The Formation of Pyrrolid-2-one-5-carboxylic Acid at the *N*-Terminus of Immunoglobulin G Heavy Chain

By D. I. STOTT* and A. J. MUNRO†

Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, U.K.

(Received 4 February 1972)

We propose that pyrrolid-2-one-5-carboxyl-tRNA is not involved in the initiation of protein synthesis in eukaryotic cells and that the *N*-terminal pyrrolid-2-one-5-carboxylic acid group of an IgG (immunoglobulin G) (that secreted by the mouse plasmacytoma Adj PC5) is formed by the enzymic cyclization of the *N*-terminal glutamine of the heavy chain of the completed IgG molecule and that the cyclization takes place inside the cell. We base these conclusions on the following evidence. (1) Pyrrolidonecarboxyl-tRNA was not found in incorporation experiments with rat liver preparations and [U-¹⁴C]-pyrrolidonecarboxylic acid, glutamic acid and glutamine, even though an incorporation extent of less than 2% of the total products could have been detected. (2) Double-labelling experiments showed that less than 8% of the nascent peptides of heavy chains (those obtained by precipitation by the antibody to Fc fragment) began with pyrrolidonecarboxylic acid. (3) Further double-labelling experiments showed that 60–66% of the heavy chains of the completed intracellular IgG molecule began with pyrrolidonecarboxylic acid after both 1 and 5 h of labelling. (4) The IgG, after secretion by plasmacytoma Adj PC5, was found to have the sequence [Glu-Val-Gln-Leu- at the *N*-termini of the heavy chains.

Pyrrolid-2-one-5-carboxylic acid has been found at the *N*-termini of the polypeptide chains of immunoglobulins and a number of other proteins and peptides (Blombäck, 1967). This has led certain authors (Moav & Harris, 1967; Bernfield & Nestor, 1968; Rush & Starr, 1970) to suggest that pyrrolidonecarboxyl-tRNA might be involved in the initiation of protein synthesis in eukaryotic cells. Other stages of protein synthesis at which *N*-terminal pyrrolidonecarboxylic acid might be formed are: (i) during synthesis of the nascent polypeptide chain on the ribosome; (ii) during and/or after assembly of the protein molecule inside the cell; (iii) after secretion of the protein by the cell. We have attempted to determine at which of these stages *N*-terminal pyrrolidonecarboxylic acid is formed by using a cell-free rat liver preparation and the cells of mouse myeloma Adj PC5, which secretes IgG‡ and a κ Bence-Jones protein.

In approaching this problem great care must be taken to prevent the cyclization of glutamine to pyrrolidonecarboxylic acid, which occurs readily at elevated temperatures in mildly acidic or alkaline

solutions, though not at neutral pH (Chibnall & Westall, 1932; Vickery *et al.*, 1935; Hamilton, 1945; Gilbert *et al.*, 1949). That *N*-terminal pyrrolidonecarboxylic acid is not an artifact, however, is suggested by the existence of several enzymes that catalyse the formation of pyrrolidonecarboxylic acid, the most notable of these being glutamine cyclotransferase. This enzyme catalyses the conversion of glutamine into pyrrolidonecarboxylic acid and glutamine peptides into pyrrolidonecarboxylic acid peptides (Messer, 1963; Messer & Ottesen, 1964, 1965). Our results indicate that the *N*-terminal pyrrolidonecarboxylic acid group of the IgG secreted by mouse myeloma Adj PC5 is formed by the enzymic cyclization of the *N*-terminal glutamine on the heavy-chains of fully assembled IgG inside the cell.*

Materials and Methods

Solutions and reagents

NEK medium contained 0.15M-NaCl, 10mM-EDTA and 10mM-potassium phosphate buffer, pH7.0. NLM medium contained 75mM-NaCl, 10mM-MgCl₂ and 25mM-KH₂PO₄, pH6.5. PBS medium contained 0.15M-NaCl and 10mM-potassium phosphate buffer, pH7.4. TKM medium contained 20mM-tris, 0.1M-KCl and 5mM-MgCl₂, pH7.8. DL-[G-³H]Valine, L-[U-¹⁴C]valine and L-[U-¹⁴C]glutamine were purchased from The Radiochemical

* Present address: Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, U.K.

† Present address: Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, U.K.

‡ Abbreviation: IgG, immunoglobulin G.

Centre, Amersham, Bucks., U.K. Carboxypeptidase A (66 units/mg; di-isopropyl phosphorofluoridate-treated) was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A., Pronase (B grade; 45 proteolytic units/mg) from Calbiochem, Los Angeles, Calif., U.S.A., and Nonidet P-40 from Shell Chemicals (U.K.) Ltd., London S.E.1, U.K.

Pyrrolidonecarboxylic acid

L-[U-¹⁴C]Pyrrolidonecarboxylic acid was prepared from L-[U-¹⁴C]glutamine (50 μ Ci, 38.8 mCi/mmol) by the method of Dearborn & Stekol (1950) and purified by passage through a column (1.5 $\times$ 1.2 cm) of sulphonated polystyrene in the acid form, followed by paper electrophoresis at pH 3.5, 5000 V, 35 min. The [U-¹⁴C]pyrrolidonecarboxylic acid was eluted with 0.1 M-NH₃ and freeze dried. Its radioactive purity measured by paper electrophoresis (as above) was greater than 97%.

Plasma-cell tumour Adj PC5

This tumour, which secretes IgG_{2a} and a κ Bence-Jones protein, was kindly provided by Dr. M. Potter of the National Cancer Institute, Bethesda, Md., U.S.A. It was grown and maintained by serial subcutaneous transplantation on Balb/c mice. Intracellular and secreted myeloma protein were labelled for various times with either L-[U-¹⁴C]valine (3 μ Ci/ml) or DL-[G-³H]valine (500 μ Ci/ml) by the method of Knopf *et al.* (1967). At the end of each series of incubations samples of the medium were tested for bacterial contamination on nutrient agar at 37°C for 10 days and were always negative.

Purification of Adj PC5 IgG, heavy-chain and N-terminal peptides

Adj PC5 IgG was purified from four sources: (1) the serum of mice that bore plasma-cell tumour Adj PC5, to provide unlabelled secreted IgG; (2) solid tumours, to provide unlabelled intracellular IgG; (3) medium in which cells had been incubated *in vitro* with [U-¹⁴C]valine, to provide ¹⁴C-labelled secreted IgG; (4) cells incubated *in vitro* with [G-³H]valine, to provide ³H-labelled intracellular IgG.

The only difference in the purification procedure for IgG from each of the four sources was in the initial preparation of the material. All procedures were done at 0–4°C unless otherwise stated. Serum required no preliminary treatment. Solid tumours, which had been stored at –20°C, were thawed quickly by rinsing three times in NLM medium at 37°C, chilled to 0°C, an equal volume of 1% (v/v) Nonidet P-40 in NLM medium at 0°C was added and the tumours were blended in a Waring Blender for 4 min.

Cell debris was removed by centrifugation (10000g for 10 min) and the supernatant was filtered through glass-wool to remove lipid. Incubation medium was centrifuged (10000g for 10 min) to remove cell debris, and Adj PC5 serum (5 ml) was added as a source of carrier IgG. The ³H-labelled cells, stored at –20°C, were thawed rapidly and suspended in cold NLM medium at 5×10^8 – 1×10^9 cells/ml. Nonidet P-40 was added to the cell suspension to a final concentration of 1% (v/v) and the suspension was homogenized in a Dounce homogenizer, having a clearance of 25 μ m (0.001 in), with six strokes of the ball plunger. Cell breakage was 90–100%. Nuclear and cell debris were removed by centrifugation (10000g for 10 min) and ribosomes were removed by centrifugation of the supernatant (165000g for 2.5 h, Spinco type 50 angle-head rotor). This procedure is modified from that of Knopf *et al.* (1967). Adj PC5 serum (5 ml) was added to the 165000g supernatant as a source of carrier IgG. From this point onwards the procedures were identical for material from all four sources. IgG was purified by (NH₄)₂SO₄ precipitation followed by chromatography on DEAE-cellulose and CM-cellulose, as described by Knopf *et al.* (1967). The purity of unlabelled IgG was tested by immunoelectrophoresis with development against anti-(normal mouse serum). The ³H- and ¹⁴C-labelled IgG preparations were tested by precipitation with 5% (w/v) trichloroacetic acid and anti-(Adj PC5 IgG). More than 95% of the trichloroacetic acid-precipitable radioactivity in each sample was also precipitable by anti-IgG, after correction for non-specific precipitation. Purified ¹⁴C-labelled secreted IgG was combined with purified ³H-labelled intracellular IgG to provide a standard for correction of losses of ³H-labelled material during subsequent procedures.

Heavy chains were prepared by reduction and alkylation of IgG, and the chains were separated by gel filtration on Sephadex G-100 in 1 M-propionic acid as described by Notani *et al.* (1966). Samples of ³H/¹⁴C-labelled heavy chain were analysed for contamination by light chains by disc electrophoresis on polyacrylamide gels containing 0.1% sodium lauryl sulphate, 8 M-urea and 10 mM-sodium phosphate buffer, pH 7.2 (modified from Summers *et al.*, 1965; Choules & Zimm, 1965). Less than 3% of the total radioactivity was found in the light-chain region. The blocked N-terminal heavy-chain peptides were prepared by the method used for rabbit IgG by Wilkinson *et al.* (1966). Heavy chains were digested with Pronase, the digest was passed through a column of sulphonated polystyrene and the pyrrolidonecarboxylic acid peptides, which were not retained by the column, were separated from the glycopeptide by gel filtration on Sephadex G-25. The pyrrolidonecarboxylic acid peptides were then separated by paper electrophoresis at pH 3.5. The position of

radioactive peptides was determined by running marker peptides, previously prepared by the same method, on side strips that were then stained by the starch-iodide method of Rydon & Smith (1952). The unstained peptides were eluted with 0.1M-NH₃ and freeze-dried.

Analysis of peptides

Peptides were hydrolysed in 6M-HCl under vacuum in a sealed tube at 110°C for 24h, and analysed on a Beckman amino acid analyser. Digestion with carboxypeptidase A was done at an enzyme/substrate ratio of 1:2 (w/w) in 0.2M-N-ethylmorpholine-acetate buffer, pH8.4, at 37°C for various times. Control samples containing no enzyme or no substrate were treated in the same way. The digests were terminated by freezing and either were analysed by paper electrophoresis at pH2.0 or peptides with N-terminal pyrrolidonecarboxylic acid were removed by loading the digest on a column (1.5cm × 1.2cm) of sulphonated polystyrene. Pyrrolidonecarboxylic acid peptides were washed through with water and amino acids were eluted with 2M-NH₃. The water eluate was acid-hydrolysed as above and analysed with the ammonia eluate by paper electrophoresis at pH3.5.

The N-terminal amino acid of the heavy chain of intracellular Adj PC5 IgG was determined by the method of Fraenkel-Conrat (1954).

Antisera

Antisera were raised in white or red New Zealand rabbits by intramuscular injection of 5–10mg of pure sterile antigen in Freund's complete adjuvant every 2 weeks for 6 weeks and then every 3 months or whenever the titre of antibody fell significantly.

Rabbit anti-(Adj PC5 Fc fragment) was prepared by absorption of rabbit anti-(Adj PC5 IgG) with Adj PC5 Fab (pepsin) fragment and had no detectable anti-(Fab fragment) activity on analysis by immunodiffusion. IgG with anti-(Fc fragment) activity was purified from the antiserum by precipitation with (NH₄)₂SO₄ at 35% saturation and chromatography on DEAE-cellulose (Levy & Sober, 1960).

Nascent peptides

Ribosomes prepared from cells incubated *in vitro* with [³H]valine were resuspended in 1.5ml of TKM medium at 0°C, layered on top of 3.5ml of 1M-sucrose in TKM medium and centrifuged at 100000g at 2°C in a Spinco SW50 rotor for 14h (modified from the method of Munro *et al.*, 1964). The pellet was resuspended in TKM medium and washed a second time by the same procedure. The washed

ribosomes were dissociated in 2.0ml of medium containing 0.1% sodium lauryl sulphate, 6M-urea and 20mM-potassium phosphate buffer, pH7.4, and dialysed against PBS medium at 4°C. The sodium lauryl sulphate precipitated out during dialysis and was removed by centrifugation at 10000g for 30min. Sodium dextran sulphate was added to a final concentration of 100μg/ml to inhibit ribonuclease. Samples (10μl) were removed for precipitation with trichloroacetic acid and cetyltrimethylammonium bromide. The remainder was precipitated with rabbit IgG anti-(Adj PC5 Fc fragment) at 37°C for 1h followed by excess of sheep anti-(rabbit IgG), incubation being continued for a further 1h at 37°C and then overnight at 4°C. A 10μl control sample was treated in the same manner with normal rabbit IgG instead of anti-(Fc fragment). A precipitate of bovine serum albumin-anti-(bovine serum albumin) (0.5mg) suspended in PBS medium (0.5ml) was added to each sample as carrier, and the precipitates were washed five times with PBS medium. The pellet from the main sample was resuspended in 1.0ml of water and portions were removed from both the pellet and supernatant for measurement of radioactivity. The ¹⁴C-labelled Adj PC5 heavy chain prepared from the IgG secreted by cells incubated with [¹⁴C]valine for 5h was added to the anti-(Fc fragment) pellet to give a ³H/¹⁴C ratio of approx. 10:1, and 2mg of unlabelled heavy chain was also added as carrier. The mixture was then fully reduced and alkylated by a modification of the procedure of Beale & Feinstein (1969). The sample was incubated at 37°C for 1h in medium containing 50mM-dithiothreitol, 6M-guanidinium chloride, 10mM-EDTA and 0.5M-tris, pH8.5, under N₂. The solution was then adjusted to 0.15M with respect to iodoacetamide, and its incubation continued at 4°C for 1h followed by exhaustive dialysis against water at 4°C. The N-terminal pyrrolidonecarboxylic acid peptides were prepared by digestion with Pronase, chromatography on sulphonated polystyrene and paper electrophoresis at pH3.5, as for whole heavy chain, except that gel filtration on Sephadex G-25 was omitted since insufficient glycopeptide was present to interfere with the subsequent electrophoresis.

Determination of radioactivity of protein and peptides

(i) ³H- or ¹⁴C-labelled protein samples that had been precipitated at 0°C with 5% (w/v) trichloroacetic acid were filtered and washed on glass-fibre paper discs (Whatman GF/C) with 5% trichloroacetic acid followed by ethanol-ether (1:1, v/v) and ether, dried, immersed in 3ml volumes of toluene containing 2,5-diphenyloxazole (5g/l) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (50mg/l) and their radioactivities measured in a liquid-scintillation counter.

(ii) ^3H - or ^{14}C -labelled samples that had been precipitated with antiserum were incubated at 37°C for 1 h and at 4°C overnight in NEK medium. Control tubes contained quantities of bovine serum albumin and anti-(bovine serum albumin), instead of the appropriate antiserum, calculated to give the same weight of antigen-antibody precipitate. Precipitates were collected on glass-fibre discs by filtration and washed six times with NEK medium, once with ethanol-ether etc., as for the trichloroacetic acid precipitates.

(iii) ^3H -labelled peptidyl-tRNA attached to ribosomes was precipitated with cetyltrimethylammonium bromide and washed on glass-fibre discs (Jackson & Hunter, 1970; R. Jackson, unpublished work).

(iv) $^3\text{H}/^{14}\text{C}$ -labelled heavy chains and peptides were freeze-dried in scintillation bottles, dissolved in 0.1 M-NaOH (0.5 ml), neutralized with 0.22 M-acetic acid (0.25 ml), and 10 ml of Triton-toluene scintillator (2,5-diphenyloxazole, 15 g; toluene, 2 litres; Triton-X100, 1 litre) was added to each sample. The radioactivities of the samples were counted on a Beckman LS150 liquid-scintillation counter with channel settings such that, with standard samples, not more than 0.5% of the ^3H counts appeared in the ^{14}C channel and not more than 30% of the ^{14}C counts appeared in the ^3H channel. Corrections were made for variations in counting efficiency by the external-standard ratio method.

Results

Incorporation of [^{14}C]pyrrolidonecarboxylic acid into tRNA

With a cell-free system containing rat liver cell sap prepared by the method of Munro *et al.* (1964) and rat liver tRNA, [^{14}C]pyrrolidonecarboxylic acid was found to be incorporated into tRNA to form glutamyl- and glutaminyl-tRNA. Incorporation was inhibited by 90% in the presence of unlabelled glutamic acid at 100 times the concentration of [^{14}C]pyrrolidonecarboxylic acid, but not in the presence of unlabelled glutamine, suggesting that pyrrolidonecarboxylic acid is incorporated into tRNA via glutamic acid. These results are in agreement with the findings of Rush & Starr (1970) for rabbit liver. The latter authors also found free pyrrolidonecarboxylic acid on hydrolysis of charged tRNA, and Moav & Harris (1967) also claimed to detect it bound to tRNA. Unlike these authors, we have been unable to find any pyrrolidonecarboxylic acid bound to tRNA, although less than 2% of the total product could have been detected in this form. We suggest that the pyrrolidonecarboxylic acid that the above authors found was an artifact resulting from the spontaneous cyclization of glutamine.

N-Terminal sequence of the heavy chain of Adj PC5 IgG prepared from serum

After digestion of the heavy chain of myeloma protein Adj PC5 with Pronase, two peptides (α and β) with blocked *N*-terminal residues were prepared. From amino acid analysis and digestion of these peptides with carboxypeptidase A, the following sequences were deduced:

where [indicates a blocked α -amino group. It was deduced that the blocked *N*-terminal residues were pyrrolidonecarboxylic acid from the following evidence: (1) the peptides were not retained by a column of sulphonated polystyrene; (2) they did not react with ninhydrin, but gave a positive starch-iodide reaction; (3) peptide β was digested with carboxypeptidase A and washed through a sulphonated polystyrene column with water. The peptide in the water eluate was identical with synthetic [Glu-Val (kindly given by Dr. E. M. Press) in (a) starch-iodide reaction, (b) amino acid composition (namely Glu:Val, 1:1), (c) electrophoretic mobility at pH 6.5 ($m = 0.67$ and 0.7 respectively relative to pyrrolidonecarboxylic acid), (d) mobility by thin-layer chromatography in silica gel in butan-1-ol-acetic acid-water (4:1:5, by vol.) ($R_F = 0.48$ for both).

N-Terminal residue of the heavy chain of intracellular Adj PC5 IgG

N-Terminal analysis of heavy chain prepared from unlabelled intracellular IgG revealed a major product with the same mobility ($R_F = 0.66$) as the phenylthiohydantoin derivative of glutamine, and one minor product with the mobility ($R_F = 0.38$) of the phenylthiohydantoin derivative of histidine, on paper chromatography in heptane-butan-1-ol-75% (v/v) formic acid (40:30:9, by vol.). The minor product is probably due to an impurity and there were no other detectable products. This suggests that the *N*-terminal residue of some of the heavy chains of intracellular Adj PC5 IgG is glutamine.

Pyrrolidonecarboxylic acid formation in nascent peptides

The following procedure showed that the pyrrolidone ring of pyrrolidonecarboxylic acid is not cleaved during digestion with Pronase. Pyrrolidonecarboxylic acid (2 mg/ml) was digested with Pronase (0.4 mg/ml) in 0.2 M-*N*-ethylmorpholine-acetate buffer, pH 8.4, at 37°C . Samples (50 μl) were removed after 24 and 45 h and subjected to paper electrophoresis at pH 3.5 at 4000 V for 25 min. The anodic half of the paper was stained with starch-iodide

reagent and the cathodic half with ninhydrin. No ninhydrin-positive material was detectable, but a strong spot with the mobility of pyrrolidonecarboxylic acid was present. Cleavage of less than 0.2% of the pyrrolidonecarboxylic acid would have been readily detectable.

The percentage of nascent heavy-chain peptides in the pyrrolidonecarboxylic acid form in Adj PC5 plasma cells was measured in the following manner. Ribosomes were isolated from Adj PC5 cells that had been incubated with [^3H]valine for 1 h. They were washed, the peptidyl-tRNA was released, portions were treated with trichloroacetic acid and cetyltrimethylammonium bromide, and the precipitates were assayed for radioactivity. Cetyltrimethylammonium bromide specifically precipitates RNA, but not protein, giving a measure of the purity of the peptidyl-tRNA (Jackson & Hunter, 1970). Some 93% of the trichloroacetic acid-precipitable radioactivity was precipitated by cetyltrimethylammonium bromide, showing that no more than 7% of the radioactivity was due to contamination by intracellular proteins not attached to tRNA; 11% of the peptidyl-tRNA was precipitated with rabbit anti-(Fc fragment) compared with 5.3% precipitated with non-immune rabbit IgG and anti-(rabbit IgG) in control samples. The peptidyl-tRNA precipitated by rabbit anti-(Fc fragment) was combined with [^{14}C]valine-labelled heavy-chain from secreted IgG, and samples of this mixture of undigested chains were assayed for ^3H and ^{14}C . The rest of the mixture was digested with Pronase, and the pyrrolidonecarboxylic acid peptides were purified and assayed for ^3H and ^{14}C . Scheme 1 shows that the $^3\text{H}/^{14}\text{C}$ ratio of the pyrroli-

donecarboxylic acid peptides divided by the $^3\text{H}/^{14}\text{C}$ ratio of the undigested peptide chains gives a measure of the percentage of anti-(Fc fragment)-precipitable nascent heavy-chain peptides in the pyrrolidonecarboxylic acid form. This gives a maximum value, however, since nascent peptides from 50% up to 100% of completed chain length may be precipitated by anti-(Fc fragment). Assuming an equal distribution, the average length of the nascent peptides precipitated by anti-(Fc fragment) will be approximately three-quarters of that of the ^{14}C -labelled heavy-chains, and a correction factor of $\frac{3}{4}$ should be applied. This is demonstrated by the hypothetical example given in Scheme 1. Table 1 shows that only 4.2% of the ^3H -labelled *N*-terminal peptides co-purified with peptides α and β . Since less than 50% of the anti-(Fc fragment) precipitate consisted of non-specifically precipitated radioactive material, and since a correction factor should be applied, it is concluded that the maximum possible proportion of anti-(Fc fragment)-precipitable nascent peptides in the pyrrolidonecarboxylic acid form is 8%.

Pyrrolidonecarboxylic acid formation in intracellular IgG

The percentage of heavy chains of IgG in the pyrrolidonecarboxylic acid form inside Adj PC5 plasma cells was measured by the same method as was used for nascent heavy-chain peptides (Scheme 1). In this case, however, all the chains are complete and no correction factor is required, provided that the chains are uniformly labelled. For this reason labelling times considerably greater than the time required

Scheme 1. Hypothetical example of the $^3\text{H}/^{14}\text{C}$ ratio of pyrrolidonecarboxylic acid peptides purified from a mixture of [^3H]valine-labelled anti-(Fc fragment)-precipitable nascent peptides and heavy chains from [^{14}C]valine-labelled secreted IgG

Percentage of anti-(Fc fragment)-precipitable nascent peptides in the pyrrolidonecarboxylic acid form, in this hypothetical example:

$$\frac{{}^3\text{H}/{}^{14}\text{C} (\text{Glu peptides})}{{}^3\text{H}/{}^{14}\text{C} (\text{undigested peptide chains})} \times \frac{3}{4} \times 100 = \frac{1}{1} \times \frac{4}{9} \times \frac{3}{4} \times 100 = 33\%$$

Table 1. Percentage of anti-(Fc fragment)-precipitable nascent peptides in the pyrrolidonecarboxylic acid form in Adj PC5 plasma cells incubated for 1 h with [^3H]valine

Sample	$^3\text{H}/^{14}\text{C}$ ratio	% in [Glu] form	Average % in [Glu] form $\pm 2\sigma$	% in [Glu] form corrected for chain length $\pm 2\sigma$
Nascent chains	12.2			
α	0.36	2.9	4.2 ± 0.8	3.2 ± 0.6
β	0.66	5.4		

Table 2. $^3\text{H}/^{14}\text{C}$ ratios of heavy-chains and peptides α and β prepared from the intracellular IgG of Adj PC5 plasma cells incubated for 1 or 5 h with [^3H]valine, mixed with IgG secreted by Adj PC5 plasma cells incubated with [^{14}C]valine for 5 h

Labelling time (h)	Sample	$^3\text{H}/^{14}\text{C}$ ratio	% in [Glu] form	Average % in [Glu] form $\pm 2\sigma$
1	Heavy chain	21.0		
	α	13.9	61	64 ± 8.6
	β	12.9	66	
5	Heavy chain	10.1		
	α	6.73	66	66 ± 2.7
	β	6.72	66	

for the synthesis of one heavy chain or light chain were chosen.

It has been shown that pyrrolidonecarboxylic acid is not formed spontaneously from *N*-terminal glutamine during the isolation procedure (Wilkinson *et al.*, 1966; Press *et al.*, 1966; Ikenaka *et al.*, 1966; O'Donnell, 1968). We have also checked the extent of conversion of glutaminy glycine into pyrrolidonecarboxylglycine on passage through a column of sulphonated polystyrene and incubation in 1M-propionic acid. Under the conditions of the isolation procedure, less than 2% of the glutaminy glycine was converted on passage through a column of sulphonated polystyrene, and 8.4% of the glutaminy glycine was converted on incubation in 1M-propionic acid at 22°C for 24h. Since propionic acid columns took only 16h to run and the heavy-chain fractions were immediately pooled, cooled to 0°C and dialysed, we conclude that less than 8% cyclization to pyrrolidonecarboxylic acid took place at this stage.

To measure the percentage of heavy chains of intracellular IgG in the pyrrolidonecarboxylic acid form, IgG was purified from Adj PC5 plasma cells incubated for 1 and 5 h with [^3H]valine, and also from the medium of Adj PC5 plasma cells incubated for 5 h with [^{14}C]valine. More than 95% of the total radioactivity was specifically precipitable with anti-(Adj PC5 IgG) in each case. The ^3H -labelled IgG preparations were each combined with ^{14}C -labelled IgG, the chains were separated, the pyrrolidonecarboxylic acid *N*-terminal peptides α and β were isolated and their $^3\text{H}/^{14}\text{C}$ ratios were measured with

samples of the undigested heavy chains. About 60–66% of the *N*-terminal peptides of ^3H -labelled intracellular IgG from cells incubated for both 1 and 5 h co-purified with the pyrrolidonecarboxylic acid peptides α and β (Table 2). This suggests that pyrrolidonecarboxylic acid is formed on the assembled IgG molecule inside the plasma cell.

Discussion

Great caution is required in experiments on the formation of pyrrolidonecarboxylic acid, owing to the tendency of glutamine to cyclize spontaneously at low or high pH values and elevated temperatures. The results of other workers (Wilkinson *et al.*, 1966; Press *et al.*, 1966) and investigations reported here suggest, however, that the occurrence of pyrrolidonecarboxylic acid at the *N*-terminal position of the polypeptide chains of immunoglobulins is not an artifact. The *N*-terminal pyrrolidonecarboxylic acid residue of IgG is not formed during the isolation procedure, nor is it cleaved by Pronase. Since the *N*-terminal pyrrolidonecarboxylic acid residue is not an artifact it is probably formed by enzyme action, as pyrrolidonecarboxylic acid is not formed spontaneously from glutamine at a significant rate under physiological conditions (Gilbert *et al.*, 1949).

We have been unable to detect any formation of pyrrolidonecarboxyl-tRNA in rat liver, and suggest that previous reports of its presence in rabbit lymph-node cells (Moav & Harris, 1967) and liver (Rush &

Starr, 1970) were due to the spontaneous formation of pyrrolidonecarboxylic acid during handling procedures. It is also doubtful whether the formation of pyrrolidonecarboxyl-tRNA in heterologous systems (Bernfield & Nestor, 1968) is of any physiological significance. Moreover, Baglioni (1970) found glutamic acid (or glutamine) at the *N*-termini of some of the nascent peptides in the cells of mouse myeloma Adj PC5, and we have found that less than 8% of the anti-(Fc fragment)-precipitable nascent heavy-chain peptides are in the pyrrolidonecarboxylic acid form. In addition, we found glutamine at the *N*-termini of some of the heavy chains of intracellular IgG whereas 60–66% of them are in the pyrrolidonecarboxylic acid form after labelling for 1 or 5 h. Thus it is unlikely that immunoglobulin synthesis is initiated by pyrrolidonecarboxyl-tRNA and there is now evidence that protein synthesis is initiated by Met-tRNA_F in at least some eukaryotic cells (Brown & Smith, 1970; Smith & Marcker, 1970; Jackson & Hunter, 1970; Wigle & Dixon, 1970; Housman *et al.*, 1970; Shafritz & Anderson, 1970). We therefore propose that glutamine is incorporated at the *N*-termini of the nascent heavy chains of immunoglobulin, possibly after initiation of protein synthesis by Met-tRNA_F. The *N*-terminal glutamine is then cyclized by an enzyme to pyrrolidonecarboxylic acid on the completed immunoglobulin molecule inside the cell.

We express our gratitude to Mr. J. Britten for his technical assistance with the rabbits, Miss J. Amery and Mr. E. Bailey for their care of the mouse colony, Dr. A. Feinstein for his gift of sheep anti-(rabbit IgG) and Dr. E. M. Press for her gift of [¹⁴C]-Glu-Val. We thank the Medical Research Council for its financial support, and D. I. S. thanks them for the Research Studentship awarded by them.

References

- Baglioni, C. (1970) *Biochem. Biophys. Res. Commun.* **38**, 212
- Beale, D. & Feinstein, A. (1969) *Biochem. J.* **112**, 187
- Bernfield, M. R. & Nestor, L. (1968) *Biochem. Biophys. Res. Commun.* **33**, 843
- Blombäck, B. (1967) *Methods Enzymol.* **11**, 398
- Brown, J. C. & Smith, A. E. (1970) *Nature (London)* **226**, 610
- Chibnall, A. C. & Westall, R. G. (1932) *Biochem. J.* **26**, 122
- Choules, G. L. & Zimm, B. H. (1965) *Anal. Biochem.* **13**, 336
- Dearborn, R. J. & Stekol, J. A. (1950) *U.S. Patent* 2 528 267
- Fraenkel-Conrat, H. (1954) *J. Amer. Chem. Soc.* **76**, 3606
- Gilbert, J. B., Price, V. E. & Greenstein, J. P. (1949) *J. Biol. Chem.* **180**, 209
- Hamilton, P. B. (1945) *J. Biol. Chem.* **158**, 375
- Housman, D., Jacobs-Lorena, M., Rajbhandary, U. L. & Lodish, H. F. (1970) *Nature (London)* **227**, 913
- Ikenaka, I., Bammerlin, H., Kaufmann, H. & Schmid, K. (1966) *J. Biol. Chem.* **241**, 5560
- Jackson, R. & Hunter, A. (1970) *Nature (London)* **227**, 672
- Knopf, P. M., Parkhouse, R. M. E. & Lennox, E. S. (1967) *Proc. Nat. Acad. Sci. U.S.* **58**, 2288
- Levy, H. B. & Sober, H. A. (1960) *Proc. Soc. Exp. Biol. Med.* **103**, 250
- Messer, M. (1963) *Nature (London)* **197**, 1299
- Messer, M. & Ottesen, M. (1964) *Biochim. Biophys. Acta* **92**, 409
- Messer, M. & Ottesen, M. (1965) *C. R. Trav. Lab. Carlsberg* **35**, 1
- Moav, B. & Harris, T. N. (1967) *Biochem. Biophys. Res. Commun.* **29**, 773
- Munro, A. J., Jackson, R. J. & Korner, A. (1964) *Biochem. J.* **92**, 289
- Notani, G. W., Munro, A. J. & Knopf, P. M. (1966) *Biochem. Biophys. Res. Commun.* **25**, 395
- O'Donnell, I. J. (1968) *Aust. J. Biol. Sci.* **21**, 1327
- Press, E. M., Piggot, P. J. & Porter, R. R. (1966) *Biochem. J.* **99**, 356
- Rush, E. A. & Starr, J. L. (1970) *Biochim. Biophys. Acta* **199**, 41
- Rydon, H. N. & Smith, P. W. G. (1952) *Nature (London)* **169**, 922
- Shafritz, D. A. & Anderson, W. F. (1970) *Nature (London)* **227**, 918
- Smith, A. E. & Marcker, K. A. (1970) *Nature (London)* **226**, 607
- Summers, D. F., Maizel, J. V., Jr. & Darnell, J. E., Jr. (1965) *Proc. Nat. Acad. Sci. U.S.* **54**, 505
- Vickery, H. B., Pucher, G. W., Clark, H. E., Chibnall, A. C. & Westall, R. G. (1935) *Biochem. J.* **29**, 2710
- Wigle, D. T. & Dixon, G. H. (1970) *Nature (London)* **227**, 676
- Wilkinson, J. M., Press, E. M. & Porter, R. R. (1966) *Biochem. J.* **100**, 303