller, 1954). Nuclei and whole cells were removed by centrifuging at 500 *g* for 10 min. at 1°.

This finding is similar to that of Keston & Dreyfus (1951) and Ranney & London (1951), and even the small amount of radioactivity found in the pneumococcal antibody was presumably due to coprecipitation of some labelled γ-globulin. No exchange occurred of amino acids in the medium with protein added to the incubation mixture. When ^{14}C-labelled antibody to pneumococci polysaccharide was added to granuloma slices from an animal immunized against ovalbumin, and reisolated after incubating for 1 min., and 6 hr. in the absence of added [^{14}C]amino acids, the radioactivities of the antibody protein were 1870 and 1880 counts/min./cm.^{2} respectively.

Secretion of antibody into incubation medium by slices

In the experiments discussed above, antibody isolated at the end of an experiment included antibody secreted into the medium as well as intracellular antibody. In order to study secretion and total formation of labelled antibody separately, slices of spleen were incubated with [^{14}C]glycine and after various time intervals the cellular components were sedimented by centrifuging for 15 min. at 15 000 *g*. Equal amounts of a γ-globulin concentrate containing antiovalbumin were added to the supernatant and to the tissue sediment (washed once with the medium). Antibody was isolated directly from the supernatant in the usual way, and from the sediment after further addition of 3–4 ml. of buffer solution, followed by freezing and thawing and homogenization to break up the cells. The radioactivities of the extracellular and intracellular antibody, as isolated, are shown in Fig. 2. Since in each case the antibody formed by the tissue slices was diluted with a very much larger amount (6 mg.) of added antibody, the observed radioactivities are not the actual radioactivities of the antibody formed but give an indication of the amounts of [^{14}C]glycine incorporated. After 30 min. radioactivity was present in the intracellular antibody, but no radioactive protein had been secreted into the medium. After 3 hr. the radioactivity of the intracellular antibody increased only slightly, whereas that of the secreted antibody continued to rise at a constant rate for 4 hr. These findings are compatible with the idea that the total pool of intracellular antibody remained constant, while the specific radioactivity gradually rose by formation of new molecules with the same specific activity as that of the intracellular free amino acids. The radioactivity of the extracellular antibody represents the accumulation of all the antibody secreted, with gradually increasing specific activity. Between the third and the fourth hours, when the radioactivity of intracellular antibody remained fairly constant, the accumulation of radioactivity in the secreted antibody was equal to about half the radioactivity of the antibody in the cells. If the specific activity of secreted and intracellular antibody were equal during this period (which would be approximately true on the assumptions stated above) this would mean that an amount of antibody equal to the intracellular pool would be secreted in about 2 hr. This value is in good agreement with the time of about 2 hr. deduced for secretion rates *in vivo* by Humphrey & Sulitzeanu (1957).

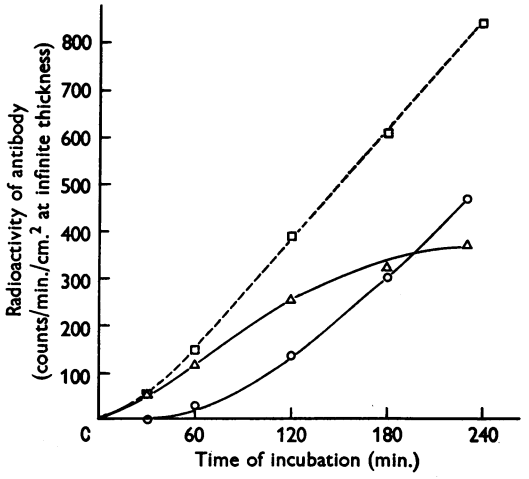


Fig. 2. Incorporation of [^{14}C]glycine into intracellular and secreted antiovalbumin by spleen slices. ○, Secreted antibody isolated from medium; △, intracellular antibody extracted from spleen slices after homogenization; □, combined intracellular and extracellular antibody. Slices of spleen (0·5 g.) from a rabbit immunized intravenously were incubated in the usual medium containing 3 μc of [^{14}C]glycine. Antiovalbumin carrier (6 mg.) was added before isolation of each antibody fraction.

The general pattern of secretion of radioactive antibody agrees with the results obtained by Miller, Bly, Watson & Bale (1951), who studied incorporation of [^{14}C]amino acids into albumin by isolated perfused rat liver, and with our own findings on antibody formation by perfused rabbit lung (B. A. Askonas & J. H. Humphrey, unpublished work). This suggests that, for a while at least, slices behave similarly to whole organs during perfusion.

Relative ability of various tissues to form antibody and other γ-globulin after immunization by different methods

Tissue slices were incubated with ^{14}C-labelled glycine or valine for 3 hr. periods and the antibody and other γ-globulin then isolated. Within each experiment the results on the various tissues are comparable, but because the amount of

intracellular plus carrier antibody varied from animal to animal the results of one experiment cannot be directly compared with those of another. The relative synthetic abilities of tissues/unit weight were compared and the contributions by the organs tested to the overall synthesis of specific antibody and other γ-globulin in the whole animal could be assessed. Whereas the weights of spleen, lung, liver, kidney and individual lymph glands can be determined easily, those of the total bone marrow and total lymph glands cannot. For these last two tissues we have therefore assumed weights of 30 g. of bone marrow and 3 g. of lymph glands. These values are based on the estimates that total bone marrow is equal to 1·5–1·7% of the body weight (Nye, 1931–32; Dietz, 1944) and that total lymphoid tissue is equivalent to about 0·15% of body weight (Yoffey & Courtice, 1956); they are lower than the estimates quoted to allow for the probability that not all the bone marrow and lymphoid tissue was as active as the femoral marrow and the enlarged lymph glands examined.

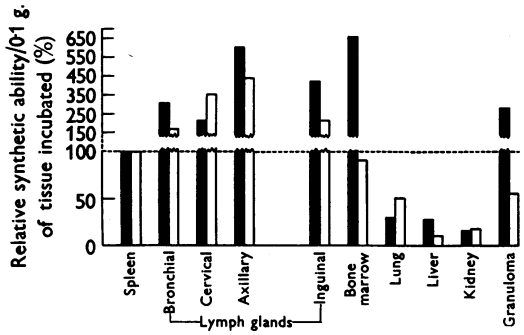
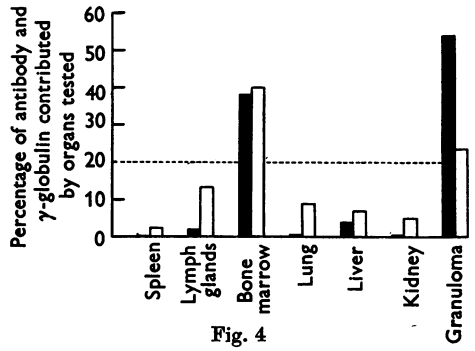


Fig. 3



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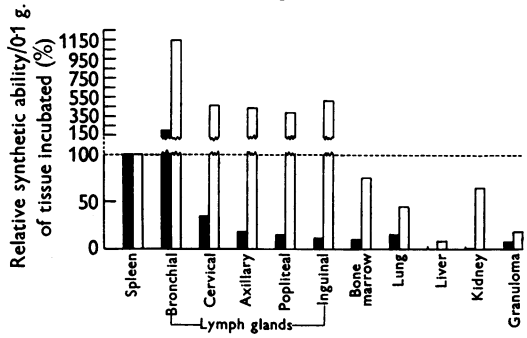

Fig. 5

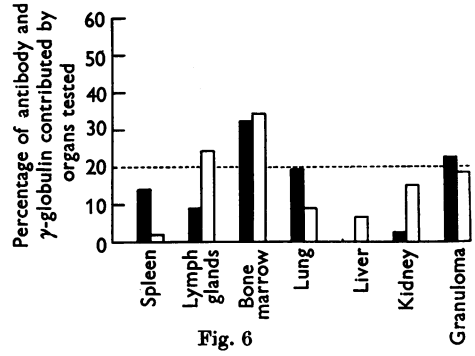

Fig. 6

Fig. 3. Relative synthetic ability of various tissues/unit weight. Black columns, antiovalbumin; white columns, γ-globulin fraction not precipitable with antigen. The rabbit was immunized intramuscularly with ovalbumin in Freund's adjuvant $2\frac{1}{2}$ months previously. Serum-antibody content was 5 mg./ml. Slices of all tissues were incubated in the usual medium, containing 2 μc of [^{14}C]glycine, for 3 hr. at 37°. Radioactivity of the isolated proteins after incubation of 0·1 g. of sliced spleen was chosen arbitrarily to represent 100% activity.

Fig. 4. Proportion of antibody and γ-globulin formed by each organ tested. Black columns, antiovalbumin; white columns, other γ-globulin fraction. Rabbits were immunized intramuscularly with ovalbumin in Freund's adjuvant. The relative contribution made by each organ was calculated by considering the total weight of the organ in the rabbit and the relative synthetic abilities of the tissues (cf. Fig. 3).

Fig. 5. Relative synthetic ability of various tissues/unit weight. Ovalbumin was administered in Freund's adjuvant, and later intravenously. Black columns, antiovalbumin; white columns, other γ-globulin fraction. The rabbit was injected twice with alum-precipitated ovalbumin intravenously 4 weeks after intramuscular administration of ovalbumin in Freund's adjuvant. The antiovalbumin content of the serum was 5·6 mg./ml. Conditions of incubation and expression of results were as in Fig. 3.

Fig. 6. Percentage of antibody and γ-globulin contributed by organs tested. Ovalbumin was administered in Freund's adjuvant and intravenously. Black columns, antiovalbumin; white columns, other γ-globulin fraction. Relative synthetic abilities of the tissues/unit weight for the same animal are presented in Fig. 5.

It must be recognized that these are approximations, but any errors are unlikely to affect the overall picture very greatly. The results below are typical of those found in repeated experiments.

Ovalbumin in Freund's adjuvant. Rabbits which had been injected either intramuscularly or intraperitoneally 2–3 months previously with ovalbumin in adjuvant were examined. Before the granulomatous tissue at the injection site was tested, it was freed from the thick viscous fluid containing necrotic cells which accumulates within it. The results of a typical experiment are shown in Figs. 3 and 4. In this rabbit, which had 5 mg. of specifically precipitable antibody/ml. of serum, the antibody-forming activity/unit weight of tissue in the spleen was low, but nevertheless was three to six times that of lung, liver or kidney. Granuloma tissue, however, was three times, lymph glands two to six times and bone marrow six times as active as spleen. The production of other γ-globulin followed a similar pattern to that of specific antibody, with the exceptions that granuloma and bone marrow were less active/unit weight than spleen. On the basis of their total weight in the whole animal, the granuloma tissue contributed a major part of the specific antibody, and bone marrow most of the other γ-globulin.

Ovalbumin in Freund's adjuvant plus intravenous alum-precipitated ovalbumin. It was observed that if animals previously injected intramuscularly with ovalbumin in adjuvant were given a short intravenous course of alum-precipitated ovalbumin, the antibody content of the serum rose rapidly to a level some five times as high. Since this suggested that the pattern of production by different tissues might have changed, this possibility was investigated. A rabbit which had received ovalbumin in adjuvant intramuscularly 4 and 5 weeks previously was given two intravenous injections of 1·5 mg. of alum-precipitated ovalbumin intravenously.

The serum content of precipitable antibody rose from 1·1 mg./ml. before the intravenous injections to 5·6 mg./ml. 1 week later. The findings in this animal are presented in Figs. 5 and 6. It will be seen that, in contrast with the previous experiment, the spleen, per unit weight, was now very much more active in forming antibody than any tissue examined except the bronchial lymph gland. As regards overall antibody production, granuloma tissue was now relatively less important, and both spleen and lung contribute significant amounts, in addition to bone marrow. The change in the pattern of overall production of other γ-globulin was much less.

Intravenous immunization with a single antigen. Figs. 7 and 8 illustrate the results obtained with tissues from a rabbit which had received nine intravenous injections of alum-precipitated ovalbumin (total 8 mg.) during the course of 3 weeks. The serum taken 2 days after the last injection contained 1·2 mg. of precipitable antibody/ml. In this animal the bone marrow formed most of the specific antibody and other γ-globulin. The apparent contribution of the liver may be spurious, since the specific radioactivities of the antibody isolated from liver slices was so low as to be of doubtful significance.

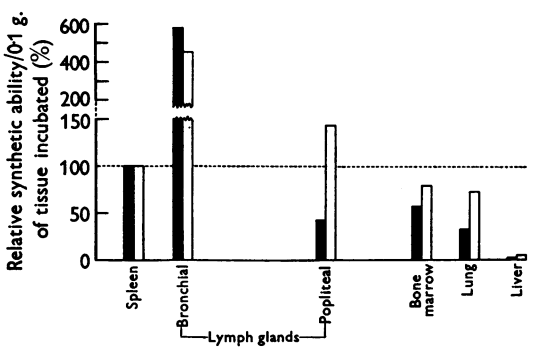

Fig. 7

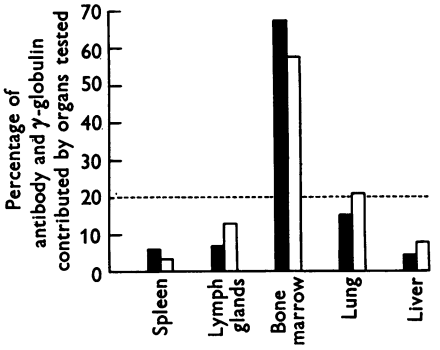

Fig. 8

Fig. 7. Relative synthetic ability/unit weight of various tissues from an intravenously immunized rabbit. Black columns, antiovalbumin; white columns, other γ-globulin fraction. Nine intravenous injections of alum-precipitated ovalbumin were given over a period of 3 weeks; the serum contained 1·2 mg. of antiovalbumin/ml. Conditions of incubation and expression of results were as in Fig. 3, except that 3 μc of L-[^{14}C]valine was used for the incubation.

Fig. 8. Proportion of antibody and γ-globulin formed by organs tested, after intravenous immunization of a rabbit. Black columns, antiovalbumin; white columns, other γ-globulin fraction. For conditions of immunization with alum-precipitated ovalbumin and relative synthetic ability of the tissues/unit weight, see Fig. 7.

Intravenous immunization with two antigens. In order to compare the production of antibody by different tissues against two different antigens in the same animal, a rabbit was injected intravenously on alternate days with formalin-killed pneumococci type 3 or alum-precipitated ovalbumin. Four days after a 3 weeks' course of such injections the serum (1 ml.) contained 2 mg. of antibody to ovalbumin and 18 mg. of antibody to capsular polysaccharide. Tissue slices were incubated in the usual way, except that the added carrier contained known amounts of antibody to capsular polysaccharide, antiovalbumin and other γ-globulin. The two antibodies were isolated successively from the same incubation mixtures. The results of this experiment are shown in Figs. 9

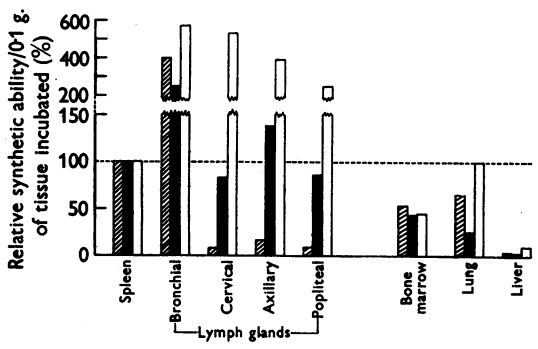

Fig. 9

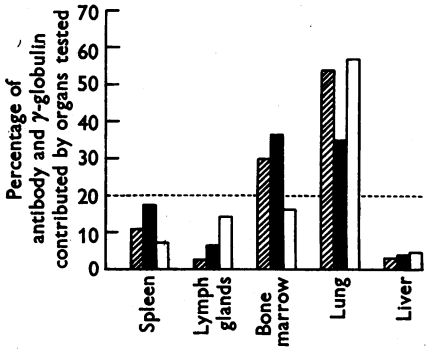

Fig. 10

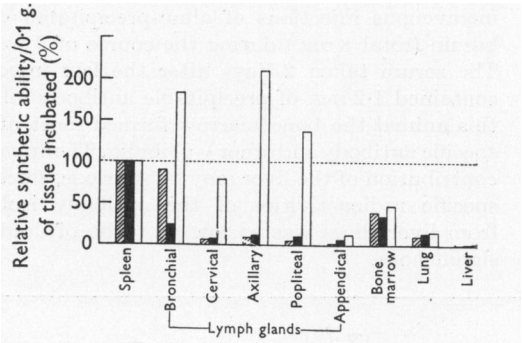

Fig. 11

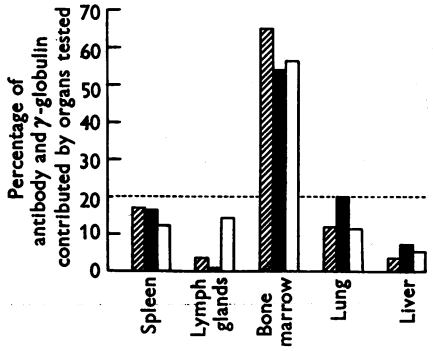

Fig. 12

Fig. 9. Relative synthetic ability of various tissues in a rabbit immunized intravenously with two antigens. Striped columns, antibody against capsular polysaccharide; black columns, antiovalbumin; white columns, other γ-globulin fraction. The rabbit received intravenous injections of formolized type 3 pneumococci and alum-precipitated ovalbumin on alternate days for 3–4 weeks. Antibody levels: 2 mg. of antiovalbumin and 18 mg. of antibody to capsular polysaccharide/ml. of serum. Conditions of incubation and expression of results were as in Fig. 3. L-[^{14}C]Valine (2 μc) was used for the incubation.

Fig. 10. Proportion of antibody and γ-globulin formed by different organs tested (intravenous alum-precipitated ovalbumin and type 3 pneumococci). Striped columns, antibody against capsular polysaccharide; black columns, antiovalbumin; white columns, other γ-globulin fraction. For conditions of immunization of the rabbit and relative synthetic ability of the tissues/unit weight see Fig. 9.

Fig. 11. Relative synthetic ability of various tissues in a rabbit immunized intravenously with two antigens. Striped columns, antibody against capsular polysaccharide; black columns, antiovalbumin; white columns, other γ-globulin fraction. The rabbit received intravenous injections of formalin-killed type 3 pneumococci and alum-precipitated ovalbumin as in Fig. 9, after a course of injections of pneumococci 9 months previously. Antibody levels: 4·3 mg. of antiovalbumin and 7·6 mg. of antibody to capsular polysaccharide/ml. of serum. Conditions of incubation and expression of results were as in Fig. 3.

Fig. 12. Proportions of antibody and γ-globulin formed by different organs tested after intravenous immunization with alum-precipitated ovalbumin and type 3 pneumococci. Striped columns, antibody against capsular polysaccharide; black columns, antiovalbumin; white columns, other γ-globulin fraction. See Fig. 11 for conditions of immunization of the rabbit and relative synthetic ability of the tissues/unit weight.

and 10. The most interesting feature, as regards the relative synthetic activities/unit weight of tissue, is the marked variation between the different lymph glands examined, which must presumably reflect some differences in access of the two antigens to these glands. A striking aspect of Fig. 10 is the very large contribution made by the lung to the overall synthesis of antibody to capsular polysaccharide and of γ-globulin, both being more than half of the total. In this rabbit both the lung and spleen were hypertrophied to three to four times their normal weights, a finding which is not uncommon when rabbits are immunized intravenously with pneumococci, and which has been observed by Humphrey & Sulitzeanu (1958) in their study of rates of antibody synthesis *in vivo*.

In animals in which such hypertrophy did not occur the picture after intravenous immunization was somewhat different. Figs. 11 and 12 illustrate an experiment carried out with tissues from a rabbit which had received one course of injections of pneumococci 9 months before a course of alternating injections of pneumococci and alum-precipitated ovalbumin as described above. The antibody content of the serum at death was 7·6 mg. of antibody to capsular polysaccharide/ml. and 4·3 mg. of antiovalbumin/ml. In this animal the contribution of the lung to antibody and γ-globulin synthesis was relatively small. It is also noticeable that the relative synthetic abilities of the lymph glands examined were much more alike.

DISCUSSION

Significance of incorporation of [^{14}C]*amino acids into proteins in systems* in vitro

We have assumed that incorporation by tissue slices of ^{14}C-labelled amino acids into specifically isolated proteins represents a true synthesis of these proteins. There has been much discussion (see, for example, Tarver, 1954) of whether such an assumption is justified, or whether incorporation does not rather represent exchange of amino acids of the proteins without true synthesis. Our control experiments showed that tissues from rabbits immunized against ovalbumin, but not against pneumococci, incorporate [^{14}C]glycine into antiovalbumin, but not into pneumococcal antibody added to the reaction mixture. Thus exchange between extracellular protein and the amino acids of the medium can be ruled out. Radioactivity in the antibody was not due to non-specific protein co-precipitated with the antigen–antibody precipitate, since no radioactivity was found in antibody isolated after incubation of liver slices; yet liver slices are known to be active in protein synthesis. Furthermore, breaking up of the cells, or even slight damage, prevented incorporation into specific antibody and other γ-globulin, even when incorporation into insoluble tissue protein still occurred. For these reasons we consider that the incorporation of ^{14}C into specific soluble proteins which we observed was in fact a measure of true synthesis.

Since the slices were incubated for only 3 hr. no attempt was made to demonstrate net formation of antibody, such as was shown by Stavitsky (1955), and by Steiner & Anker (1956), using conditions under which lymph cells and spleen cells remained active for 1–3 days. However, it was possible to calculate the amount of antibody which might be formed, as the following example shows.

After incubation of spleen slices with 2·8 μc of [^{14}C]glycine for 3 hr., 5 mg. of antiovalbumin (including carrier antibody) was isolated, having a radioactivity equivalent to 7500 counts/min./cm.^{2} (at infinite thickness) for 1 g. of incubated tissue.

Since our counter gave 1000 counts/min. for a 1 cm.^{2} standard containing 10^{-3} μc/mg. (at infinite thickness), the isolated antibody had an activity of $7{\cdot}5 \times 10^{-3}$ μc/mg., i.e. 5 mg. of antibody contained $37{\cdot}5 \times 10^{-3}$ μc. The medium contained 2·8 μc of [^{14}C]glycine and a total of 120 μg. of glycine. Thus 1·3 % of the glycine (or 1·6 μg.) was incorporated into the antibody. Since rabbit antibody contains 5 % of glycine (Smith, McFadden, Stockell & Buettner-Janusch, 1955), about 32 μg. of antibody would have been newly formed by 1 g. of spleen. This is probably an underestimate, since it ignores dilution of added isotope by intracellular amino acids. Antibody produced by cells cultured for 2 days in medium containing [^{14}C]glycine was found by Steiner & Anker (1956) to have about half the isotope concentration, calculated on the assumption that all the glycine residues of the antibody were derived from the added labelled glycine. But even if the intracellular glycine from which the new antibody was formed had a lower specific activity than the glycine in the medium, only microgram quantities would have been newly formed by 0·5 g. of spleen, and so small an increase would be difficult to detect under our conditions of isolating the antibody by precipitation with antigen.

Mechanical damage to the cells stopped incorporation of [^{14}C]amino acids into specific antibody or other γ-globulin, while considerable uptake into the insoluble tissue protein persisted. When the cells were homogenized in various media with a Potter–Elvehjem-type homogenizer with a polytetrafluoroethylene pestle, no incorporation into these soluble proteins could be demonstrated, and only a much smaller incorporation

into the insoluble proteins. Whole cells therefore seem to be necessary for formation of specific soluble proteins, or at least for their release from subcellular particles. Other workers studying subcellular fractions of tissues have found similar results. Littlefield, Keller, Gross & Zamecnik (1955) found that during the first 20–30 min. the insoluble protein of liver microsomes became labelled *in vitro*, but that there was very little activity in the soluble proteins. Stephenson, Thimann & Zamecnik (1956) likewise found that the microsomal fraction of tobacco leaves infected with tobacco-mosaic virus incorporated radioactive amino acids into insoluble proteins but not into those of the virus. In contrast with our findings, Ogata, Ogata, Mochizuki & Nishigama (1956) report that cell-free homogenates of popliteal lymph glands (obtained by grinding with glass powder) incorporated [^{14}C]glycine into antibody to a greater extent and for a longer time than into tissue protein or nucleoprotein fractions. These results are at variance also with the findings of other workers who have studied synthesis of soluble proteins in broken-cell systems.

The exact significance of the shape of the curves obtained when incorporation of [^{14}C]amino acids into antibody is plotted against time (Figs. 1, 2) is difficult to assess. Since the antibody was isolated by addition of a very large excess of carrier the ordinates represent total incorporation. There is undoubtedly a lag period before the appearance of radioactive antibody and γ-globulin, lasting about 10–15 min. (Figs. 1, 2). How much of this is due to the time required for the cells to recover after being chilled and handled, and how much to preliminary formation of cell-bound compounds as postulated by Peters (1953), it is impossible to state.

Variation in sites of synthesis with different methods of immunization

In untreated rabbits, slices of various tissues (spleen, lymph glands, bone marrow) have been shown to incorporate ^{14}C-labelled amino acids into the γ-globulin fractions normally found in the serum, whereas slices of liver and kidney showed no such activity (Askonas, Humphrey & Porter, 1956). The same tissues that form the γ-globulin normally found in serum may be stimulated to form specific antibodies after administration of antigen, and in general we have found that stimulation of production of specific antibody was accompanied by an equal or even greater production of γ-globulin not precipitable with antigen. The question arises, how much of the other γ-globulin is in fact unprecipitated antibody against the specific antigen or impurities. In unpublished experiments we found that animals immunized as described formed negligible amounts of precipitable antibody against pneumococcal antigens other than the capsular polysaccharide, or against possible contaminants of ovalbumin such as conalbumin or ovomucoid. Using the technique of Farr (1956) we failed to detect significant non-precipitating antibodies against thrice recrystallized ovalbumin. There are no grounds therefore for stating that a substantial part of the other γ-globulin was specific antibody against the injected antigen, and its nature remains uncertain.

The major sites of antibody formation varied with the route of injection of the antigen. However, most active synthesis took place in the tissues which may be expected to take up the largest amount of antigen after injection by different pathways, provided that these tissues are capable of undergoing the type of cellular proliferation required for antibody formation. The liver is an exception: antigen accumulates in the Kupffer cells, but induces barely any antibody formation. A possible explanation is that antigen uptake by Kupffer cells is so efficient as to preclude its uptake by other types of cells, e.g. the primitive reticulum cells, described by Marshall & White (1950), which may be a necessary preliminary to antibody synthesis.

When ovalbumin was administered in Freund's adjuvant intramuscularly, the spleen and lung made only traces of antibody (Figs. 3, 4). After two intravenous injections of antigen, the spleen had a higher synthetic ability than the other tissues tested except for the bronchial lymph node (Figs. 5, 6). The relative contribution of the spleen and lung increased from a trace to 15 and 20% respectively. The responses shown by the spleen, lung and bronchial lymph node suggest that these tissues contained cells which had been 'conditioned' by trace amounts of antigen released from the adjuvant site but that proliferation of plasma-type cells and the production of large amounts of antibody by these tissues could take place only when more antigen was taken up by these tissues themselves (i.e. after intravenous injection).

Even when two particulate antigens, one in the form of killed bacteria and one adsorbed on to particles, are injected by the same route, intravenously, it is clear that their subsequent distribution must become different, since quite large variations were observed in the synthetic abilities of tissues for the two antibodies (Fig. 9). The lung is the main capillary filter bed in the body, and it is not surprising that the draining lymph nodes regularly showed such high activity. We are unable to generalize, however, about what conditions will cause the enormous proliferation of

antibody-forming cells in the lung itself, which was frequently, but not always, observed in animals given prolonged intravenous injections of pneumococci.

SUMMARY

1. Incorporation of [^{14}C]amino acids into specific antibody and other γ-globulin *in vitro* by tissue slices, from immunized animals, occurred at a constant rate for a few hours after a short lag period provided that the cells were undamaged. After mild damage no incorporation into secreted proteins occurred, although insoluble tissue proteins still became labelled. Under our conditions, procedures which broke up the cells completely prevented incorporation of added [^{14}C]amino acids into specific protein. Evidence is given that incorporation of the ^{14}C-label into antibody is due to synthesis and secretion of antibody by the tissue slices.

2. By comparing the rates of incorporation of [^{14}C]glycine or valine *in vitro* by different tissues taken from rabbits immunized by different routes, it was possible to assess the relative contributions of these tissues to overall synthesis of specific antibody and other γ-globulin in the whole animal.

3. Rabbits immunized by intramuscular injection of ovalbumin in Freund's adjuvant mixture synthesized most of their antibody in the local granuloma, but, after further intravenous injection of alum-precipitated antigen, the spleen, lung and bone marrow became important sites of synthesis. When ovalbumin or pneumococci, or both, were injected intravenously the bone marrow, or in some animals the lungs, made most of the antibody. In rabbits immunized simultaneously with two different antigens, tissues varied considerably in their ability to synthesize each kind of antibody.

4. Tissues stimulated to produce specific antibody also produced increased amounts of other γ-globulin which was not identified as specific antibody.

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