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Studies on the Asymmetrically Attached Oligosaccharide of Rabbit Immunoglobulin-G

I. BIOSYNTHESIS AND STABILITY OF THE C_2-OLIGOSACCHARIDE

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Summary. Two populations of IgG molecules have been isolated from rabbit serum: Type 1, which is devoid of C_2-oligosaccharide, and Type II, which has the C_2-oligosaccharide attached to only one of the H-chains (Fanger & Smyth, 1970b). By labelling with different isotopes, it was possible to study the stability of the two IgG Types *in vitro* and *in vivo*; conclusions could then be drawn concerning biosynthesis of the carbohydrate moiety.

The C_2-oligosaccharide is not affixed to or deleted from the IgG molecule during its life span in the circulation; carbohydrate attachment takes place exclusively within the biosynthetic cell. Furthermore, a symmetrical molecule with the C_2-oligosaccharide on both H-chains does not appear to be formed *in vivo*, although this molecule could easily be formed *in vitro*. It follows that the order of events in biosynthesis of IgG is that first the polypeptide chains are assembled and then the carbohydrate moiety is added to complete the molecule.

No exchange of half molecules takes place between different IgG molecules in the serum but *in vitro* this exchange could be induced under mild conditions. Thus after secretion from the cell the IgG molecule retains its structural integrity, both with regard to its constituent polypeptide chains and to its characteristic complement of oligosaccharides.

INTRODUCTION

The C_2-oligosaccharide is an intriguing feature of the molecular structure of rabbit IgG. Being attached to only one of the two H-chains, it confers asymmetry on an otherwise symmetrical molecule. In addition, the C_2-oligosaccharide is present on 80 per cent of the IgG molecules in pooled serum; the remainder lack this carbohydrate (Fanger and Smyth, 1970a). With the ability to separate the molecules that bear the C_2-oligosaccharide from those that do not (see Fig. 3), it has become possible to study the behaviour of the two IgG Types *in vitro* and *in vivo* and hence obtain an insight into the molecular events involved in biosynthesis of the carbohydrate moiety.

EXPERIMENTAL METHODS AND RESULTS

Materials

Rabbit IgG was isolated from pooled rabbit serum (New Zealand and Californian Strains, obtained from White Cloud Farm, Tring, Hertfordshire) by gel filtration on

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Sephadex G-200, chromatography on DEAE-cellulose and chromatography on CM-cellulose (Fanger and Smyth, 1970b). Subsequent separation of IgG fractions was carried out at 4° by stepwise chromatography on DEAE-cellulose at pH 7. Type 1-IgG was eluted in 0·005 M phosphate and Type 2-IgG in 0·02 M phosphate (Fig. 1).

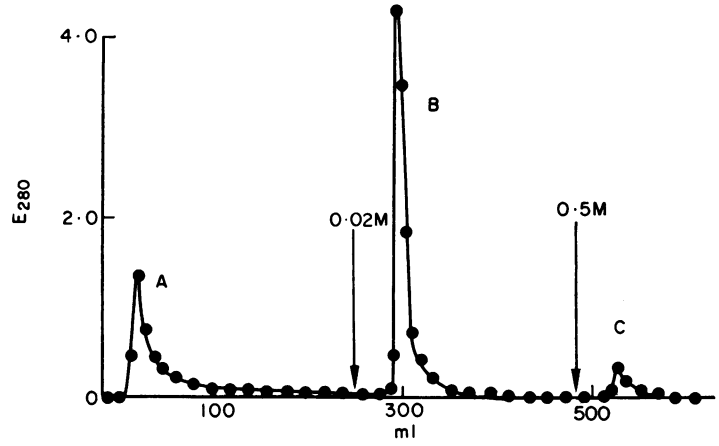


FIG. 1. Isolation of Type 1-IgG and Type 2-IgG by chromatography on DEAE-cellulose. Rabbit IgG (200 mg), obtained by chromatography on DEAE-cellulose in 0·02 M sodium phosphate buffer, pH 7, and then on CM-cellulose (Fanger and Smyth, 1970b), was divided into two fractions (A and B) by rechromatography at 4° on the same DEAE-cellulose column (20×2·5 cm) with 0·005, 0·02 and 0·5 M phosphate as eluents; fraction C represents residual IgG remaining on the column in the 0·02 M eluent. The arrows indicate the points at which the 0·02 and 0·5 M eluents were applied.

Amino sugar analysis

The method employed is described in detail elsewhere (Fanger and Smyth, 1970b); an analysis comparing Type 1-IgG with Type 2-IgG is shown in Fig. 2. Type 1-IgG is almost devoid of galactosamine whereas Type 2 contains one residue of galactosamine per two H-chains.

Iodine labelling of Type 1- *and Type* 2-*IgG*

Labelling was carried out by the method of McFarlane (McFarlane, 1958). Type 1-IgG or Type 2-IgG (6 mg) was treated with [^{131}I] or [^{125}I]iodide (100 μCi), respectively, in the presence of iodine monochloride at pH 8·5. The labelled protein was separated from iodide by gel filtration on Sephadex G-25. To ensure that the chromatographic behaviour of the Type 1- or Type 2-molecules was not changed by iodination, the two labelled proteins were separately chromatographed at 4° on a column (20×2·5 cm) of DEAE-cellulose with 0·005 M sodium phosphate and 0·02 M sodium phosphate pH 7·0 as eluents (Fig. 3). Radioactivity was measured with the aid of a well-type Packard scintillation counter.

In vivo *stability of Type* 1- *and Type* 2-*IgG*

The experiment was designed to determine whether exchange of half molecules of Type 1- and Type 2-IgG takes place in the serum. [^{131}I] Type 1-IgG (1·6 mg containing $3·9\times10^6$ counts/min/mg in 2 ml of 0·005 M sodium phosphate buffer, pH 7) was mixed with [^{125}I] Type 2-IgG (1·6 mg containing $2·4\times10^6$ counts/min/mg in 2·3 ml of 0·02 M phosphate

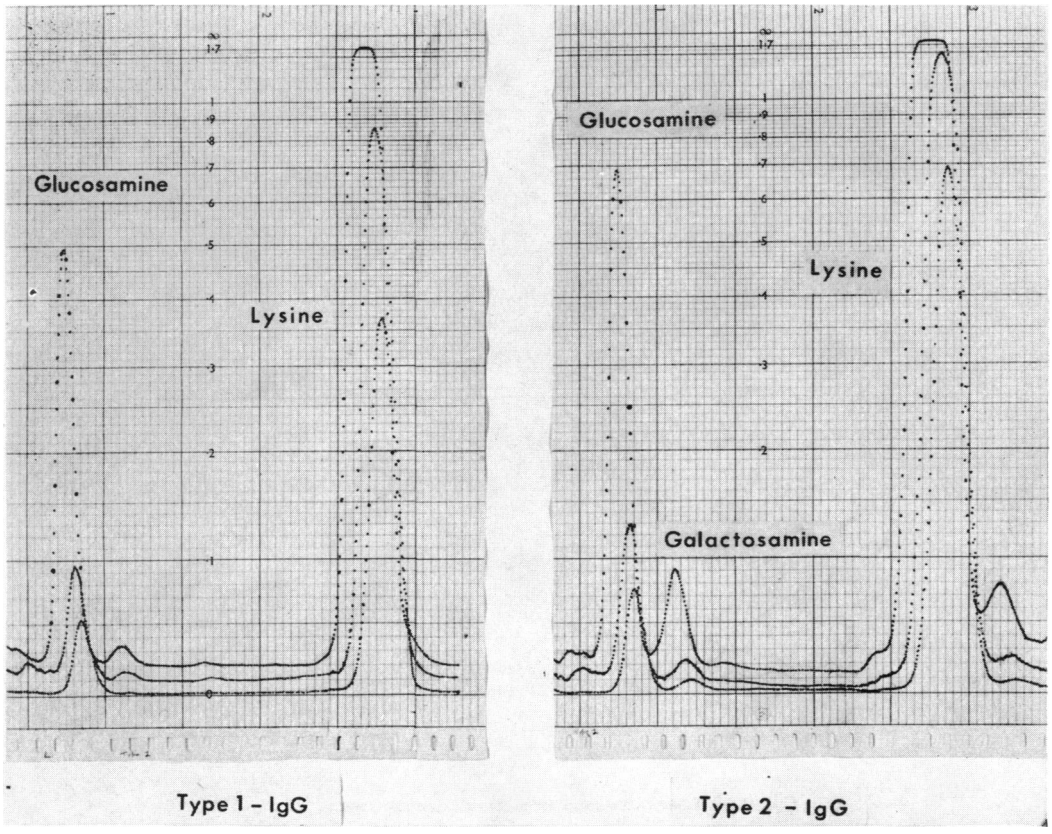


FIG. 2. Amino-sugar analysis of Type 1- and Type 2-IgG. Type 1-IgG (a) or Type 2-IgG (b) (2 mg) was hydrolysed in 6 N HCl at 110° for 6 hours. Residual basic peptides were removed on a column of Dowex 2-X4 (Fanger and Smyth, 1970b) and analysis was carried out on a column (20 × 0·9 cm) of the amino acid analyser using 0·35 M sodium citrate, pH 5·28, as eluent.

buffer, pH 7) and a 4-ml portion was injected through the ear vein of a New Zealand rabbit; a 0·2 ml portion was retained as a control. The serum of the rabbit had previously been analysed to ensure that it contained both Type 1- and Type 2-IgG and would therefore contain enzymes conceivably involved in carbohydrate attachment or removal.

At 3, 6 and 14 days after injection, 15 ml of blood was taken from the ear vein and the serum was separated. The IgG was isolated by gel filtration on Sephadex G-200 and the 7S-fraction was subjected to DEAE-cellulose chromatography; Type 1- and Type 2-IgG were resolved by the routine procedure (see Fig. 1). After 14 days, the distribution of radioactivity between Peak 1 and Peak 2 was: 92 per cent of the ^{131}I radioactivity in the effluent was eluted with the IgG fraction that emerged in the 0·005 M phosphate eluate; 94 per cent of the ^{125}I radioactivity in the effluent was eluted with the IgG fraction that emerged in the 0·02 M phosphate eluate. These results are given in Table 1 together with the results obtained by analysis of IgG isolated from serum after 3 and 6 days. Included in the Table 1 are the results obtained when the control mixture of [^{131}I] Type 1- and [^{125}I] Type 2-IgG, added to unlabelled 'bulk IgG' to match the composition of the 16 day IgG solution, was chromatographed as above.

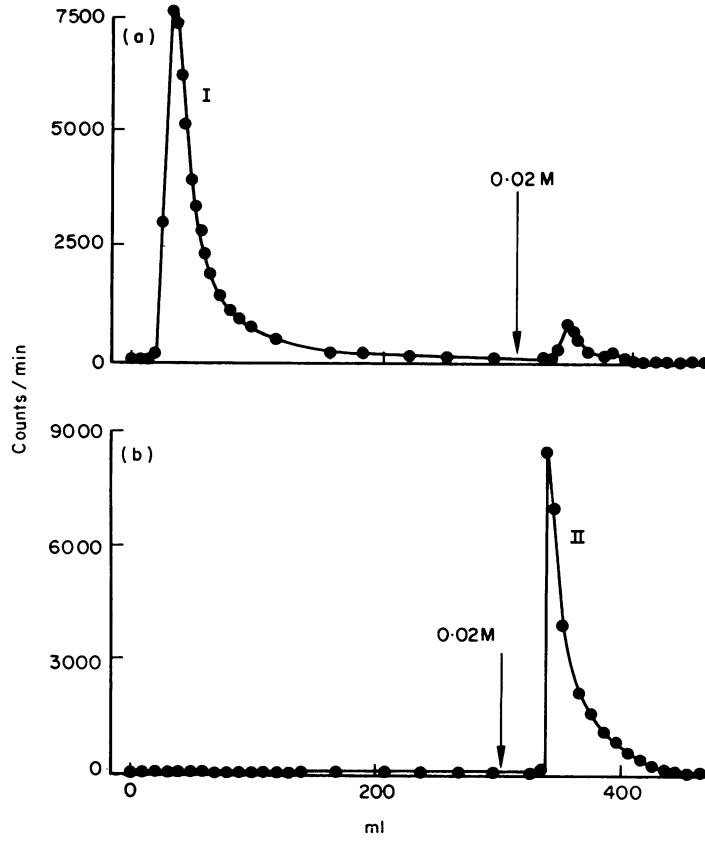


FIG. 3. Chromatography of ^{131}I Type 1-IgG and ^{125}I Type 2-IgG on DEAE-cellulose. (a) ^{131}I Type 1-IgG, (b) ^{125}I Type 2-IgG. ^{131}I Type 1-IgG (5 mg containing 2×10^4 counts/min/mg) or ^{125}I Type 2-IgG (20 mg containing 6×10^3 counts/min/mg) was added to a column ($20 \times 2{\cdot}5$ cm) of DEAE-cellulose at 4° with 0·005 M phosphate buffer, pH 7, as eluent; at 300 ml, as indicated by the vertical arrow, the eluate was changed to 0·02 M phosphate. Radioactivity in effluent fractions was determined with the aid of a well-type Packard scintillation counter.

TABLE 1

CHROMATOGRAPHY OF A MIXTURE OF ^{131}I TYPE 1-IgG AND ^{125}I TYPE 2-IgG ON DEAE-CELLULOSE AFTER ISOLATION FROM SERUM

	Distribution of ^{131}I (per cent)		Distribution of ^{125}I (per cent)	
	Type 1	Type 2	Type 1	Type 2
3 days	95	5	3	97
6 days	94	6	2	98
14 days	92	8	6	94
Control	93	7	2	98

A mixture of ^{131}I Type 1- and ^{125}I Type 2-IgG was injected into a New Zealand strain rabbit. At intervals, blood was taken and IgG isolated from the serum. The IgG was resolved into Type 1- and Type 2-molecules by chromatography on DEAE-cellulose and the ^{131}I and ^{125}I radioactivity associated with each type was measured.

It is clear that the distribution of radioactivity between the two chromatographic fractions is the same when a mixture of the two IgG Types is chromatographed directly or when the mixture is first injected into the circulation of the rabbit, reisolated, and then chromatographed (see Table 1). In each case, the labelled IgG molecules retained their initial chromatographic behaviour.

In order to verify the results obtained with the New Zealand strain rabbit, the same protocol was applied to two Himalayan rabbits; the two IgG-Types were again shown to retain their structural integrity in the serum.

In vitro *stability of Type* 1- and *Type* 2-*IgG*

(1) *Hybridization between half molecules of Type* 1- *and Type* 2-*IgG*. The experiment was designed to determine whether combination would take place *in vitro* between a basic half molecule and an acidic half molecule. The reaction conditions were chosen to bring about selective reduction of the inter H-chain disulphide bridges (Hong and Nisonoff, 1965) and the resulting half molecules were allowed to reassociate to form hybrids. It was anticipated that the combination of a basic half molecule from Type 1-IgG with an acidic half molecule from Type 2-IgG would provide a hybrid four-chain molecule with altered chromatographic properties (Fig. 4).

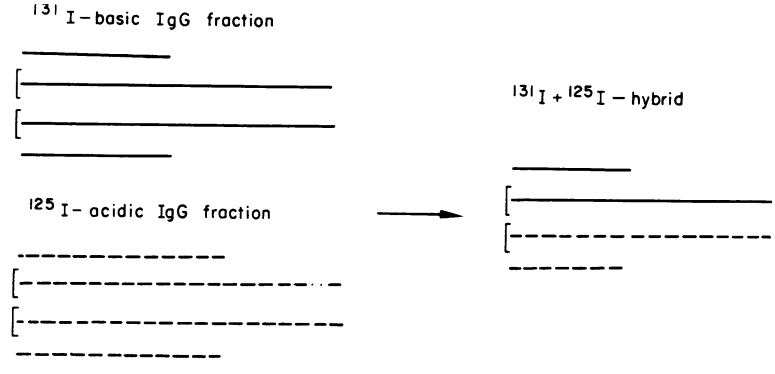


FIG. 4. Diagram illustrating the formation of a hybrid IgG molecule by the association of half molecules from a mixture of basic and acidic IgG fractions.

TABLE 2

In vitro HYBRIDIZATION OF HALF MOLECULES FROM ^{131}I TYPE 1-IgG AND ^{125}I TYPE 2-IgG

	Distribution of ^{131}I (per cent)		Distribution of ^{125}I (per cent)	
	Type 1	Type 2	Type 1	Type 2
Hybridized mixture	58	42	41	59
Control	94	6	2	98

A mixture of ^{131}I Type 1-IgG (0·2 mg, 7×10^5 counts/min/mg) and ^{125}I Type 2-IgG (0·4 mg, 2×10^5 counts/min/mg) was added to an unlabelled mixture of the two IgG Types (30 mg; ratio of Type 1 to Type 2 was 1 : 4) and reduction was carried out in 0·0014 M mercaptoethanol at pH 8. The solution of half molecules was dialysed at 4° for 8 hours against 0·15 M NaCl containing 0·02 M phosphate buffer, pH 7, then overnight against 0·005 M phosphate prior to chromatography on DEAE-cellulose.

[^{131}I] Type 1-IgG (0·2 mg containing 7×10^5 counts/min/mg) was mixed with [^{125}I] Type 2-IgG (0·4 mg containing 2×10^5 counts/min/mg) in 5 ml of 0·2 M sodium phosphate buffer, pH 8. IgG (30 mg), containing unlabelled Type 1- and Type 2-IgG in the 1 : 4 ratio present in pooled serum, was added. Mercaptoethanol was added to a final concentration of 0·0014 M and the solution was maintained at 30° for 2 hours. The solution was dialysed for 8 hours at 4° against a buffer of 0·15 M sodium chloride containing 0·02 M phosphate at pH 7 and then overnight against 0·005 M phosphate buffer, pH 7. After centrifugation, chromatography was performed at 4° on DEAE-cellulose with 0·005 M and 0·02 M phosphate buffers. The distribution of radioactivity between the two chromatographic fractions is given in Table 2. In a control experiment, the mixture of the two IgG Types was treated identically but with omission of the reducing agent.

The distribution of radioactivity on chromatography of the hybridized mixture of Type 1- and Type 2-IgG was markedly different from the control value (Table 2), and indicates that exchange of half molecules has taken place. This *in vitro* result contrasts with the unchanged chromatographic behaviour that was exhibited by the [^{131}I] Type 1- and the [^{125}I] Type 2-IgG molecules isolated from serum in the *in vivo* experiment (Table 1). Thus it appears that exchange of half molecules can take place *in vitro* but does not *in vivo*. The reproducibility of the behaviour of the two IgG types in the control experiments emphasizes the considerable change that was noted with the *in vitro* hybridized mixture, care being taken in all experiments to employ the exact concentration of the two eluents.

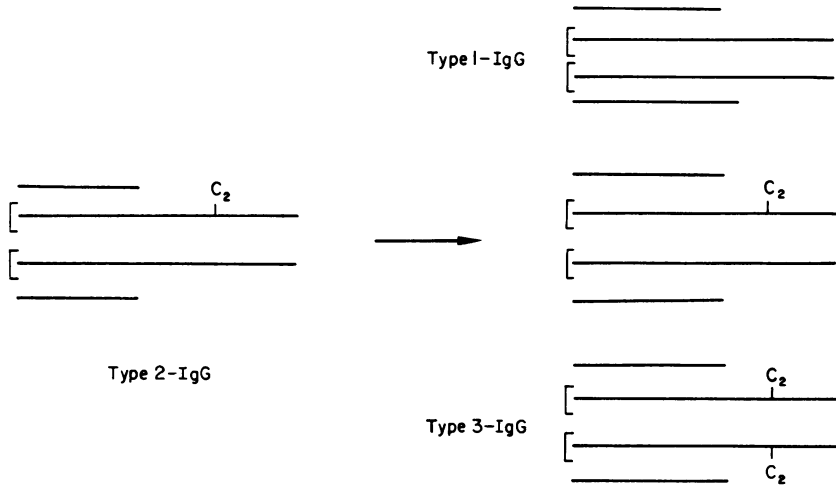


FIG. 5. Diagram illustrating hybridization between half molecules of Type 2-IgG, forming an IgG molecule with the C_2-oligosaccharide on both H-chains (Type 3-IgG).

The results of these experiments are consistent with the finding of Nisonoff and his colleagues (Seth, Nisonoff and Dray, 1965) that under similar reducing conditions a half molecule carrying a single H-chain allotype is able to combine with a half molecule carrying a different allotype.

(2) *Hybridization between half molecules of Type 2-IgG.* With the finding that half molecules from acidic and basic IgG fractions are able to associate, hybridization was attempted on the asymmetric Type 2-molecule alone (Fig. 5), to determine whether a symmetrical di-C_2 molecule (Type 3-IgG) could be formed *in vitro*.

Type 2-IgG (22 mg) was reduced in 0·0014 M mercaptoethanol at pH 8 for 2 hours. After dialysis for 8 hours at 4° against 0·15 M NaCl containing 0·02 M phosphate buffer, pH 7, and 0·005 M phosphate buffer overnight, chromatography was performed on DEAE-cellulose with 0·005 M phosphate, 0·02 M phosphate and 0·5 M phosphate buffers as eluents. The high ionic strength phosphate was employed to elute the most acidic IgG fraction formed during the hybridization. The IgG species eluted in each of the three chromatographic positions was analysed for amino-sugar content. The fraction emerging in 0·005 M phosphate contained 0·20 residue galactosamine per half molecule; that in 0·02 M phosphate, 0·6 residue galactosamine; and the most acidic fraction, eluted in 0·5 M phosphate, contained 0·77 residue galactosamine. The values were calculated relative to glucosamine = 5·0 (Fanger and Smyth, 1970a).

The galactosamine is present exclusively in the C_2-oligosaccharide of IgG and hence the values reflect the number of C_2-oligosaccharide chains per half molecule of IgG. It is clear that if all the molecules in a given fraction were asymmetric, containing the C_2-oligosaccharide on only one of the two H-chains, then the galactosamine content would be 0·5 residue per half molecule of IgG. Since the most acidic IgG fraction contained 0·77 residue of galactosamine, a significant proportion of these molecules must contain C_2 on both H-chains. On the other hand, the IgG molecules eluted to 0·005 M phosphate contained a low galactosamine content; the majority of these molecules were devoid of the C_2-oligosaccharide.

(3) *Removal of sialic acid from Type 2-IgG.* The experiment was designed to determine whether removal of the sialic acid component from Type 2-IgG would result in an alteration in chromatographic properties. Type 2-IgG (18 mg) was dialysed at pH 2·4, 4°, for 12 hours, and then dialysed against 0·005 M phosphate buffer, pH 7 overnight. After centrifuging the solution, chromatography was performed on DEAE-cellulose under the standard conditions. The distribution of radioactivity between the two fractions revealed a significant change in the chromatographic behaviour of the Type 2-molecules, 24 per cent of which chromatographed in the 'Peak 1' position after incubation at acid pH. This result is consistent with the view that removal of the C_2-oligosaccharide, which contains two sialic acid residues, from Type 2-IgG would cause a proportion of the molecules to become sufficiently basic to chromatograph in the 'Peak 1' position. Attempts to remove sialic acid from the intact Type 2 molecule with neuraminidase were not successful, although sialic acid could be partially removed from the isolated C_2-glycopeptide on treatment with this enzyme.

DISCUSSION

A major fraction of the IgG molecules in rabbit serum contains the C_2-oligosaccharide attached to one H-chain only (Fig. 6) while a minor fraction is devoid of the C_2-oligosaccharide (Fanger and Smyth, 1970b). This diversity with respect to the C_2-carbohydrate

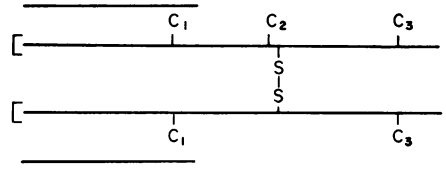


FIG. 6. Chain structure of the Type 2-IgG molecule, illustrating the distribution of the C_1-, C_2- and C_3-oligosaccharides.

could arise either in the serum or in the biosynthetic cell. The existence of two distinct populations of IgG molecules in the serum raises the possibility that one might be the precursor of the other, the carbohydrate being removed from Type 2-IgG or added to Type 1-IgG during circulation. Another possibility is that in the cell only symmetrical molecules are synthesized and asymmetry could arise by exchange of half molecules in the serum. If this were the case, the C_2-free molecules in the serum might exchange rapidly with di-C_2 molecules, thus accounting for the absence of a di-C_2 molecule in the circulation.

Within the cell, on the other hand, half molecules with the C_2-oligosaccharide might associate preferentially with half molecules devoid of C_2, forming the asymmetric molecule; alternatively, the carbohydrate might be added only after the four chain molecule has been assembled.

The mechanisms differ if the C_2-oligosaccharide can undergo addition or deletion by serum enzymes, if exchange of half molecules can take place in the circulation, and if preferential association takes place between half molecules. Accordingly, we investigated the *in vivo* and the *in vitro* stability of the two IgG Types.

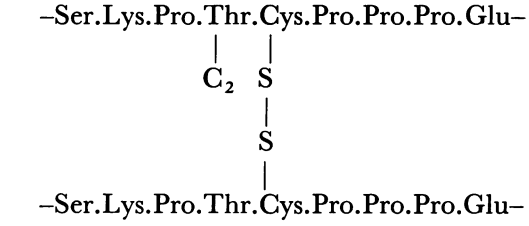
A basic population of IgG molecules which lacks the C_2-oligosaccharide (Type 1-IgG) and an acidic population which carries the C_2-oligosaccharide (Type 2-IgG) were labelled with different isotopes and injected into an animal; the two IgG Types were reisolated from the serum at intervals and examined to find whether each isotope was still attached to its initial IgG molecule. During 14 days in the circulation, both Type 1- and Type 2-IgG were found to retain their respective radioactive labels (Table 1). Had exchange taken place between half molecules of the basic and acidic IgG populations, a change would have been expected in the distribution of label on chromatography, due to the formation of hybrids. Indeed, such an exchange between half molecules was readily induced *in vitro* by subjecting a mixture of Type 1- and Type 2-IgG to mild reducing conditions, which can give rise to half molecules, and then allowing reassociation to take place with the formation of four-chain molecules (Table 2). That no change in chromatographic behaviour was seen in the *in vivo* experiment, when Type 1- and Type 2-IgG were reisolated from the circulation, establishes that exchanges of half molecules do not occur in the serum.

It is further seen that the C_2-oligosaccharide is not added to the molecules of Type 1-IgG or removed from Type 2-IgG during circulation. When Type 2-IgG was exposed to mild acid conditions *in vitro*, which removes a portion of the sialic acid component, a significant proportion of the molecules exhibited an altered chromatographic behaviour. As no change was found with Type 2-IgG *in vivo*, it would appear that the initial carbohydrate moiety remains attached to the parent IgG molecule throughout its time in the circulation. Preservation of chromatographic properties was exhibited also by Type 1 molecules during circulation; hence the acidic C_2-oligosaccharide does not become attached to the molecule of Type 1-IgG in the serum. These experiments demonstrate that the IgG molecule retains both its initial polypeptide chains and its initial complement of oligosaccharide throughout its life span in the circulation. It is concluded that the carbohydrate moiety is added to the IgG molecule intracellularly.

The Type 2-IgG molecule in the serum contains the C_2-oligosaccharide on one H-chain only. The question arises: is the C_2-oligosaccharide attached to the H-chain on the ribosome or is it attached to the H-chain after assembly of the polypeptide chains has been completed? If the former, dimerisation of H-chains, which takes place at an early stage in bio-

synthesis (Askonas and Williamson, 1968), would at once lead to the formation of a symmetrical molecule containing two C_2-oligosaccharide chains; yet strikingly no di-C_2 molecule has been found in the serum. It might still be possible that the symmetrical association of two C_2-containing chains is prevented by steric hindrance, the two oligosaccharides presenting an obstruction to coupling of the H–H disulphide bridge. However, the successful reconstitution of C_2-containing half molecules *in vitro* to form di-C_2 molecules shows that the symmetrical structure can form without difficulty. That the di-C_2 molecule is not found *in vivo* suggests that the C_2-oligosaccharide is added to the H-chain after the H-chain dimerises or after the polypeptide chains have associated to form a four-chain molecule.

In the primary structure of the hinge region of rabbit IgG (Smyth and Utsumi, 1967; Givol and Delorenzo, 1968; Cebra, Porter and Steiner, 1968), the C_2-oligosaccharide is coupled through a threonine residue immediately adjacent to the inter H-chain disulphide bridge (Fanger and Smyth, 1970b) and it is reasonable to consider this a congested area with respect to enzyme action.



Thus the absence of a symmetric di-C_2 molecule *in vivo* could be explained on the basis that attachment of the second C_2-oligosaccharide by an enzyme may be prevented by the presence of the first oligosaccharide and the neighbouring disulphide bridge. Alternatively, the enzyme responsible may recognize only a symmetrical region lacking carbohydrate; after attachment of the first C_2-oligosaccharide, the region next to the H–H disulphide bridge is rendered asymmetric and the enzyme might have less affinity for the new substrate. In either case, it is clear that the C_2-oligosaccharide is not attached to the H-chain until after it has associated with a second H-chain during the assembly of the IgG molecule.

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