Kinetics of Formation of Cx-Reactive Protein. (27581)

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Tillett(1,2) first described the existence of a protein in sera of patients suffering from pneumonia and other infectious diseases which was not found in normal human sera. This protein could be precipitated from serum by addition of the C-polysaccharide of the pneumococcus. It was therefore termed "C-reactive protein" (CRP) and its properties have been studied by several investigators(3, 4,5,6).

A similar and analogous protein has been demonstrated in acute phase rabbit serum(7), and this substance has been called "Cx-reactive protein" since it reacts with the Cx-polysaccharide of the pneumococcus. A close biological similarity exists between human C-reactive protein and rabbit Cx-reactive protein(8), but the source, function and mode of formation of these proteins is still obscure. Since the presence of CxRP appears to be part of a general non-specific response to infection and other stimuli, it was thought that it would be of value to study the kinetics of formation of this protein by observing the incorporation of C^{14} labelled amino acids into CxRP and serum albumin.

Experimental. While CxRP can be assayed by addition of Cx-polysaccharide in the presence of 0.001 M Calcium ion, or by addition of sheep anti-rabbit CxRP serum, determination of specific radioactivity depends on accurate knowledge of the exact amount of CxRP being counted. Cx-polysaccharide lends itself to a quantitative determination of CxRP and has been used in this study.

Serum from rabbits fed on commercial diet was tested for the presence of CxRP by using an absorbed sheep anti-rabbit CxRP serum. Rabbits shown to be free of CxRP were divided into 2 groups. One group of 3 rabbits (#203, 204, 305) was prelabelled with glycine-1-C^{14} given intravenously 14-16 hours prior to challenge with an intramuscular injection of typhoid-paratyphoid vaccine (Lederle). A second group of 5 rabbits (#205, 206, 207, 210, 302) was given typhoid vaccine intramuscularly 14-16 hours prior to receiving glycine-1-C^{14} intravenously. This latter group is accordingly termed "postlabelled". In all rabbits, the dose of typhoid vaccine was 1.0 ml injected in 3 separate sites. Administration of typhoid vaccine serves to induce the formation of CxRP. CxRP was detectable in the serum of these rabbits 5-7 hours after induction on qualitative assay using antiserum.

Blood samples were drawn from the marginal ear vein for the initial samples of serum and by cardiac puncture for later samples. The blood was permitted to clot for one hour, centrifuged and the sera frozen. Sera drawn within 3 hours after giving isotope were dialyzed overnight against physiological saline to remove free amino acid. Cx-polysaccharide used in these experiments was kindly supplied by Schieffelin and Co. CxRP was precipitated by the serial addition of Cx-polysaccharide to the serum until the supernate failed to react with an absorbed sheep anti-rabbit serum, indicating that all CxRP had been removed. The precipitate was collected, washed 3 times with 0.02 M $CaCl_2$, then dialyzed versus 0.05 M sodium citrate and then against 0.05 M $CaCl_2$ in the cold. The precipitate was then dialyzed against distilled water and trichloroacetic acid (TCA) was added to a final concentration of 10%. The mixture was heated to 80°C for 5 minutes, then refrigerated for 2 hours. The precipitate was plated on tared Millipore filter discs, washed twice with distilled water, dried at 70°C for one hour, weighed and counted in a Geiger-Muller tube.

In general, samples were counted for a time sufficient to accumulate 1280 counts; accordingly the error in counting ranged from −2.8% to +2.8% (the error in counting is equal to the square root of number of counts).

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The weight of the protein on the plate was calculated by difference (error was less than ± 0.01 mg) and specific activities (counts/min/mg protein) were corrected by self-absorption coefficients.

Only sera which had been treated with Cx-polysaccharide and shown not to react against sheep anti-rabbit CxRP serum were used for the assay of serum proteins. To 0.2 ml of serum was added 0.2 ml of saturated ammonium sulphate solution at room temperature; the mixture was allowed to stand one hour at room temperature and was then centrifuged and the "globulin" precipitate removed. To 0.1 ml of the supernate was added 0.1 ml of saturated ammonium sulphate solution. This was allowed to stand for one hour at room temperature. The precipitate (albumin) from this fraction was collected, washed twice with 80% ammonium sulphate, dialyzed against distilled water and TCA was added to a final concentration of 10%. The TCA mixtures were then treated as above. All precipitates were plated on tared Millipore filter discs, washed twice with distilled water, dried at 70°C and counted in a G-M tube.

Results. Rabbits whose body proteins had been prelabelled by an injection of glycine-1-C^{14} given 14-16 hours prior to induction of CxRP formation with typhoid vaccine yielded a CxRP which had a specific activity higher than that of serum albumin early in the response (Fig. 1). CxRP and serum albumin then declined at similar rates.

A careful study of rate of incorporation of glycine into CxRP and serum albumin was carried out at close intervals after giving the isotope intravenously to 2 rabbits (#207, 210) which had received typhoid vaccine 14 hours before and were actively producing CxRP. In each rabbit, labelled glycine appeared in the CxRP considerably later than in the albumin (Fig. 2). Specific activity of the CxRP of rabbit #210 then rose steeply to a maximum and declined at a rate comparable to that of the prelabelled rabbit (Fig. 3). Rabbit #206 exhibited comparable curves except that the early intervals after administration of isotope were not studied (Fig. 4).

Discussion. If CxRP were formed solely by *de novo* synthesis, then CxRP formed by the prelabelled animal should have relatively little radioactivity, while CxRP formed by the postlabelled rabbit should have a specific activity higher than that of serum albumin. Since in this study, CxRP formed by the prelabelled animal has a specific activity higher than that of serum albumin initially, some CxRP must have been present in body tissues, in some form, before induction. The diminishing specific activity seen later is consistent with dilution by newly synthesized, and therefore nonradioactive, CxRP.

The possibility (in the prelabelled experiments) that CxRP derives its activity from glycine released from breakdown of serum proteins seems remote in view of the known half-life of rabbit serum proteins which is considerably greater than the intervals considered here. As to free serum glycine being the source, one does not expect high levels of labelled amino acids in the serum or extracellular fluid 12 hours after injection. Furthermore, as shown in Fig. 3, an animal already making CxRP is given glycine-1-C^{14} by vein and incorporates considerably more labelled amino acid into the CxRP fraction than into the glycine. In this situation, albumin does not appear to be a precursor for CxRP. Of note are the similarities of these curves, after 8 hours, to the curves depicted in Fig. 1. At this point, these are nearly comparable experimental situations. The difference in initial labelling of CxRP and albumin in Fig. 1 is made more evident by considering the molar composition of glycine in each of these fractions. CxRP contains 0.17 *micro*mole of glycine per milligram of protein,† whereas albumin contains 0.33 *milli*mole of glycine per milligram of protein(10). This would tend to augment the difference in labelling noted between these 2 substances.

Green and Anker(9) have demonstrated that glycine is incorporated into serum proteins only after a definite lag phase has

† This determination was kindly performed by Dr. H. Rosen, Walter Reed Army Inst. of Research.

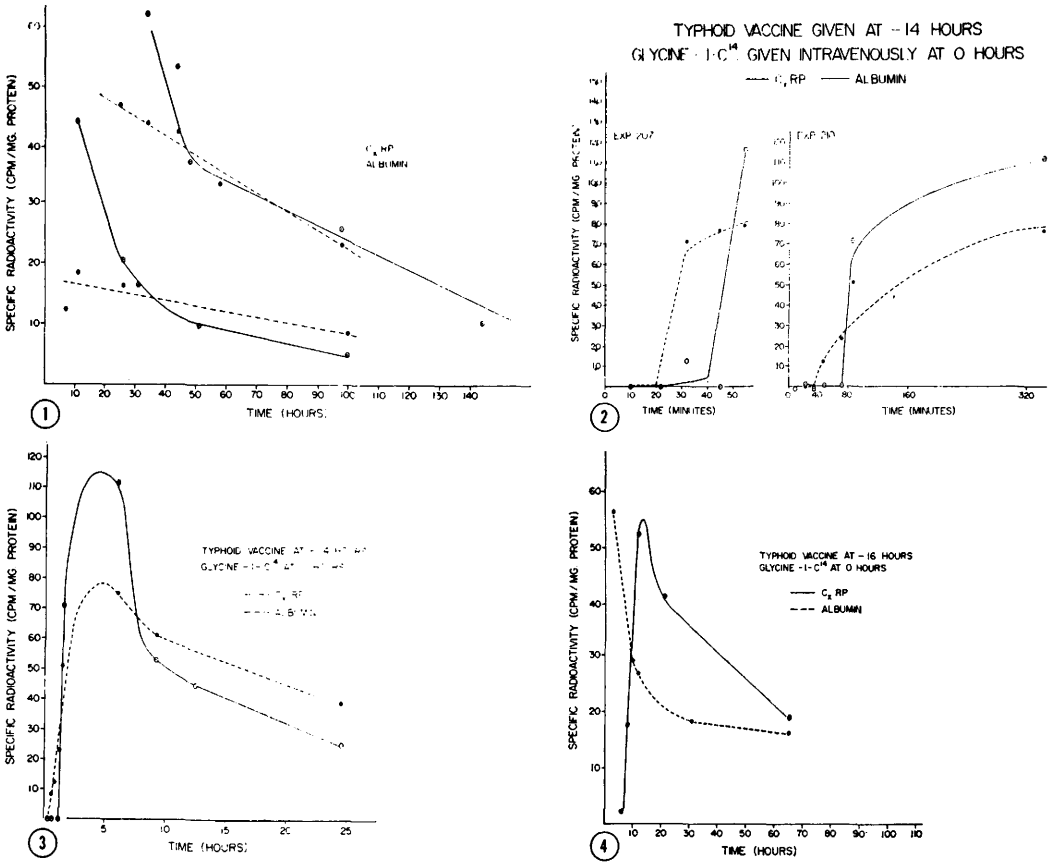

FIG. 1. *Specific activity of CxRP formed by prelabelled rabbits.* Rabbit #305 (top curves) received 150 μc of glycine-1-C^{14} and rabbit #204 (bottom curves) received 187 μc of glycine-1-C^{14} intrav. at zero time. Data show levels of radioactivity of CxRP and of serum albumin, and are consistent with the hypothesis that some CxRP must have been formed before rabbits received typhoid vaccine. Diminishing specific activity seen later is consistent with dilution by newly synthesized, and therefore, non-radioactive, CxRP.

FIG. 2. Rabbit #207 received 140 μc of glycine-1-C^{14} and rabbit #210 received 67 μc of glycine-1-C^{14} 14 hr after receiving typhoid vaccine intramusc. Incorporation of glycine-1-C^{14} into CxRP and albumin was studied at close intervals following administration of isotope. In both rabbits, glycine-1-C^{14} appeared in CxRP considerably later than in albumin.

FIG. 3. *Specific activity of CxRP formed by postlabelled rabbit.* Further data obtained from rabbit #210 (see Fig. 2). The general similarity between specific activities of serum albumin and CxRP are consistent with appreciable *de novo* synthesis of CxRP. Its specific activity rose initially, as newly synthesized labelled protein was added to animal's total circulating CxRP. After first few hours, further synthesis of unlabelled CxRP resulted in a reduction in specific activity, while after the tenth hour both albumin and CxRP declined in specific activity at about the same rate. Another rabbit (#205) not shown here, gave similar results.

FIG. 4. Rabbit #206 received 67 μc of glycine-1-C^{14} intrav. 16 hr after receiving typhoid vaccine intramusc. Same model as that in Fig. 2 and 3 except that early intervals after inj. of isotope were *not* studied. The pronounced lag in appearance of radioactivity in CxRP of this animal is consistent with the idea that labelled amino acid must have been incorporated into a tissue precursor, from which circulating CxRP was derived much later.

elapsed. Studies of the comparative lengths of the lag phase seen during incorporation of glycine-1-C^{14} into CxRP and serum albumin, in the acute phase rabbit have demonstrated that CxRP has a longer lag period than does serum albumin (#207, 210). The results of these experiments are consistent with the idea that CxRP is not formed on challenge of the rabbit, but rather exists in some form in tissue prior to breakdown of a precursor molecule to give circulating CxRP. The existence of a tissue precursor for a plasma protein is,

of course, not unique. It seems incorrect, in the light of this study, to consider CxRP to be made only when an animal is "sick".

In the postlabelled rabbit (#210), the general similarity between the specific activities of serum albumin and CxRP are consistent with some *de novo* synthesis of CxRP. The specific activity of the CxRP rose initially as newly synthesized labelled protein was added to the animal's total circulating CxRP. After the first few hours, further synthesis of unlabelled CxRP resulted in a reduction in specific activity, while after the tenth hour both the albumin and CxRP declined in specific activity at about the same rate.

Summary. Studies on the kinetics of formation of CxRP in rabbits have been carried out. By measuring relative specific activities of CxRP and serum albumin in rabbits given glycine-1-C^{14} and induced with typhoid vaccine to form CxRP, evidence has been obtained to suggest that CxRP is being manufactured constantly and appears in the serum on challenge.

I wish to acknowledge the kind and generous counsel of Dr. Chandler A. Stetson in the design and execution of these experiments.

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Utilization of Free and Esterified Cholesterol-4-C^{14} for Corticoid Biosynthesis by Hog Adrenal Homogenates.* (27582)

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It has been shown(1) that hog adrenal homogenates produce corticoids in the presence of reduced triphosphopyridine nucleotide (TPNH) and oxygen. During steroidogenesis, the level of endogenous free cholesterol declined by an amount comparable to the amount of steroid produced. Adrenal free cholesterol fell to about 50% of the original level during a 2-hour incubation period. However, addition of exogenous free cholesterol to the homogenates initially or after 2 hours, failed to increase steroid production. The level of cholesterol esters in the homogenates remained constant throughout the incubation period. These findings are in agreement with those of Péron and Koritz(2), who, using rat adrenal homogenates, suggested that the failure of exogenous free cholesterol to increase corticoid output could be explained by enzyme saturation by endogenous cholesterol or by an inability of exogenous cholesterol to enter the biosynthetic pathway for corticoid synthesis. Later Péron(3), concluded that mixing of endogenous and exogenous free cholesterol did take place in rat adrenal homogenates. Addition of 117 μg of cholesterol-4-C^{14} to an incubation mixture containing about 35 mg of rat adrenal tissue with a TPNH generating system gave 1.88% of the total C^{14}-radioactivity in eluates of steroid zones after chromatography.

The present work was undertaken to study further the interconversions of free and esteri-

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