ON THE SYNTHESIS OF ANTIBODY PROTEIN*

by

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It is generally agreed that amino acid composition[1], end groups[2], molecular weight[3], electrophoretic mobility[4], metabolic fate[5] and antigenic properties[6] of rabbit antibodies and rabbit γ globulins are identical**. The formation of antibodies could therefore occur by one or a combination of the following processes: a. a change of the specific surface configuration and folding of the γ globulin molecules, b. a partial breakdown of γ globulin and resynthesis of the parts into antibodies, c. a *de novo* synthesis of antibodies from amino acids. The work reported here supports the last possibility. It is also shown that incorporation of labelled amino acids into antibody protein begins immediately after administration of the antigen and precedes the appearance of precipitable antibody in the circulation by several days.

METHODS

Syntheses. Carboxyl labelled ^{14}C-glycine with a specific activity of 0.1 mC/mM was prepared as previously described[7].

Mixtures of amino acids were obtained by growing the fresh water algae, *Scenedesmus* D-3***, in the presence of D_2O, $^{15}NH_4Cl$ or $^{14}CO_2$. The algae were extracted with alcohol-ether (1:1) and with hot aqueous 5% trichloroacetic acid. The residual protein was then hydrolyzed in 6 N HCl for 3 days, the digest concentrated to dryness, taken up in water, filtered, and neutralized with NaOH. This highly coloured solution of amino acids was found to be non-toxic to rabbits when given orally or intravenously. The ^{14}C mixture contained a quantity of amino acids equivalent to 0.22 mM of amino nitrogen and 0.02 mC of ^{14}C per ml; the ^{15}N mixture was equivalent to 1.4 mM of amino nitrogen per ml and the nitrogen contained about 35 atoms per cent excess ^{15}N; the deuterio amino acids were present at a concentration corresponding to 0.22 mM of amino nitrogen per ml and had about 60 atoms per cent excess ^{2}H.

Analytical procedures. In some experiments, 0.5 g of benzoic acid was added daily to the diet and hippuric acid isolated from the urine. The isotope concentration of the glycine portion of this compound was regarded as being equal to that of the glycine of the so-called "metabolic pool" of the animal[8].

In experiment A, glycine was isolated by chromatographic separation on Dowex 50 after acid hydrolysis[9].

In experiments A and B, ^{14}C compounds were burned, the $^{14}CO_2$ converted to barium carbonate and this counted in a gas flow counter. In experiments C to F the proteins were prepared for direct counting by the following procedures. The protein was precipitated with 5% trichloroacetic acid, washed twice with acetone and thoroughly dried over P_2O_5 at 100° in vacuo for 24 hours. The fine powder thus obtained was suspended in ether in a counting cup. The ether was allowed to evaporate leaving an even layer of material. Finely powdered hippuric acid was plated from ether suspension and counted. Deuterium and ^{15}N concentration were determined in the mass spectrometer.

In experiments A to D, the ^{14}C concentrations of the glycine portions of the various materials are expressed as counts/min for an infinitely thick sample of barium carbonate of 3.5 cm^2 area. This permits a direct comparison of the ^{14}C concentration of the glycine portion of the different

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** The following discussion applies to rabbit antibodies. Antibodies of other species fall into different classes with respect to their chemical properties.

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compounds. In experiment A, the glycine was isolated and burned. In experiments B to D the measured counts were multiplied by 1.6 for the proteins counted directly, and by 0.7 for the hippuric acid counted directly. The conversion factors were determined by comparing the direct counts for one of the samples with the counts obtained after isolating and burning the glycine. In experiments E and F the counts/min for an infinitely thick sample of the whole protein of 3.5 cm^2 area are given.

Since the antigen-antibody precipitate formed at the equivalence ratio contains 90 % antibody and 10 % ovalbumin[10], the isotope concentration of the antibody was obtained by multiplying the isotope concentration of the total precipitate by 1.1.

Immunization. Recrystallized ovalbumin (Armour) was used as antigen in all experiments. Previously immunized rabbits were used after their antibody titer had either disappeared or declined to a very low level. With such animals, one or two injections of antigen in an emulsion of mineral oil stabilized with Tween 80, produced sufficient antibody to permit its isolation and isotopic analysis. The immune responses studied were therefore secondary respones.

Isolation of proteins. Antibody titer was estimated by determination of the equivalence ratio[11]. After addition of the optimal proportion of antigen the precipitate was allowed to stand for several hours and then washed three times with cold 0.9 % NaCl[12]. Contamination of the precipitate with serum protein was negligible since if precipitation of unlabelled antibody was carried out in the presence of added labelled serum proteins, less than 4 % of the precipitate consisted of the added isotopic plasma proteins.

After removal of antibody, the remaining serum proteins were precipitated with ammonium sulfate at 33 and 50 % saturation to give respectively a γ globulin and a remaining globulin fraction, with the albumin remaining in the supernatant[13]. In experiments A and B it was found that the non-antibody γ globulin fraction had the same ^{14}C concentration as the remaining globulins, so that in experiments C and D serum proteins were fractionated only into albumins and globulins by precipitation of the latter with 50 % saturated ammonium sulfate. In experiments E and F the plasma proteins were not separated.

EXPERIMENTAL

Experiment A. 250 mg of carboxyl labelled glycine was given intraperitoneally to a rabbit and 5 mg of ovalbumin were injected intravenously after 36, 48 and 60 hours. The isotope concentrations of the serum albumin, globulin, the excreted hippuric acid

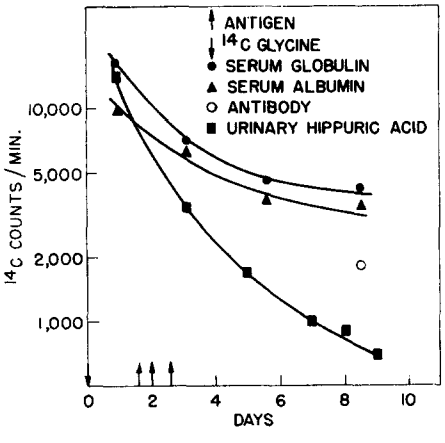

Fig. 1. Expt. A: The arrows indicate the injection times. The ordinate gives the ^{14}C concentration of the glycine portion of the compounds as cts/min of an infinitely thick sample of $BaCO_3$ of 3.5 cm^2 area.

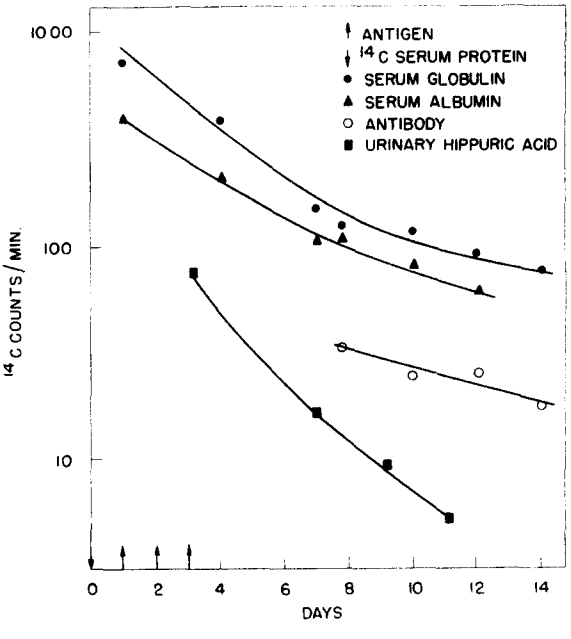

Fig. 2. Expt. B: Symbols as in Fig. 1.

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and anti-ovalbumin antibody isolated on the 8th day after the injection of the glycine are given in Fig. 1.

Experiment B. Labelled plasma proteins were obtained from a donor rabbit 40 hours after the administration of 250 mg of labelled glycine. Twenty ml of the donor's plasma were then injected intravenously into the experimental animal and 5 mg of antigen each administered 1, 2 and 3 days later. The results obtained are shown in Fig. 2.

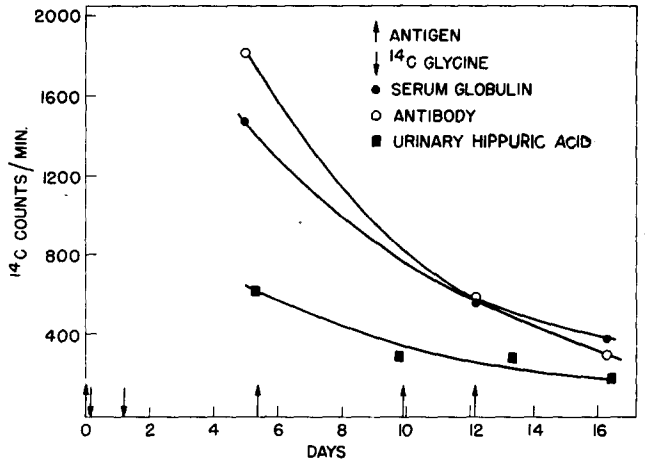

Fig. 3. Expt. C: Symbols as in Fig. 1.

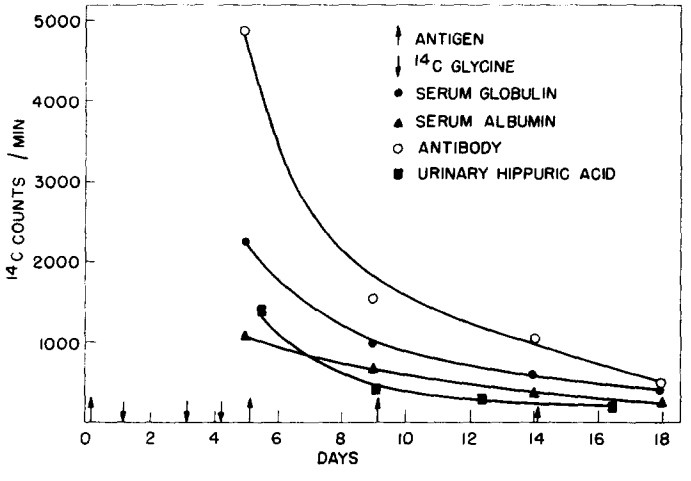

Fig. 4. Expt. D: Symbols as in Fig. 1.

Experiments C and D. 10 mg of ovalbumin were administered at the beginning of the experiments. After 2 and 26 hours in experiment C and after 24, 72 and 100 hours in experiment D, 25 mg each of ^{14}C labelled glycine were given. Periodically, blood samples were removed and 20 mg of ovalbumin again injected (Figs. 3 and 4).

Experiment E. 23, 7 and 23 ml of the mixtures of amino acids labelled with ^{14}C, ^{15}N and ^{2}H, respectively, were injected 6, 40 and 90 hours respectively after the adminis-

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tration of 3 mg of ovalbumin. Samples of plasma protein and antibody were analyzed for the various isotopes (Fig. 5).

Experiment F. After injection of 10 mg of ovalbumin, 0.5 ml of the mixture of amino acids labelled only with ^{15}N was injected every 12 hours throughout the remainder of the experiment. Beginning on the third day, 1 ml of the mixture of labelled amino acid containing only the ^{14}C label was administered in addition to the ^{15}N amino acids. Samples of plasma protein were analyzed at intervals for the two isotopes as shown in Fig. 6. Between the 5th and 6th day an antibody sample was also analyzed.

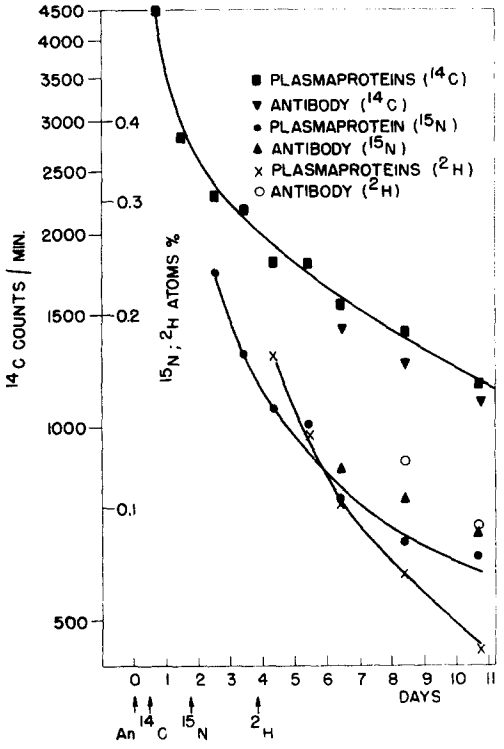

Fig. 5. Expt. E: The ordinate gives the ^{14}C concentration of the whole proteins as cts/min of an infinitely thick sample of 3.5 cm^2 area; the ^{15}N and ^{2}H concentrations are given as atoms per cent excess for the whole protein.

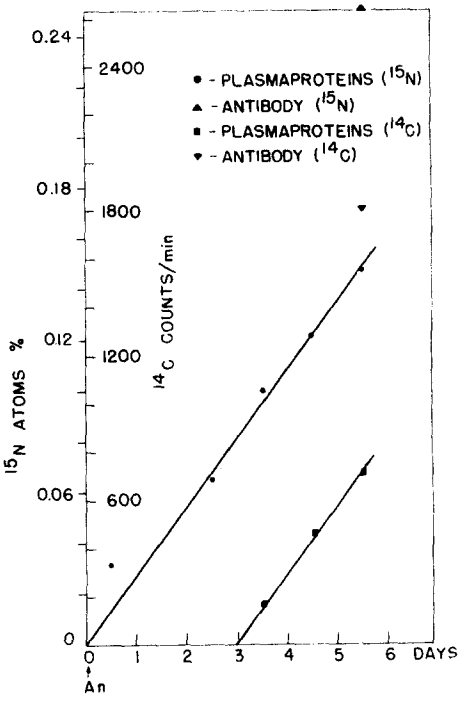

Fig. 6. Expt. F: Ordinate as in Fig. 5.

RESULTS

In experiments A and B (Fig. 1 and 2) where labelled material was administered before the antigen injection, the isotope concentration of the resulting antibody was lower than that of any of the serum protein fractions at any time during the period following antigen administration. However, the isotope concentration of the antibody glycine was the same as the hippuric acid glycine at a time about 4 days earlier.

In experiments C and D (Fig. 3 and 4) the procedure was modified in that antigen was administered before the labelled material. In both experiments the first sample of antibody was found to have a higher isotope concentration than that of the corresponding

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serum globulin sample. In experiment D (Fig. 4) the first blood samples were taken about 24 hours after the last of the glycine injections, so that the isotope concnetration of the serum globulin must have been near the maximum at this time. Although the hippuric acid glycine was not analyzed prior to the time the first blood sample was taken, the isotope concentration of the non-protein glycine must have been very high shortly after glycine administration[8].

After the first blood samples were taken, in experiments C and D, 3 injections of large amounts of antigen were given at intervals of a few days in order to remove part of the circulating antibody. The antibody recoverable a few days after each antigen injection was therefore a mixture of newly-formed antibody and that which had not been removed by the antigen. By this procedure the labelled antibody was replaced by unlabelled antibody faster than seems possible by turnover alone as observed in experiments B and E (Figs. 2 and 5). In experiment C (Fig. 3) it was found that the last antibody sample had a lower isotope concentration than the serum globulin of the same day and approached that of the urinary hippuric acid.

The results of experiments A to D inclusive therefore indicate that the isotope concentration of the antibody glycine depends on the isotope concentration of the non-protein glycine rather than on the isotope concentration of the glycine in the serum proteins.

Assuming that incorporation of labelled amino acids denotes synthesis of antibody protein, experiments E and F were designed to determine approximately the rates at which antibody protein is made from amino acids at different times after antigen injection and before appearance of circulating antibody.

In experiment E (Fig. 5) three different labelled amino acid mixtures (^{14}C, ^{15}N and ^{2}H) were administered separately at increasing time intervals after antigen injection. The concentration of each isotope in the final circulating antibody is proportional to 3 factors: the length of time during which the labelled amino acids were present in the pool, the concentration of the isotope in the pool, and the quantity of antibody protein synthesized in the presence of each isotope compared to the total amount of antibody protein made, *i.e., the relative rate of synthesis.*

In order to estimate the relative rate of synthesis it is therefore necessary to recalculate the data in such a way as to eliminate the effect of the first two factors. The major portion of isotope incorporation into antibody as well as into plasma proteins probably occurs within 18 hours after injection of the labelled amino acids, since at this time the isotope concentration of the metabolic pools becomes negligible[14]. This result presumably applies to all three isotopes in an identical manner. In the steady state plasma proteins are synthesized at a constant rate and the isotope concentration of the plasma proteins is proportional to the isotope concentration of the amino acid pool. The ratio of the isotope concentration of the antibody to the isotope concentration of the plasma protein therefore depends only on the relative rate of antibody protein synthesis*.

The results obtained with the three isotopic markers can be directly compared, provided that the three isotopes are treated similarly by the animal. The data for the

* It has been shown that in rats intracellular proteins of the liver and other organs turn over at about the same rate as plasma proteins[15]. If similar rates also apply to rabbit antibody protein prior to its appearance in the blood, then the ratio of the isotope concentrations of antibody protein to plasma protein will be independent of the turnover rates.

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plasma proteins were therefore replotted in Fig. 7 so that the scales for the isotope concentrations are comparable and the time scale is based on the time of injection of the respective isotopic amino acids. It can be seen that, for the purposes of this discussion, the rates of decay do not differ appreciably for the three isotopes. Once the antibody is present in the bloodstream, the three isotopic markers are lost from it at the same rates as from the plasma proteins (Fig. 5).

The values for the antibody/plasma-protein ratios are 0.9 for ^{14}C, 1.15 for ^{15}N and 1.54 for ^{2}H, the labelled amino acids having been administered at 10, 41 and 89 hours, respectively, after antigen injection. Using these values the amounts of antibody protein synthesized at the different times were determined by graphic integration*. It was estimated that during the 6½ days from antigen injection to maximum titer, 10, 13 and 17% of the total antibody protein were synthesized on the 1st, 2nd and 4th day respectively. These results are given in Fig. 8.

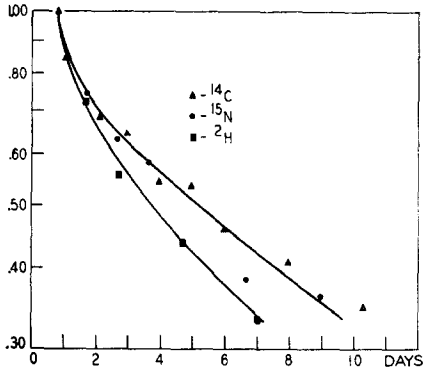

Fig. 7. Expt. E: Relative isotope concentrations of the plasma proteins. The value obtained 24 hours after injection of the corresponding amino acid mixture is taken as 1.

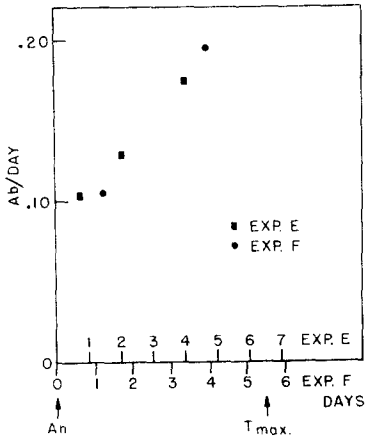

Fig. 8. Expts. E and F: Fraction of total antibody protein synthesized per day.

In experiment F (Fig. 6) the labelled amino acids were injected every 12 hours so as to maintain the isotope concentration of the pool approximately constant**. Therefore the isotope concentration, i, of the serum proteins will rise linearly at first and then approach asymptotically the isotope concentration of the amino acid pool. The net rate of incorporation of isotope into the serum protein, di/dt will be proportional to $(I - i)$, where I is the isotope concentration of the amino acid pool. Although I

* Plotting the ratios against the time of isotope administration a straight line is obtained which can be extrapolated to the time of maximum titer. The product of each isotope ratio and the time the label is present in the pool yields the amount of antibody protein synthesized in the presence of a particular isotope. The area under this line from zero time to maximum titer gives the total quantity of antibody produced.

** If labelled amino acids are given to an animal, the isotope concentration in the amino acid pool will rise sharply and then fall to a few per cent of the peak value within 12 hours[14]. In experiment F the amount of labelled amino acids remaining in the metabolic pool at the time of a subsequent injection is relatively small and it it therefore assumed that the average daily isotope concentration of the pool will not rise appreciably during the time of the experiment.

The intermittent rather than a continuous administration of isotopic amino acids is not considered to introduce an appreciable error in the interpretation of the results.

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cannot be measured directly it may be assumed that it is about equal to the antibody ^{15}N concentration, if during the period of ^{15}N administration antibody protein was synthesized only from the amino acids of the metabolic pool as indicated by experiments A to D. From the integral form $i = I\ (1 - e^{-kt})$ of the first order rate equation k can be calculated and is constant within the experimental error having a mean value of 0.14 ± 0.02. Since k is the fraction of protein synthesized per day, the half-life of the protein is about 5 days. This value is in agreement with the half-life calculated from rate of decline in experiment B and the assumption that the antibody ^{15}N concentration is equal to I is therefore supported.

The rate of incorporation of ^{14}C into the serum proteins in experiment F should be identical to that of ^{15}N. This assumption is confirmed by experiment E (Fig. 7) in which the decay rates of the serum protein for the two isotopes were identical. Since the ^{14}C concentration of the amino acid pool could also not be measured directly, advantage was taken of the identity of the rates. A scale for the ^{14}C values of the plasma proteins was therefore adopted in Fig. 6 so that they paralleled the corresponding ^{15}N values. The ratio of the two scales then gives the ratio of the isotope concentrations of the amino acid pools and the ^{14}C concentration of the pool can be calculated as 2640 counts. Since the ^{14}C labelled amino acids were not present throughout the entire period of antibody synthesis, the ^{14}C concentration of the antibody is less than that of the amino acid pool. The ratio of the ^{14}C concentration of the antibody to that of the amino acid pool represents the fraction of antibody protein synthesized during the period of administration of ^{14}C. Since the ^{14}C concentration of the antibody was 1810 counts this fraction is calculated to be 69.% Therefore, during the first 60 hours after antigen injection 31% of the antibody protein was synthesized, while during the following 72 hours, the remaining 69% was produced*. It should be noted that antibody first became detectable in the blood after the 3rd day, and that maximum titer was reached on the 5th day. The average quantities of antibody protein synthesized per day in the two time periods are also given in Fig. 8. They agree reasonably well with results of experiment E.

DISCUSSION

The results of experiments A to D are strong evidence for the conclusion that no major component of each of the plasma protein fractions (*i.e.*, albumin; non-antibody γ-globulin and remaining globulin) is a direct intermediate in the incorporation of glycine into antibody protein[16]. On the other hand, the isotope concentration of the glycine in the metabolic pool after antigen injection seems to determine the isotope concentration of the antibody glycine. The calculations based on the data of experiment F also show that most of the antibody protein is formed by *de novo* synthesis from amino acids after antigen administration. It must be pointed out that our results do not preclude the participation of a minor fraction which is normally present in the plasma proteins as an intermediate in antibody synthesis. This is considered to be

* If intracellular turnover of antibody protein before its release into the circulation occurs, the ^{15}N concentration of the antibody would be unaffected. However, ^{14}C free antibody synthesized during the first period would incorporate ^{14}C due to turnover in the second period, and the ^{14}C content of the antibody would be greater than would correspond to the quantity of antibody protein synthesized in the second period. The calculated amount of antibody synthesized during the first period represents therefore a minimum value.

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unlikely, however, since the rate of turnover of this protein fraction would have to be several times that of the major plasma protein components whose turnover rates are among the fastest known[15].

Experiments E and F show that the incorporation of amino acids into antibody protein begins shortly after antigen injection and that the rate of incorporation increases until the antibody appears in the bloodstream.

Presumably, once maximum titer is reached, the rate of antibody synthesis falls so that it just replaces the degradative loss of circulating antibody[5]. It should be noted that several days elapse before the amino acids which are removed from the metabolic pool shortly after antigen administration appear as circulating antibody. However, once the peak titer has been reached, there is no appreciable delay between the administration of isotopic glycine and isotope appearance circulating in the antibody[5].

It seems reasonable to suppose that the synthesis of antibody protein takes place intracellularly. However, only small amounts of serologically active antibody have been found in tissues before the appearance of circulating antibody. It is therefore conceivable that an inactive precursor is formed and subsequently converted to serologically active antibody at or about the time of antibody release into the circulation. This process could possibly be analogous to the activation of proteolytic enzymes on release into the intestinal tract. Antibodies have previously been considered to appear in the circulation as they are synthesized and the exponential rate of appearance has led to the assumption that the synthesis is related to an autocatalytic process[17]. The work presented here suggests that antibody release is separated in time from the synthesis so that the rate of antibody appearance in the circulation is an expression of antibody release from individual cells, as has already been suggested[18]. A situation which is at least mathematically analogous exists for the liberation of bile pigments from red cells[19].

SUMMARY

1. On the basis of the relative isotope concentrations of serum globulin and antibody under different experimental conditions it is concluded that serum globulin is not a direct precursor of serum antibody. It appears that antibody is synthesized de novo from amino acids.

2. When amino acid incorporation is used as a criterion of antibody protein synthesis it is concluded that antibody release does not immediately follow the synthesis. At least one-third of the antibody protein was synthesized before antibody was detectable in the circulating blood.

RÉSUMÉ

1. Les concentrations relatives en isotope de la sérumglobuline et d'un anticorps dans diverses conditions expérimentales, permettent de conclure que la sérumglobuline n'est probablement pas un précurseur de l'anticorps sérique. Il semble que l'anticorps est synthétisé de novo à partir d'aminoacides.

2. Quand l'incorporation des aminoacides est prise comme critère de la synthèse de la protéine anticorps, il apparaît que la libération de l'anticorps ne suit pas immédiatement sa synthèse. Le tiers au moins de la protéine anticorps est synthétisée avant que l'anticorps soit décelable dans le sang circulant.

ZUSAMMENFASSUNG

1. Auf Grund der relativen Isotopenkonzentrationen von Serumglobulin und Antikörper unter verschiedenen Versuchsbedingungen wurde geschlossen, dass das Serumglobulin kein Vorgänger des Serumantikörpers zu sein scheint. Es scheint, dass Antikörper von neuem aus Aminosäuren synthetisiert werden.

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2. Aus der Benützung des Aminosäureeinbaus als Kriterium für die Antikörperproteinsynthese wird geschlossen, dass eine Freisetzung des Antikörpers nicht unmittelbar auf die Synthese folgt. Zumindest ein Drittel des Eiweisses des Antikörpers wurde synthetisiert ehe der Antikörper im zirkulierenden Blut aufgefunden werden konnte.

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Note added in proof

Independent evidence that plasmaproteins are not the immediate precursors of antibody protein was recently published[20].