Rate of Elimination of Homologous Globulins (including Antibody) from the Circulation

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Schoenheimer, Ratner, Rittenberg & Heidelberger (1942) and Heidelberger, Treffers, Schoenheimer, Ratner & Rittenberg (1942) showed that pneumococcus antibodies are synthesized and eliminated from the circulation in actively immunized rabbits in much the same way as other ^{15}N -labelled plasma proteins, and also that antibodies passively transfused to a non-immune animal do not incorporate ^{15}N administered as glycine in the diet. They arrived at a half-life for antibody and normal plasma proteins of 20 days, which is much longer than the values later obtained for rabbit plasma proteins by the use of ^{14}C -labelled amino acids (cf. review by McFarlane, 1952). It is probable that the discrepancy is due to repeated utilization of labelled amino nitrogen. We thought it worth while, therefore, to repeat their work both on actively and passively immunized rabbits, but using [^{14}C]lysine and [^{14}C]phenylalanine instead of glycine.

It seemed to us particularly important to investigate whether passively transfused antibody takes up amino acid carbon. If antibody molecules take up neither nitrogen nor carbon from free amino acids, we may assume that they are not subject in the circulation to chemical reactions affecting their amino acid composition, and by analogy that normal plasma-protein molecules also enjoy the same chemical isolation. Thus the decay curve of plasma protein radioactivity will have an absolute significance and if amino acids are not re-incorporated into newly formed protein ought to be identical with that of antibody concentration. In fact, our results show that they are never precisely the same, implying either that the rabbit does, in fact, discriminate in some way between the two so far as elimination from the circulation is concerned or that there is a small source of error, e.g. re-incorporation, inherent in the transfused ^{14}C -labelled protein technique which has not hitherto been recognized.

An additional objective was to find out whether transfused antibody is eliminated in the hyper-immune animal at the same rate as in the normal, since the demonstration that antibody elimination is independent of the rate of its synthesis is not only intrinsically interesting, but makes it possible, as is shown, to use available data to calculate absolute rates of antibody formation.

METHODS

Animals. Male albino rabbits bred in the National Institute for Medical Research were used throughout. Their weights lay between 2.3 and 2.7 kg., and they were maintained on pelleted diet no. 18 (Bruce & Parkes, 1946) supplemented with hay.

Blood samples. Following the transfusion or injection, 3–6 ml. blood samples were taken from the marginal vein of the ear over periods in some cases as long as 3 weeks. Serum was fractionated, either before or after removal of antibody, by addition of 27% (w/v) aqueous Na_2SO_4 solution, buffered with potassium phosphate ($I=0.1$) at pH 7.8 to give a concentration of 18%. The precipitate was separated by centrifugation, dissolved in water and reprecipitated at 18% (in some instances 16%) Na_2SO_4 . After removal of Na_2SO_4 by dialysis the resulting globulin solution was regarded as γ -globulin, although electrophoretic analysis indicated that about 5% of β -globulin was also present. The γ -globulin, after removal of antibody, is termed 'normal globulin' without prejudice to the question whether or not it contains antibodies against unknown antigens (Grabar, 1950).

Immunization. Formalin-killed type III pneumococci were used as antigen throughout. They were injected intravenously thrice weekly for 3–4 weeks or sometimes longer (cf. Kauffman, Bjørneboe & Vammen, 1938). Some animals received second courses after a 3-month interval, and among them remarkably high antibody titres (up to 40 mg. antibody/ml. serum) were obtained.

^{14}C -labelled proteins. 170 mg. (102 μC) DL-[carboxy- ^{14}C]lysine monohydrochloride (Arnstein, Hunter, Muir & Neuberger, 1952) was given subcutaneously to a rabbit 5 days after the last injection of pneumococci, and the animal was bled out and perfused with Ringer's solution 9.5 hr. later. The serum and perfusate were fractionated with Na_2SO_4 , the thrice-precipitated total-globulin precipitate containing 25% (w/w) of specifically precipitable antibody. The radioactivities of the antibody and normal globulin were 720 and 400 counts/min./mg. C, respectively. A second animal received 140 mg. DL-[carboxy- ^{14}C]phenylalanine supplied by the Radiochemical Centre, Amersham, and of much higher specific activity. This was given in divided doses over a period of 48 hr., beginning 2 days after the last injection of pneumococci. The rabbit was bled out and perfused 15 hr. later and the serum proteins were fractionated as before. The globulin precipitate contained 40% (w/w) specifically precipitable antibody, and the radioactivities of antibody and normal globulin were 6320 and 4840 counts/min./mg. C, respectively.

Transfusions of labelled proteins. The labelled antibodies and normal globulins prepared as described were injected in 0.9% NaCl solution into the marginal ear vein of rabbits.

Usually 0.3–0.4 g. antibody and 0.5 g. normal globulin were injected in 10 ml. of solution, and the injection was made over a period of 2 min.

Separation of antibody. Preliminary experiments with pneumococcal fractions showed that at least 90% of the antibodies produced in response to formalinized pneumococcus type III were specifically precipitable with nitrogen-free purified capsular polysaccharide prepared by Dr P. G. H. Gell. Furthermore, this antigen–antibody system has the advantage that it forms a tight, stringy precipitate which separates out rapidly and almost completely even at high dilution, and the amount of antibody carried down in the zone of optimum proportions is little altered by up to 50% excess of antigen. This makes quantitative precipitation of antibody from small amounts of serum relatively easy and rapid. All serum samples were allowed to stand at 2° for 2–3 days and centrifuged for 15 min. at 2000 g before use, in order to remove any non-specific precipitate. The general procedure was that of Heidelberger & Kendall (1932), except that the antibody precipitates after washing with ethanol and ether were weighed directly, or the amounts were estimated by solution in 0.1 N-NaOH and measurement of the light absorption at 280 m μ . In some experiments, and in all samples in which the amount of antibody expected was less than 1 mg./ml. serum, complement was first removed by addition of an ovalbumin–antiovalbumin system as described by Heidelberger & Mayer (1942). The antigen–antibody precipitate contained approx. 3% antigen, which is neglected in the calculations.

Protein estimations. These were made by weighing after drying, usually from the frozen state, or by measurement of light absorptions at 280 m μ . on solutions in 0.1 N-NaOH.

Measurement of radioactivity. This is described in an Addendum to this paper by Bradley, Holloway & McFarlane. Protein samples (approx. 5 mg.) were combusted at 650° in a Pt boat in a stream of O₂. The ¹⁴CO₂ was condensed in liquid air, treated to remove traces of peroxides and transferred quantitatively to a gas counting tube.

Calculation of level of circulating radioactive protein. Some of the figures show curves marked *A* (antibody) and *G* (normal globulin) which indicate the amount of transfused labelled protein in blood samples at various times. These represent values calculated from the specific activity of the material transfused and the concentration and specific activity of the same material in the samples, on the assumption that the radioactivity of the sample is due entirely to transfused molecules. The calculation is as follows: concn. of transfused labelled protein in sample = (total concn. of protein in sample) $\times$ (specific activity of sample) / (specific activity of original labelled protein).

Correction for sampling. When repeated blood samples are taken, there is a cumulative removal of substances, some of which are not being continually replaced, and failure to recognize this may significantly affect estimates of half-life. It has therefore been necessary to apply a correction factor, based on the assumption that protein transfused to 2.3–2.7 kg. rabbits becomes part of a protein pool (composed partly of blood plasma and partly of extravascular tissue fluids) equivalent to that contained in 200 ml. plasma after 15 hr. Subsequent removal of blood samples equivalent to 2 ml. plasma, for example, requires a correction of 1% for each sample in estimating the pool size in the hypothetical unbled animal, while the correction for a sample of similar volume in the first 15 hr., i.e. before lymph-plasma equilibrium is established, lies between 1 and 2%. Corrections of

this kind were applied to each value after the first on the curves of antibody concentration and of normal globulin specific activity in the passive transfusion experiments.

EXPERIMENTS AND RESULTS

Passive immunizations

Three experiments involving transfusions of labelled antibody and labelled normal globulin were made. In order to conserve radioactive material and yet reach a sufficient antibody level in the recipient rabbit's plasma to permit quantitative recovery from small samples, the transfused proteins in the first two experiments were diluted before administration with twice their weight of similarly prepared unlabelled antibody-containing globulins—the expedient presupposing that proteins from different individuals will be treated in identical fashion. Although such an assumption is reasonable, the necessity for making it was avoided in the third experiment which was carried out without preliminary dilution, and in fact the results in all three experiments were closely similar.

The first two experiments using [¹⁴C]lysine-labelled proteins gave identical results which are shown in Fig. 1. The third experiment using [¹⁴C]phenylalanine-labelled proteins also gave similar results which are shown in Fig. 2. It will be seen in each case that the antibody disappeared rapidly from the circulation during an initial phase lasting about 25 hr., and that subsequently a slower exponential elimination was established. The half-life of this elimination of antibody in the first rabbit was 150 hr. and in the second 98 hr. with no evident reason for the difference. On the other hand, the specific radioactivity of the antibody remaining in the circulation was substantially unchanged after 20 days. Variations in specific activities, which are greater in Fig. 1 than Fig. 2, were within the overall error of each experiment. We conclude, therefore, in agreement with Heidelberger *et al.* (1942) who used [¹⁵N]glycine, that transfused homologous antibody molecules do not exchange their constituent amino acids so long as they remain in the circulation. This conclusion is also reinforced by results with valine obtained by Gros, Coursaget & Macheboeuf (1952).

Since the total normal-globulin concentration in the plasma remained substantially constant throughout, the globulin radioactivity curve presumably reflects the disappearance of transfused radioactive normal globulins. The curves of antibody concentration and of normal globulin radioactivity follow similar courses, particularly during the first 5 days, prompting the conclusion that they leave the body fluids at almost the same rate. The qualification is important since closer inspection suggests a small difference in the two rates, and this is discussed in greater detail below.

A fourth experiment in this series was performed to ascertain whether preliminary loading with passively transfused antibody affects the rabbit's behaviour towards radioactive antibody given subsequently. Two intravenous injections were given 1 day apart, containing together 1.2 g. antibody and 1.36 g. normal globulin. Three days after the second injection, when equilibration in the protein pool had been established, [^{14}C]lysine-labelled antibody and labelled normal globulin were transfused. The findings are given in Fig. 3 and it will be seen that the specific radioactivity of the antibody fell slowly to about half in the course of the first 50 hr. The time taken for lymph-plasma equilibration of antibody was unusually long in this animal, possibly because of the rather large amount of protein previously transfused. The *A* and *G* derived curves show the amounts of transfused radioactive antibody and normal globulin remaining in the circulation at various times, and indicate half-lives in the steady phase of decline of 105 and 120 hr., respectively, compared with 122 hr. for the total antibody. We conclude that preliminary loading with passively transfused antibody does not significantly affect the rate of elimination of labelled antibody given subsequently.

Active immunization

Although normal animals eliminate transfused homologous antibody and transfused normal globulin at approximately the same rate, an animal

Fig. 1. Homologous [^{14}C]lysine-labelled antibody and similarly labelled normal globulin in normal rabbit. 340 mg. antibody (186 counts/min./mg. C) and 495 mg. normal globulin (200 counts/min./mg. C) dissolved in 10 ml. injected intravenously into 2.3 kg. rabbit. First samples after 15 min.

Fig. 2. Homologous [^{14}C]phenylalanine-labelled antibody and similarly labelled normal globulin in normal rabbit. 380 mg. antibody (6320 counts/min./mg. C) and 520 mg. normal globulin (4840 counts/min./mg. C) dissolved in 8.2 ml. injected intravenously into 2.5 kg. rabbit. First sample after 15 min.

Fig. 3. Effect of previous loading with unlabelled antibody. Total of 1.195 g. antibody and 1.366 g. normal globulin injected intravenously in two equal doses 4 and 3 days beforehand into 2.7 kg. rabbit; antibody level at start of experiment, 5.0 mg./ml. 64 mg. [^{14}C]lysine-labelled antibody (728 counts/min./mg. C) and 182 mg. normal globulin (400 counts/min./mg. C) in 10 ml. injected intravenously. First sample 15 min. later. *A* and *G* are calculated curves showing concentrations of labelled antibody and normal globulin remaining in plasma (see Methods).

which is actively synthesizing antibody might behave differently in this respect. A rabbit was therefore hyperimmunized by two courses of pneumococcus type III injections and, 3 days after the last injection, was transfused with [^{14}C]lysine-labelled antibody and similarly labelled normal globulin. Fig. 4 summarizes the results, and it will be noted that this rabbit had so much antibody in the serum before transfusion (35 mg./ml.) that the subsequent transfusion only raised the plasma antibody level by some 10%. This high dilution of the injected labelled material meant a consequent reduction in the accuracy of counting, but nevertheless the results are accurate enough to show that in conditions in which both antibody and normal globulin are being steadily replaced, the specific radioactivity of both falls in the same general way. The calculated *A* and *G* curves show the usual features of initial rapid dilution followed by steady decline with half-lives of 126 and 112 hr., respectively. Since the antibody pool was several times greater than the normal-globulin pool the animal must have been manufacturing antibody several times faster than normal globulin, and yet there is no very evident difference in the rates of elimination of the two.

DISCUSSION

The results show that circulating ^{14}C -labelled antibody molecules retained their specific radioactivity with remarkable constancy for as long as it was

possible to observe them and over a period (21 days) during which their concentration fell to less than 5% of the initial value. This observation, taken in conjunction with the demonstration by Heidelberg *et al.* (1942), and by Gros *et al.* (1952) that pneumococcus antibodies passively transfused to non-immune rabbits do not incorporate isotope-labelled glycine or valine administered in the diet, appears to establish beyond doubt that there is no mechanism for disintegrating or synthesizing pneumococcus antibody molecules or for translating amino acids from one such molecule to another within the plasma and lymph of the normal rabbit.

Normal rabbit plasma proteins are probably equally insusceptible to chemical reactions, but to obtain absolute proof of this requires an assurance, which is available in the case of passively transfused antibody, that no new production of labelled molecules is taking place. In the absence of this assurance it is necessary to proceed by analogy, i.e. by comparing the elimination rates of labelled normal globulin and homologous antibody in the same rabbit, and the interpretation will be correspondingly confused if there are physicochemical differences between the two which affect their rates of elimination. There are, in fact, no known differences of this kind between rabbit pneumococcus antibody and normal rabbit γ -globulin, the current view being that they differ only in the finer details of molecular structure or surface configuration (cf. Haurowitz, 1952) but, of course, it is always possible that rate of elimination from the plasma is more sensitive to smaller chemical differences than is generally believed.

Before discussing the results of experiments designed to make this comparison, it is desirable to explain the basis of the so-called *A* and *G* curves. The rate of decline of specific radioactivity after transfusing labelled proteins to an immune animal depends not only on the rate of elimination of the transfused labelled molecules but also on the rate at which new unlabelled ones are being discharged into the circulation. If an equilibrium exists, i.e. the animal is producing unlabelled protein and eliminating total protein of the same kind at equal rates, then the decline of specific radioactivity reflects only the elimination rate. Equilibrium may be disturbed in either of two ways, however, (a) by unbalanced production of unlabelled molecules, e.g. when the antibody level is rising or falling, or (b) by the appearance of a new source of labelled molecules, e.g. by re-incorporation of the label. Useful, if limited, information about the unbalanced system can be obtained if simplifying assumptions are made, especially that (b) is non-existent. In this case, for example, by postulating that labelled antibody molecules in the plasma at a given time were all part of an original transfusion,

Fig. 4. Homologous [^{14}C]lysine-labelled antibody and normal globulin in hyperimmune rabbit. 2.5 kg. rabbit immunized with two courses pneumococcus injections (last injection 3 days beforehand). 64 mg. antibody (728 counts/min./mg. C) and 182 mg. normal globulin (400 counts/min./mg. C) in 10 ml. injected intravenously. First sample 15 min. later. *A* and *G* are calculated curves showing concentrations of labelled antibody and normal globulin remaining in plasma (see Methods).

it is possible to estimate the labelled antibody concentration and obtain the so-called *A* curve. In Fig. 3 this turns out to be very different from the antibody specific-radioactivity curve, but as might be expected very similar to that of the normal globulin.

When the various values relating to transfused labelled proteins are plotted semi-logarithmically the curves are linear, except during the first 20–40 hr. The work of Wasserman & Mayerson (1951) shows that the initial non-linear portion reflects mainly the equilibration of labelled proteins between blood and lymph while the linear portion is shown by Miller, Bly, Watson & Ball (1951) to reflect the transference of labelled protein molecules from lymph to tissue cells and their replacement by newly formed unlabelled ones.

By extrapolating the linear portion of the passive immunization curves to zero time, estimates are available of a hypothetical concentration of antibody and of a hypothetical specific activity of normal globulin which would have resulted if the transfused labelled proteins had become instantly dispersed throughout the plasma and lymph at uniform concentration. For example, in Fig. 2 both the antibody concentration and the globulin specific activities fall to hypothetical values which are 60 % of the measured starting values. This means that two-fifths of the labelled antibody has passed out of the circulation, and similarly that two-fifths of the labelled globulin has also passed out and been replaced in the circulation by the same amount of unlabelled globulin. The information which may be deduced from these figures is that the lymph volume is sufficient to accommodate two-thirds of the antibody or normal globulin in the plasma, though what this volume is will, of course, depend upon the mean concentrations of these proteins in the lymph. It seems reasonable to suppose that in the animal referred to in Fig. 2 the actual lymph volumes and the ratios of lymph concentration of each protein to its plasma concentration, were the same. In the animal referred to in Fig. 1, on the other hand, it appears that at equilibrium the lymph held an amount of normal globulin approximately equal to that in the plasma, whereas it held rather more (approx. 10 %) than an equal amount of antibody for reasons which are not understood.

The results of the passive immunization experiments (Figs. 1 and 2) also show that the slopes of the curves of antibody concentration and normal-globulin specific activity are similar, but probably not identical. Superposition shows the apparent half-life of globulin activity to be 135–145 hr. (elimination rate 11.5 % per day), while that of antibody concentration is 120–130 hr. (elimination rate 12.5 % daily). A fractional difference in the same sense is also evident in the derived *A* and *G*

curves of Fig. 3, but not in those of Fig. 4. More experiments are necessary to establish the significance of this difference, but assuming it to be real, an obvious explanation is that labelled amino acids liberated by the breakdown of transfused antibody and normal globulin are being re-incorporated into plasma proteins. Since the passively immunized animal is not making new antibody, similar re-incorporation cannot affect the slope of the *A* curve in Fig. 3. Correspondingly, since the actively immunized animal of Fig. 4 is making new antibody, we expect (and find) little or no difference between the *A* and *G* curves. They need not be identical in this case, however, because the new production of antibody, although initially faster than that of globulin, did not balance its elimination, the plasma titre falling during the experiment. The general conclusion may be drawn that re-incorporation of the isotope label may significantly affect the results of experiments using transfused labelled proteins, and that the use of a serological label is free of this possible source of error in the measurement of elimination rates.

We have also observed the course of labelling of plasma proteins in normal and immune rabbits using [^{14}C]amino acids with results which will be published in due course. In these experiments similar exponential decay curves have been obtained in the period 20–200 hr., but with somewhat longer corresponding half-lives, suggesting that the re-incorporation of labelled amino acids proceeds on a larger scale, as might be expected. However, the apparent rates of elimination are not so much longer as to suggest that the rabbit discriminates in any notable way between antibody which it has received from another animal and that made by itself. Any major effect of this kind would, of course, seriously qualify the more general conclusions in this paper.

Although the work described above has been confined to pneumococcus antibodies, the elimination rate after passive transfusion in rabbits is much the same for other antibodies. Earlier work by one of us (J.H.H.) showed that when mixtures of homologous antisera, prepared against human group *A* erythrocytes and against Forssman antigen, were transfused, with or without changes in blood volume, the half-lives of the second stage of elimination in nine rabbits averaged 120 hr., with a range of 96–132 hr. These antibodies are often found in normal rabbits, and are to that extent less artificial than pneumococcus antibodies, yet their behaviour is so alike that it seems safe to say that an average elimination rate of 12.5 % daily applies generally to antibodies in rabbits.

In the active-immunization experiment the plasma antibody concentration was as high as 35 mg./ml. and in the passive ones lower than one-

Table 1. *Serum antibody levels and mean rates of antibody production in a hyperimmunized rabbit*

The protein in the plasma and lymph spaces of this 2.5 kg. rabbit was found to be equivalent to the protein in 200 ml. of its plasma. Immunizing injections of formalin-killed pneumococcus type III were given on the 1st, 3rd, 5th and 10th days and totalled 10^{10} organisms.

Date	mg. antibody/ml.	Mean rate of synthesis (g./day)
3. xi. 51	0.04	—
11. xi. 51	16.2	0.6
15. xi. 51	33.0	1.45
16. xi. 51	38.1	1.85
18. xi. 51	35.0	1.18
22. xi. 51	32.0	0.65

tenth of this value. Nevertheless, within the limitations of the experimental accuracy, the elimination rates are the same, viz. 12.5 % daily. This means, of course, that the absolute rate of disintegration of antibody protein in g./day is different by an order of magnitude in the rabbits immunized in these two different ways and leads us to suggest the use of the term 'rate fraction' rather than merely 'rate' to denote the regular elimination of a constant proportion of all the antibody protein in the plasma and lymph. When the rate fraction for elimination is constant, absolute rates of antibody production follow at once from a knowledge of the total weight of antibody in the plasma and lymph. So long as plasma concentrations are steady, absolute production rates of antibody or normal globulins must be in proportion to their concentrations. When the level is increasing or diminishing steadily we may assume, as a first approximation, that production over a period is that required to maintain the mean level together with that required to compensate for any net change in the pool size. An example of the application of these principles is given in Table 1, which refers to a rabbit hyperimmunized with pneumococcus type III. This animal not only produced antibody, for a short period, four times as fast as normal globulin, but during the 19-day period of observation made approx. 18 g. new antibody, in response to an antigenic stimulus of 2.5 mg. pneumococci of which not more than 10 % was antigenic capsular polysaccharide.

SUMMARY

1. Radioactive antibody and normal globulin have been prepared by injecting [*carboxy*- ^{14}C]lysine and [*carboxy*- ^{14}C]phenylalanine into rabbits hyperimmunized against pneumococcus type III. These have been transfused to normal and immune rabbits.

2. The specific radioactivity of antibody transfused into normal rabbits remained unchanged for as long as 3 weeks, indicating that molecules of this kind do not exchange their amino acid carbon while they remain in the circulation. The concentration of antibody and the specific radioactivity of transfused globulin declined at a steady rate of approximately 12.5 % per day, the globulin rate being fractionally lower than that of the antibody. It is suggested that the true rate of elimination of these globulin-like molecules is given by the decline of the serological label which is irreversibly lost rather than by isotopic labelling, even where the isotopically-labelled proteins are transfused.

3. The specific radioactivities of transfused labelled antibody and labelled normal globulin in the plasma of immune rabbits both declined at the same approximate rate of 12.5 % per day. The rates of antibody and normal-globulin elimination are shown to be approximately the same and independent of the rate of production. Hence the weight of these proteins produced in a given interval is proportional to their average plasma concentration over the same period. An example is given of the use of this information to calculate absolute rates of antibody production by a rabbit at various stages of hyperimmunization.

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ADDENDUM

Assay of ^{14}C in the Gas Phase as Carbon Dioxide

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The assay of biological materials for their ^{14}C content presents certain difficulties owing to the low energy of the radiation emitted. While the direct assay of solid substances by means of thin end-window counters, which is popularly used, is very convenient, the method has the following disadvantages: (a) the sensitivity is low owing to absorption in the window of the counter; (b) many materials, owing to their granular or hygroscopic nature, are difficult to prepare in uniform layers in a reproducible manner; (c) comparisons of carbon specific radioactivity (which are usually sought) cannot be made directly from counts on materials of different composition.

The two last difficulties can be overcome by combustion of the material, followed by absorption of the carbon dioxide in barium hydroxide, and assay of the resulting barium carbonate; but the first remains. Some improvement in efficiency can be effected by the use of windowless counters, but losses due to poor geometry and absorption by the sample itself are still large. The problem has been adequately reviewed recently (Bernstein & Ballentyne, 1950; Brown & Miller, 1947).

The difficulties outlined above have led to increasing use of assay methods employing gaseous carbon dioxide suitably mixed with other gases to give favourable counting properties, and two general methods of assay for biological materials have been described. That of Van Slyke, Steele & Plazin (1951) employs the well-known wet-combustion technique of Van Slyke & Folch (1940) for 5–15 mg. samples, followed by proportional counting of $^{14}\text{CO}_2$ activity in the presence of methane, the latter as described by Bernstein & Ballentine (1950). The second, described by Glascock (1952), is based on a standard micro-analytical dry combustion (C and H), modified to enable water and carbon dioxide to be collected by condensation instead of by chemical combination. The $^{14}\text{CO}_2$ is then assayed in a Geiger counter of the type described by Brown & Miller

(1947) using CS_2 as a charge-transfer gas. This gas, however, is inconvenient to handle in a vacuum system, and counters filled with CO_2 – CS_2 and operated in the Geiger region have been shown to be 15 % less efficient than CO_2 –methane-filled counters operated in the proportional region (Miller *et al.* 1950).

The method to be described here follows closely that of Glascock in the preparation and handling of the CO_2 , but differs from his method and that of Van Slyke *et al.* (1951) in that the CO_2 is assayed as the pure gas, i.e. at the upper end of the region of limited proportionality. Buchanan & Nakao (1952) describe an improved version of the Van Slyke technique and mention that they have used pure CO_2 although they prefer to mix it with methane. The pulses from pure CO_2 in their (metal) tube are described as ‘jagged’ and overshooting of the counting rate occurs, an experience we have not had while using the Pyrex tube described below. We take the view that the introduction of an auxiliary gas is unnecessary.

APPARATUS

$^{14}\text{CO}_2$ produced by combustion of 2–10 mg. samples in O_2 in a quartz tube is condensed in a spiral in liquid air, surplus O_2 and other gases being pumped away, and the $^{14}\text{CO}_2$ then passes into the high-vacuum part of the line for introduction into the counting tube. The quartz combustion furnace and Pyrex vacuum manifold are illustrated in Fig. 1. The simple ‘band filling’ of the combustion tube for C and H analyses (but omitting the PbO_2 , for reasons detailed below) is followed by a spiral Pyrex trap immersed in liquid O_2 .

The tubes employed (Fig. 2) have graphite cathodes and 0.1 mm. diameter tungsten centre wire (42 s.w.g.), the total volume being about 30.0 ml. Protruding glass sleeves over the electrodes help to minimize leakage across the surface of the glass at the relatively high voltages used. The tubes are usually of our own construction but correspond in general design to a larger tube, viz. type GA26, by 20th Century Electronics, Ltd. This tube and a smaller version of it made for us by that firm also both proved satisfactory.

Fig. 1. Furnace and high-vacuum line used for the combustion of ^{14}C -substances in a stream of oxygen and subsequent collection and purification of carbon dioxide. The pressure of gas is measured at constant volume in a mercury manometer before transferring the gas to a proportional counter for measurement of radioactivity. All taps and ground joints are lubricated with Apiezon 'T' grease. (Metropolitan-Vickers Electrical Co. Ltd.)

Fig. 2. Geiger tube with graphite cathode and tungsten anode for assay of radioactive carbon dioxide in the proportional region.

The operating potential on the tube is in the region of 2600 v, at which voltage plateaux 200 v long, with slopes not greater than 4%/100 v are obtained (Fig. 3). The counter pulses are fed to a 1008-type linear amplifier (Atomic Energy Research Establishment, Harwell) and thence to a scaler (type 1009). The overall gain is 5×10^4 and the pulses are fed to a discriminator which rejects all below 25 v. Tube

Fig. 3. Graph showing relationship of counting rate to anode voltage for a given sample of radioactive carbon dioxide. A working voltage is chosen in the middle of the plateau.

efficiencies are such that at least 80% of all ^{14}C disintegrations are registered. The efficiencies were determined by combustion of a standard ^{14}C -labelled material (serum albumin) the activity of which was established by comparison of a solid sample with a substandard of ^{14}C -labelled polystyrene, supplied by The Radiochemical Centre, Amersham, Bucks. The albumin is convenient for testing since it combusts easily and reproducibly and is closely similar in composition to the materials usually combusted; ^{14}C -labelled polystyrene, on the other hand, does not readily combust quantitatively. Tube efficiencies in the region of 80% which we obtain are a few per cent higher than correspond with the geometrical ratio of active to total volume of the tube, possibly reflecting an uncertainty in the

absolute value of our standard. Freedman & Anderson (1952) could not obtain better slopes than 10%/100 v using metal tubes at higher pressures of pure CO_2 .

PROCEDURE

To ensure efficient condensation of $^{14}\text{CO}_2$, the pressure in the spiral trap is maintained below 1 mm./Hg, since below this pressure the mean free path of water molecules increases rapidly and they are more likely to strike the condensing surface. During combustion the flow of cylinder O_2 is maintained at approx. 15 ml./min. with the help of tap T_2 and the visual indication of the bubbler (B.S.S. 1428; Part A1, 1950), and the excess of oxygen is pumped away via a bypass system (not shown) with the tap T_4 closed, the rest of the manifold being under high vacuum. No special care is taken to remove CO_2 and organic compounds from the O_2 used, as with the usual sample weights employed (2–10 mg.) the correction for the blank CO_2 is equivalent to less than 10 μg . of C. All heating of the combustion furnace is done electrically and the traversing section at 800–850° is electrically driven. As in Glascock's technique, 5–7 min. is required for a single combustion. Any residual CO_2 in the combustion tube is recovered by closing tap T_1 and evacuating the combustion tube via the trap. The trap is then isolated from the combustion tube and evacuated to below 10^{-5} mm. Hg by opening T_4 . T_3 is then closed and the liquid O_2 replaced by an ethanol–solid CO_2 bath. The $^{14}\text{CO}_2$ condensed in the spiral evaporates and is shared by the spiral and the rest of the manifold by opening T_3 . T_3 is then closed again, and the $^{14}\text{CO}_2$ in the line condensed in the purifier. This procedure, by which about 8% of the $^{14}\text{CO}_2$ is sacrificed in the spiral, reduces to negligible levels the transfer of impurities, including water, which are significantly volatile at the temperature of solid CO_2 . Where a value for total carbon in the sample is also sought (see below) the proportion of total CO_2 thus rejected is accurately known from a volume calibration of spiral and line separately.

The $^{14}\text{CO}_2$ is purified from volatile electronegative gases by contact for 1–2 min. with a reduced-copper wire heated electrically to redness, and is then condensed again while the non-condensable gases produced by reaction with the wire are pumped away. The $^{14}\text{CO}_2$ is then allowed to evaporate and is condensed in the manometer where its mass is measured in terms of the pressure produced in a known manometer volume. The pressure of gas in the manometer is raised to a standard value by introducing inactive CO_2 from the reservoir (via a tap with a tapering groove filed in the key for easier control). Sample $^{14}\text{CO}_2$ thus mixed with inactive CO_2 is then transferred to the counter by condensation. The pressure in the counter is 20 cm. Hg, this being the lowest pressure at which good counting properties are obtained.

Except with samples of very low activity, a complete analysis for ^{14}C activity, from commencement of combustion to completion of counting, requires 75 min. and samples can succeed one another at 40 min. intervals. The operation of the counters and of the scaling equipment is routinely checked by means of calibrated active CO_2 drawn from a reservoir. The daily background behind 1 in. Pb shielding has been 17–18 counts/min. with a variation from day to day which is just outside the statistical error.

Of eleven typical combustions of ^{14}C -labelled serum albumin, a mean specific activity of 519 ± 9.0 counts/min./mg. C was obtained, compared with a figure of 515 derived

from a solid-count comparison with the ^{14}C -substandard. The mean carbon content calculated from the recovery from the samples was $49.1 \pm 0.18\%$ compared with 49.7% determined by standard microanalytical procedure.

DISCUSSION

We have found the method of purification of the CO_2 described above to be indispensable because of the extreme sensitivity of the proportional counter to traces of electronegative gases. The function of the PbO_2 in the conventional C and H apparatus is to absorb oxides of nitrogen, sulphur and halogens and, provided the peroxide is suitably prepared, good results are obtained in gravimetric measurements. However, in our experience, traces of oxides of nitrogen (and possibly oxychlorides) escape absorption by the PbO_2 and suppress counts in the tube. For example, 1 vol. NO_2 in 10000 vol. $^{14}\text{CO}_2$ produces several per cent reduction in the counting rate. While PbO_2 of sufficiently high standard to retain even this small quantity of impurity can be prepared, its efficiency is markedly impaired after the combustion of only four to five organic samples of 5 mg. each; in addition, PbO_2 has other objectionable features, owing to its strong affinity for CO_2 and H_2O (Niederl & Niederl, 1942). The procedure described here deals adequately with the relatively high concentrations of oxides of nitrogen and sulphur in gas from the combustion of proteins when the PbO_2 is omitted altogether.

The technique is specially designed for the assay of 2–10 mg. protein samples of low specific activity such as occur in studies with [^{14}C] amino acids on small laboratory animals, where the weight of sample is limited or on humans where the activities are low. In our experience it is more accurate than solid-sample counting because of the difficulty in preparing protein films of uniform thickness free from cracking and troubles due to absorption of moisture during the counting. We have also found protein powders, especially after drying from the frozen state, to be objectionable to handle from the point of view of personal protection and of keeping the laboratory free from contamination. On the other hand, a small piece of freeze-dried 'protein cake' is easily transferred intact to the platinum boat where it is at once wetted, dried under the infrared lamp and weighed when cold. In all subsequent handling, the protein adheres tenaciously to the boat until combustion is complete. The waste active gas is finally pumped through an exhaust pipe which passes through the laboratory window. In some thousands of combustions, we have so far had no case of significant cross-contamination of samples or of a rise in the activity of the dust in the counting room. An accessory virtue of the technique lies in the fact that a micro-analysis for carbon is carried out and the information so obtained often throws

important light on the purity of the biochemical specimen.

Against these advantages has to be set the fact that the apparatus described requires the close attention of a skilled operator; unlike solid-sample counting, the technique does not lend itself to automatic operation.

SUMMARY

A technique is described for the assay of ^{14}C using carbon dioxide. The gas obtained by micro-analytical combustion is purified by contact with a hot copper filament and its radioactivity assayed in the proportional region without admixture.

We owe the construction of the counting tubes and of several gas lines to the skill of Mr D. Hart, whose services we gladly acknowledge.

Fate of Homologous Antibody in the Rabbit

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In the preceding paper (Humphrey & McFarlane, 1954) it was shown that ^{14}C -labelled antibody and ^{14}C -labelled normal γ -globulin disappear from the circulation of the rabbit at similar rates. Evidence was produced to support the view first put forward by Heidelberger, Treffers, Schoenheimer, Ratner & Rittenberg (1942) that antibody molecules in the circulation do not exchange their constituent amino acids, and it was suggested that normal globulin molecules probably also do not do so. Antibody molecules may therefore be permissible tools for use in the study of the ultimate fate of plasma globulins, the important advantage being that they can be identified and recovered from the tissues by means of specific precipitation.

The fate of transfused homologous whole-plasma proteins labelled with [^{14}C]lysine has been studied by Yuile, Lamson, Miller & Whipple (1951) in dogs. Although these workers were able to account for a high proportion of the original activity transfused, their results do not reveal how much of the radioactivity found in the tissues was in the form of unchanged transfused protein and how much was represented by newly formed tissue proteins. In addition, they did not distinguish between labelled intracellular protein and labelled plasma protein in the lymph. By transfusing ^{14}C -labelled antibody globulin and using specific antigen-antibody precipitation, it may be possible to determine how

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much of the labelled protein found in the tissues is unchanged antibody. Furthermore, if Evans Blue dye (T 1824) is injected at least 12 hr. before death, the dye content of the perfused organs provides an estimate of the maximum amount of residual lymph protein—with certain reservations which are discussed below.

There follow results of a study of this kind on a rabbit killed 8 days after receiving a transfusion of [^{14}C]phenylalanine-labelled plasma globulins taken from another rabbit immunized against the capsular polysaccharide of pneumococcus type III. It would obviously be preferable to use pure labelled antibody, but since it was impracticable to separate pneumococcus antibody from other γ -globulins in sufficient amount we were obliged to use the total γ -globulins (called hereafter ^{14}C -labelled globulin) from the plasma of an immunized rabbit. Thus, although all the transfused material had a radioactive label, only 40% was labelled serologically. We assume throughout that the ratio of ^{14}C -labelled antibody to ^{14}C -labelled normal globulin is not altered during the passage of the transfused proteins throughout the body fluids of the recipient, and that both antibody and normal globulin are broken down and/or incorporated into tissue protein at the same rate. It is a corollary of this assumption that when labelled antibody is extracted from tissues by specific precipitation, a proportionate