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Interchain Disulphide-Bond Formation in the Assembly of Immunoglobulin G

HEAVY-CHAIN DIMER AS AN INTERMEDIATE

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1. The role of disulphide-bond formation in the assembly of G_{2a} myeloma protein 5563 was studied by pulse-labelling ascitic plasma cells of tumour-line 5563 for 2–8min. with radioactive amino acids, and analysing the intracellular proteins. Myeloma-protein determinants were first purified by ion-exchange chromatography under conditions that do not dissociate non-covalently linked sub-units of immunoglobulin G. The pulse-labelled material was then analysed by electrophoresis on polyacrylamide gels in sodium dodecyl sulphate–phosphate–urea buffer, which dissociates non-covalently linked sub-units; after gel electrophoresis, radioactive protein bands were located by radioautography, and characterized immunologically after elution. 2. Two heavy-chain intermediates were detected: (i) heavy-chain dimer; (ii) the dimer with one light chain attached. Free light chains had previously been shown to be intermediates in assembly. No evidence for the presence of half-molecules (one light chain attached to one heavy chain) was obtained. The formation of the disulphide bond between the heavy chains thus appears to precede the light-chain–heavy-chain linkage in immunoglobulin G assembly.

The constituent light and heavy chains of IgG* have been shown to be synthesized separately as complete polypeptide units on different polyribosomes (Williamson & Askonas, 1967). IgG is then assembled from the light and heavy chains with the formation of interchain disulphide bonds. In a series of studies with murine plasmacytoma 5563 (Potter, Fahey & Pilgrim, 1957), which produces large amounts of a single molecular species of IgG (Awdeh, Askonas & Williamson, 1967), we have shown: (*a*) that newly synthesized light chains are autonomously released into a small pool of free light chains; (*b*) that the free-light-chain pool is maintained at a constant size by turnover of light chains as intermediates in IgG assembly (these findings were reviewed by Askonas & Williamson, 1967*a*); (*c*) that newly synthesized free light chains are randomly mixed with the pool before incorporation into IgG; (*d*) that the flow of completed heavy chains into IgG is linear, but is preceded by a short lag, suggesting a very small time-ordered pool of completed or almost completed heavy chains intermediate in IgG assembly (Williamson & Askonas, 1968*a*). In these previous studies we did not distinguish between covalent and non-covalent assembly of IgG molecules. However, the formation of the interchain disulphide bonds of IgG should permit us to identify the intermediate stages of assembly. Thus from the four-chain model possible intermediates would be half-molecules, consisting of one light chain and one heavy chain, heavy-chain dimers, or heavy-chain dimers with one attached light chain. In this report we describe our search for such intermediates within the cells.

Our approach was to expose 5563 ascitic-tumour cells to brief pulses of labelled amino acids, and to prepare the intracellular proteins. Purification of the pulse-labelled myeloma protein and any intermediates was then carried out under conditions of chromatography under which non-covalently linked sub-units are not dissociated; the purified material was then analysed by polyacrylamide-gel electrophoresis, under conditions known to dissociate non-covalently linked IgG sub-units (Williamson & Askonas, 1968*b*), followed by radioautography. Intermediates were further identified by precipitation with antisera specific for heavy chain or light chain. We found two intermediates in the assembly of G myeloma protein 5563, a heavy-chain dimer and a heavy-chain dimer with one light chain attached. The labelling of the heavy-chain dimer relative to G myeloma protein with time was shown

* Abbreviations: IgG, immunoglobulin G; SDS, sodium dodecyl sulphate.

to follow the pattern expected for an intermediate in IgG assembly. No evidence was found for half-molecules as intermediates in the assembly of IgG in this tumour. An account of preliminary results has been presented (Askonas & Williamson, 1967*b*).

MATERIALS AND METHODS

Materials. Solution E contained 10mM-tris–HCl, pH 7·5, 10mM-KCl, 1mM-$MgCl_2$. Solution PBS was 0·14M-NaCl–20mM-phosphate buffer, pH 7·4. Radioactive amino acids were obtained from The Radiochemical Centre, Amersham, Bucks. U-^{14}C-labelled algal protein hydrolysate had a specific radioactivity of 54mc/mg. atom of carbon, L-[4,5-^{3}H]leucine one of 1c/m-mole and DL-[^{3}H]valine one of 480mc/m-mole. SDS was purchased from Matheson, Coleman and Bell Inc., East Rutherford, N.J., U.S.A. Iodocetamide (British Drug Houses Ltd., Poole, Dorset) was recrystallized from ethanol. Sodium deoxycholate was purchased from British Drug Houses Ltd.

Plasma-cell tumour 5563. This tumour line, forming a G_{2a} myeloma protein, was kindly sent to us by Dr M. Potter (National Institutes of Health, Bethesda, Md., U.S.A.); an ascitic form was used for these experiments and kept in transplant by intraperitoneal transfer into C_3H/He mice.

Preparation of G myeloma protein 5563 *and its partial-reduction mixture.* G myeloma protein 5563 was prepared from the serum of tumour-bearing mice by chromatography on DEAE-cellulose (Askonas, 1961) followed by gel filtration on Sephadex G-200 as described by Williamson & Askonas (1968*b*). In the latter paper we also described the partial reduction of the G myeloma protein with 10mM-2-mercaptoethanol yielding a mixture of heavy-chain dimer, heavy-chain dimer with one light chain attached, free light chains and a small amount of free heavy chains.

Preparation of half-molecules from tumour-line 47*A*. Plasmacytoma 47A (Potter & Kuff, 1964) produces only half-molecules (one light chain linked to one heavy chain). This protein was prepared from the urine of mice bearing 47A tumours by precipitation with 50% satd. $(NH_4)_2SO_4$ at 4° and chromatography on DEAE-cellulose (Whatman DE11). The $(NH_4)_2SO_4$ precipitate was suspended in water (about 10mg. protein/ml.) and dialysed against 40mM-potassium phosphate buffer, pH 8·0, and the half-molecules were eluted in the same buffer from a column of 3g. of DEAE-cellulose.

Preparation of pulse-labelled intracellular proteins. Freshly collected ascitic tumour 5563 cells were washed with Hanks medium containing 10mg. of bovine serum albumin/ml. and 0·01% of heparin. To lyse contaminating erythrocytes, the cells were suspended for 1min. in solution E at a concentration of 5×10^8 cells/ml.; iso-osmoticity was then restored with 10× Hanks medium. The plasma cells were recovered by centrifugation and incubated with ^{3}H-labelled amino acids or U-^{14}C-labelled algal protein hydrolysate for 2–8min., at a concentration of 5×10^7 cells/ml. Incubation was terminated by chilling the tubes at 0° and adding ice-cold medium. The cells were washed once and disrupted in solution E with 0·5% (sodium deoxycholate and the intracellular protein was freed from subcellular particles by layering the extract on to a density gradient made up of 8ml. of 30%, 1ml. of 10% and 1ml. of 4% (w/v) sucrose. After centrifugation for 80min. at 104000*g*, the top layer (2–3ml.) represented the intracellular proteins. Carrier myeloma protein (1mg.) was added unless otherwise indicated.

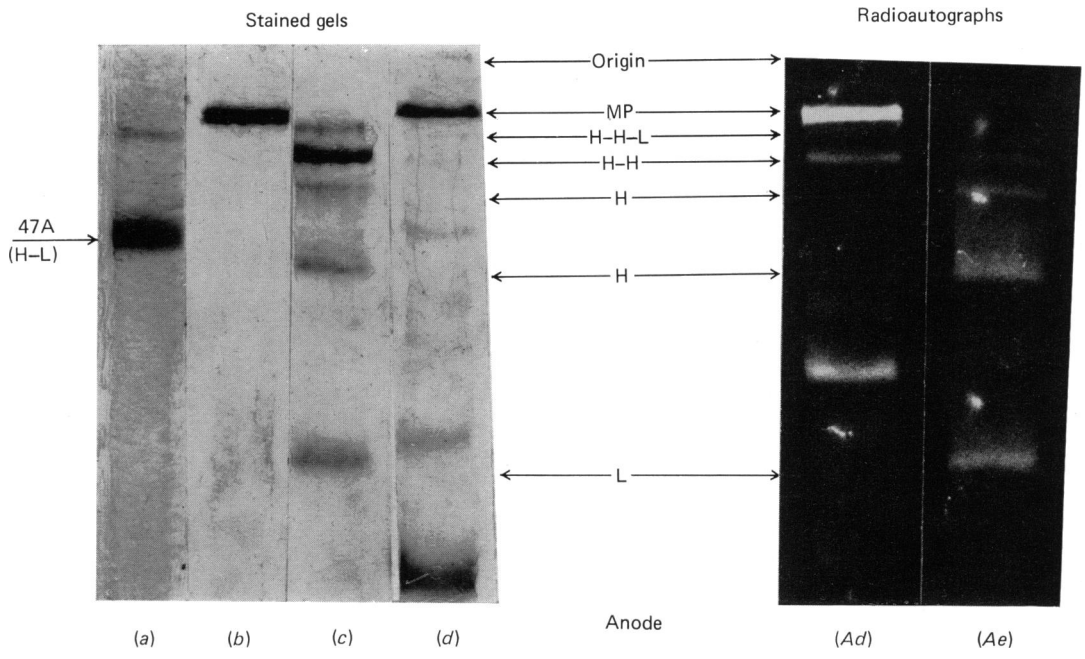
Purification of myeloma protein and possible intermediates in pulse-labelled intracellular protein extract. The intracellular protein extract was concentrated by leaving the sample in a dialysis bag in a tube filled with dry Sephadex G-100 until the volume was lowered to about 1ml., and the extract was then dialysed against 10mM-potassium phosphate buffer, pH 8·0. Chromatography was carried out on a column (1cm. × 12cm.) of ion-exchange DEAE-cellulose by using stepwise elution, first with 10mM-potassium phosphate buffer, pH 8·0, and secondly with 25mM-phosphate buffer, pH 8·0. Fractions (2ml.) were collected, and the radioactivity of protein precipitable with 7% (w/v) trichloroacetic acid was determined on samples (0·1ml.). The contents of the peak five or six tubes eluted with 25mM-phosphate buffer, pH 8·0 (containing myeloma protein or its non-covalently linked sub-units), were pooled and freeze-dried.

Polyacrylamide-gel electrophoresis and radioautography. Samples were applied with non-radioactive carrier myeloma protein, supplemented on some occasions with its partial-reduction mixture (obtained by reduction with 10mM-2-mercaptoethanol); electrophoresis was carried out in 8cm. gel columns in glass tubes (0·6cm. internal diameter). Gels [7% (w/v) acrylamide with 2·7% cross-linking] contained 0·1% SDS, 0·5M-urea and 0·1M-phosphate buffer, pH 7·2, as used by Summers, Maizel & Darnell (1965). After electrophoresis for 5hr. (10mA/column), the gels were sliced lengthwise into slabs 2mm. thick and the protein bands were stained with Amido Black.

For radioautography, individual stained sections were soaked in plasticizer for 30min. (Hermans, McGuckin, McKenzie & Bayrd, 1960), covered with cellophan, dried by suction on sintered polythene disks and mounted on glass plates. Ilford Ilflex film was used, and exposure varied from 4 to 8 weeks.

Development of radioautographs. The method was developed by Mr Richard Bowlby, to whom we are grateful for carrying out the radioautography. The X-ray films were developed in Johnson's Consol (diluted with 4vol. of water) for 5min. at 25°. The reaction was stopped by treatment with 2% (v/v) acetic acid for 2min., and the plates were washed for 10min., dipped for 2min. into a solution of 10% (w/v) KI containing one or two crystals of I_2/250ml., and then washed for 10min. and dried. This resulted in black bands on an opaque green film, which increased the contrast with small amounts of radioactivity.

Immunological identification of intermediates after polyacrylamide-gel electrophoresis. For this purpose samples (0·1ml.) of myeloma protein and non-covalently linked sub-units, purified from the pulse-labelled intracellular proteins, were applied to the gels in the presence of non-radioactive carrier proteins (50μg. of 5563 myeloma protein and 75μg. of its 10mM-2-mercaptoethanol-reduction mixture); electrophoresis was carried out for 4½hr. in columns of 5% (w/v) polyacrylamide gel in SDS–phosphate–urea as described above. The gel columns were cut into 1mm. fractions and dispersed by means of a mechanical fractionator as described by Maizel (1966). In this machine the gel was extruded through a narrow orifice in a continuous stream of phosphate-buffered saline, pH 7·4 (about 0·3ml./1mm. section). A further 0·6ml. of buffer was added



EXPLANATION OF PLATE 1

Polyacrylamide-gel electrophoresis in SDS–urea–phosphate buffer, pH 7·2 (see the text). (*a*), (*b*), (*c*) and (*d*), Photographs of stained gels: (*a*) half-molecules (L–H) isolated from the urine of mice bearing 47A plasmacytoma; (*b*) purified G myeloma protein 5563; (*c*) G myeloma protein 5563 partially reduced with 10mM-2-mercaptoethanol; (*d*) radioactive sample obtained by chromatography on DEAE-cellulose of intracellular protein after pulse-labelling of tumour 5563 cells (2×10^8) for 4min. with 20μc of ^{14}C-labelled algal protein hydrolysate. (*Ad*) Radioautograph of gel (*d*); (*Ae*) radioautograph of radioactive sample (*d*) after reduction with 0·1M-2-mercaptoethanol into heavy and light chains. H, Heavy chains; L, light chains; MP, myeloma protein.

to each fraction, the tubes were shaken and about 35–50% of the radioactive proteins were eluted overnight at room temperature. The acrylamide gel was packed by centrifugation at 2500rev./min. for 15min.; the supernatant contained the eluted radioactive protein. The radioactively labelled proteins carrying myeloma-protein determinants were precipitated with a slight excess of antiserum from rabbits hyperimmunized with purified 5563 G myeloma protein, in the presence of non-radioactive myeloma protein. This antiserum reacted with myeloma-protein 5563 Fc and Fab papain fragments, heavy chains and light chains. Occasionally the radioactivity of total protein was estimated in samples by precipitation with 7% (w/v) trichloroacetic acid. Where indicated, in other experiments one sample of the gel supernatant was treated with antiserum specific for heavy-chain determinants (anti-Fc fragment absorbed with Fab fragments). The second sample was treated with antiserum specific for light chains (absorbed with Fc fragment) to precipitate only proteins carrying light-chain determinants. After incubation at 37° for 30min. the antibody–antigen precipitates were left overnight at 4°, washed twice with cold saline and dissolved in 0·4ml. of formic acid; samples (0·3ml.) were counted in 10ml. scintillation fluid [7g. of 2,5-diphenyloxazole, 150mg. of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene, 50g. of naphthalene, 200ml. of toluene, 800ml. of A.R. dioxan and 30ml. of ethanol] in a Packard Tri-Carb scintillation counter.

RESULTS

Analysis for intracellular IgG intermediates in plasma-cell tumour 5563. On the basis of our previous studies on the time-course of labelling of the constituent chains of IgG in ascitic cells of plasmacytoma 5563 (Williamson & Askonas, 1968*a*) a 4min. radioactive pulse was chosen for preliminary examination. Labelled intracellular soluble proteins were isolated, after disruption of the cells with sodium deoxycholate, on sucrose gradients as described above and then fractionated on a column of DEAE-cellulose at pH 8·0. Free light chains were eluted with 10mM-phosphate buffer, pH 8·0, and myeloma protein with 25mM-phosphate buffer, pH 8·0 (Fig. 1*a*). To check the behaviour of possible intermediates on this column, the elution pattern of a partial-reduction mixture of myeloma protein was determined. Myeloma protein treated with 10mM-2-mercaptoethanol was used; this reduction mixture contained predominantly free light chains, heavy-chain dimers, and small amounts of heavy-chain dimers with one light chain attached and free heavy chains (see Plate 1*c*). Owing to strong interaction between the chains at neutral pH, this mixture was not fractionated on the column and was eluted in the position of myeloma protein under these non-dissociating conditions (Fig. 1*b*). When pulse-labelled intracellular proteins were fractionated by this procedure about 85% of the radioactive protein eluted with carrier myeloma protein was precipitable by antisera specific for

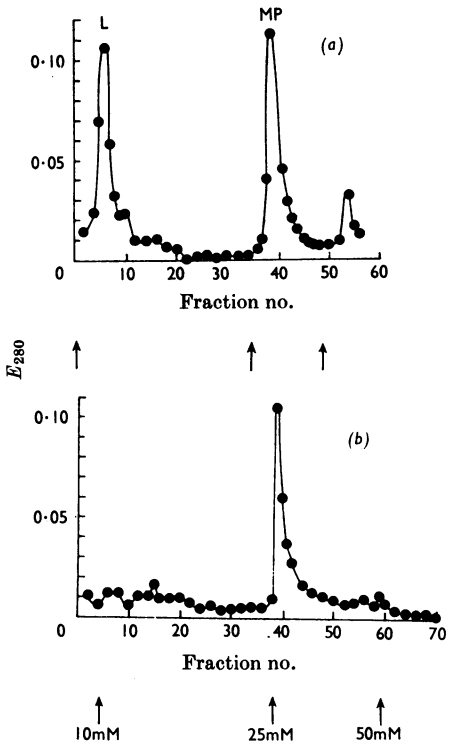


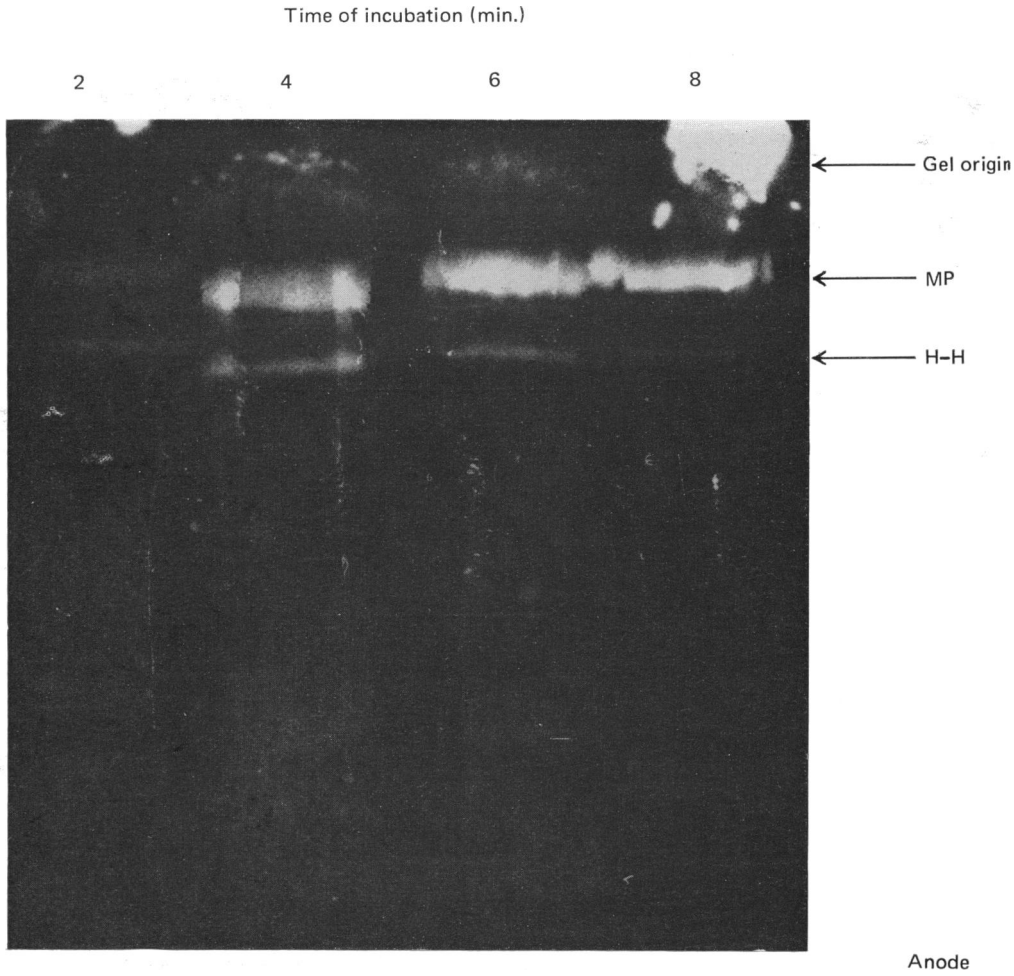
Fig. 1. Purification of myeloma-protein determinants. Chromatography was carried out on DEAE-cellulose columns (1cm. × 12cm.) by using stepwise elution with 10, 25 and 50mM-potassium phosphate buffer, pH 8·0, as indicated by the arrows. Fractions (2ml.) were collected. (*a*) G-myeloma protein 5563 (1mg.) and purified protein 5563 light chains (1mg.). (*b*) G myeloma protein 5563 after reduction with 10mM-2-mercaptoethanol and alkylation with iodoacetamide. L, light chains; MP, myeloma protein.

myeloma protein. This labelled-protein fraction containing carrier myeloma protein was then subjected to electrophoresis on polyacrylamide gels at neutral pH in the presence of SDS and urea, the conditions used for our study analysing partial-reduction products of G myeloma protein (Williamson & Askonas, 1968*b*); the position of radioactive components was determined by radioautography. The pattern of radioactive protein bands obtained after a 4min. radioactive pulse is shown in Plate 1(*Ad*), with marker proteins made visible by Amido Black staining for comparison (Plates 1*a*—1*d*). The main radioactive band coincided in its position with G myeloma protein; a further radioactive band appeared to coincide with the position of heavy-chain dimer on these gels. Contaminating radioactive proteins were present

(15% of radioactive protein was not precipitated with anti-myeloma protein) but these did not appear to correspond with any of the possible fragments of myeloma protein; the nature of these contaminants is not known, and they varied somewhat in different preparations. In particular there did not appear to be any radioactivity in the position of half-molecules, which run slower than heavy chains and faster than heavy-chain dimers in this buffer system, which suppresses charge differences and fractionates mainly by molecular sieving. The half-molecules produced by the MOPC 47A tumour line (Potter & Kuff, 1964) were used as reference compound (Plate 1*a*). Recently we obtained some half molecules (L–H) of the 5563 myeloma protein in experiments on reduction and subsequent reformation of interchain disulphide bonds. The 5563 protein half-molecules ran in the expected position on acrylamide-gel electrophoresis in SDS–urea, their position after electrophoresis being similar to that of tumour 47A half-molecules. Labelled intracellular myeloma-protein determinants isolated by the above procedure also contain as impurity a pigmented compound that runs faster than the light chains on electrophoresis but which is non-radioactive (Plate 1*d*). The radioactive bands were also found in the positions of myeloma protein and heavy-chain dimer when gel electrophoresis was carried out in 0·1M-formate buffer, pH 2·5, in 5·5M-urea (Williamson & Askonas, 1968*b*). The radioactivity pattern was the same whether or not carrier heavy-chain dimer was added for electrophoresis; we therefore endeavoured to further identify the radioactivity in the heavy-chain dimer position and to assess its importance. On reduction of the radioactive sample with 0·1M-2-mercaptoethanol, the radioactive proteins were converted into heavy and light chain with a trace of heavy-chain dimer remaining unreduced (Plate 1*Ae*). The radioactivity pattern was similar to the pattern of stained protein bands resulting from reduction of myeloma protein (Plate 3*a*). As pointed out by Williamson & Askonas (1968*b*), in this electrophoretic system heavy chains run as two bands for reasons that are not clear but may be related to the instability of heavy chains, and to differential binding of SDS to partially unfolded chains. The heavy-chain dimer appears to be more stable, and we have no evidence for more than one protein band on electrophoresis in the SDS gel. Reduction of the 5563 myeloma protein with 5mM-mercaptoethanol was shown previously to release about 50% of the light chains, leaving mainly H–H and L–H–H; these two species migrated as separate individual protein bands (Williamson & Askonas, 1968*b*).

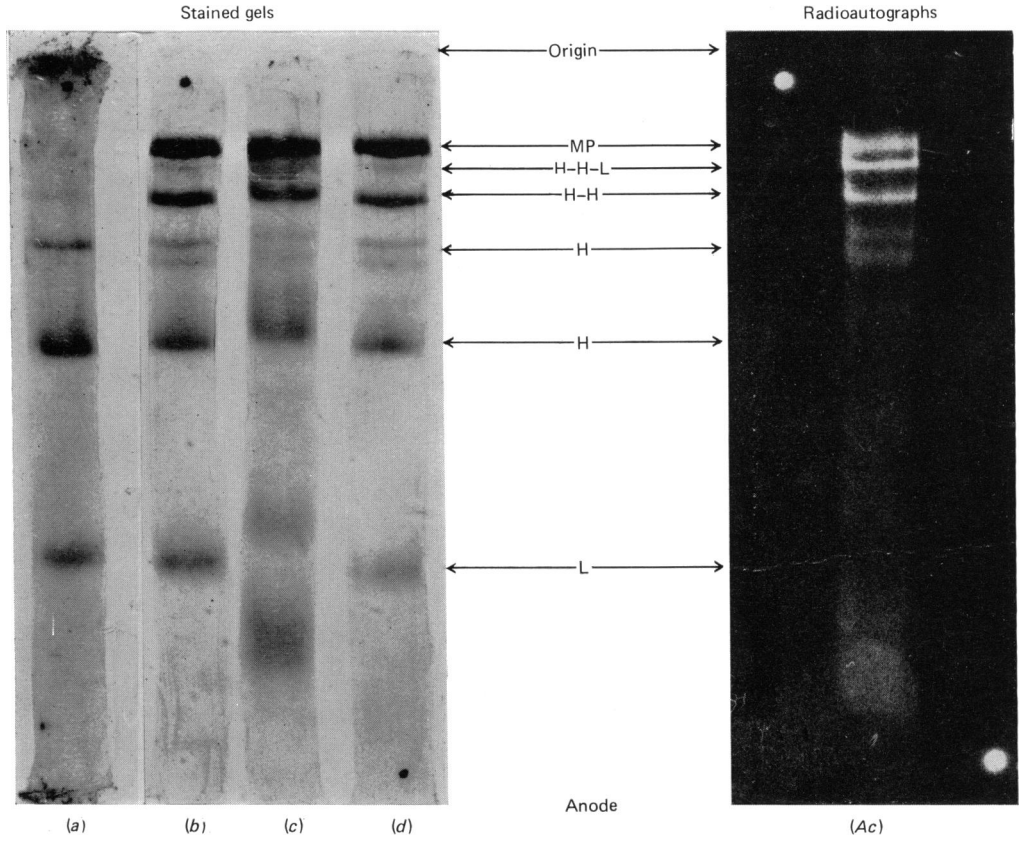
Relative labelling of intermediates and G myeloma protein with time. We wished to establish whether this new radioactive component, apparently corresponding to heavy-chain dimers, present in the pulse-labelled myeloma-protein fraction, was a true intermediate in assembly of myeloma protein or merely an artifact of the isolation procedure. Pulse-labelled myeloma protein was isolated from cells exposed to radioactive amino acids for a series of pulse times (2,4,6 and 8min.). DEAE-cellulose-purified material from each pulse time was subjected to electrophoretic analysis on polyacrylamide gels in the presence of SDS and urea. The resultant radioautographs are shown in Plate 2. For analysis of the material labelled for 4, 6 and 8min., similar amounts of radioactivity were loaded on to the gels, but owing to the small amount of radioactivity in the material labelled in the 2min. pulse, only 40% as much radioactivity was loaded for analysis of it. In each of the samples two bands of radioactivity were present, corresponding to the positions of myeloma protein and heavy-chain dimer; however, the relative intensities of radioactivity in these two protein bands varied with time. There was faint radioactivity in both protein bands after a 2min. pulse. From 4 to 8min. the intensity of radioactivity in the position corresponding to the heavy-chain dimer decreased relative to that in myeloma protein. By taking into account the fact that myeloma protein is linearly labelled with time it was possible to estimate that the maximal labelling of the heavy-chain dimer occurs between 2 and 4min. Thus the labelling pattern of this component suggests that it is a transient intermediate and not an isolation artifact due to reduction of assembled radioactive myeloma protein.

Isolation of pulse-labelled intermediates in the presence of alkylating agent. Reduction cleavage of labelled myeloma protein during the isolation procedure has been excluded above. To avoid any possible disulphide-bond formation after the breaking of the cells the experiments described above were repeated in the presence of alkylating agents. Iodoacetamide was added before disruption of the pulse-labelled cells to give a final concentration of 0·15M. In addition, to decrease loss of intermediates, the carrier myeloma protein (400μg.) added before chromatography on DEAE-cellulose was supplemented with a 10mM-2-mercaptoethanol-reduction mixture of myeloma protein (400μg.). The radioautograph resulting from electrophoretic analysis of 4min.-pulse-labelled myeloma protein isolated by this modified procedure is illustrated in Plate 3(*Ac*) together with the stained electrophoretic pattern (Plate 3*c*). The black dots on the stained gels (carrier mixture, Plates 3*b* and 3*d*) represent spots of radioactive ink to aid alignment of the autoradiograph with the stained gel. Radioactive bands corresponding to both myeloma protein and heavy-chain dimer were



EXPLANATION OF PLATE 2

Relative labelling of heavy-chain dimer with time: Radioautographs of polyacrylamide gels after electrophoresis of intracellular myeloma-protein determinants labelled with ^{14}C-labelled amino acids for various time intervals, as indicated. Electrophoresis was carried out in phosphate buffer, pH7·2, containing SDS and urea (see the text). The samples loaded contained similar amounts of radioactivity, except the 2min. sample, which contained about 40% as much radioactivity as each of the others. Ascitic tumour cells ($1{\cdot}1 \times 10^9$) were incubated at 37° with 30μc of ^{14}C-labelled amino acid mixture, and samples were removed as follows: (*a*) 2min., $4{\cdot}5 \times 10^8$ cells; (*b*) 4min., $3{\cdot}6 \times 10^8$ cells; (*c*) 6min., $1{\cdot}8 \times 10^8$ cells; (*d*) 8min., $0{\cdot}9 \times 10^8$ cells. H, Heavy chains; MP, myeloma protein.



EXPLANATION OF PLATE 3

Radioautographic analysis of alkylated intracellular myeloma-protein determinants after pulse-labelling for 4min. Polyacrylamide-gel electrophoresis was carried out in SDS–urea–phosphate, pH7·2 (see the text). (*a*), (*b*), (*c*) and (*d*): Photographs of stained gels: (*a*) G myeloma protein reduced with 0·1M-2-mercaptoethanol; (*b*) and (*d*) non-radioactive G-myeloma protein 5563 plus its 10mM-2-mercaptoethanol-reduction mixture; (*c*) radioactive sample from a 4min. pulse with carried protein as for (*b*). (*Ac*) Radioautograph of sample (*c*). The black dots on the stained gel were added with radioactive ink to ascertain the alignment of the radioautograph with the stained gels. H, Heavy chains; L, light chains; MP, myeloma protein.

found in this material; in addition another radioactive band corresponded in its position to heavy-chain dimer with a single light chain attached. This component (H–H–L) would be the expected intermediate between heavy-chain dimer and myeloma protein. However, only traces of radioactivity were found in this position when myeloma protein was isolated in the absence of alkylating agent and the carrier partial-reduction mixture (Fig. 2). There were two bands with low radioactivity in the region of one of the minor heavy-chain bands which have not been identified. This is also the position of transferrin bands on these gels, and these are frequent contaminants of myeloma protein.

Identification of intermediates by antibody–antigen precipitation. For the purpose of further identification, we wished to test the immunological reactivity of the radioactive components corresponding to H–H and H–H–L. It was found to be possible, though not without considerable loss of material, to cut up polyacrylamide gels, elute the proteins from the dispersed gel and identify radioactive proteins by precipitation with specific antisera. The results obtained using this procedure are illustrated in Fig. 2. When pure radioactive myeloma protein (prepared from the extracellular fluid of an ascitic-cell incubation *in vitro* with carrier protein) was subjected to electrophoresis, the gel subsequently sliced, dispersed and the fractions eluted, closely similar peaks of radioactivity were obtained by precipitation with either 7% (w/v) trichloroacetic acid or a specific antiserum to myeloma protein (Fig. 2*a*). In another trial experiment radioactive myeloma protein and the products of its reduction with 0·1 M-2-mercaptoethanol (light and heavy chains) were compared by electrophoresis on separate gels and identification of the radioactive proteins by precipitation with specific antisera (Fig. 2*b*). Recovery of radioactive material was low (about 35–50%); however, the recovery of radioactivity in the heavy-chain and light-chain bands was in the ratio 7:3, showing that there was no preferential loss of one or other of the chains.

In applying this method to characterization of myeloma protein determinants labelled during only a 4 min. pulse of radioactivity, there was the added difficulty of obtaining material of sufficiently high specific radioactivity. Tumour 5563 ascitic cells ($2·8 \times 10^8$) were incubated for 4 min. with 60 μc each of [^{3}H]valine and [^{3}H]leucine, and disrupted with sodium deoxycholate in the presence of 0·15 M - iodoacetamide. ^{3}H - labelled intracellular myeloma protein was isolated on DEAE-cellulose. Electrophoresis on polyacrylamide gels was carried out in the presence of carrier myeloma protein (60 μg.) and its 10 mM-2-mercaptoethanol-reduction mixture (80 μg.) in SDS–urea, pH 7·2. A separate gel was stained to establish the position of the different proteins. Samples of gel fractions containing the eluted radioactive proteins were treated with antisera specific for the heavy chain; three close peaks of radioactivity were precipitated in positions corresponding to myeloma protein, H–H–L and

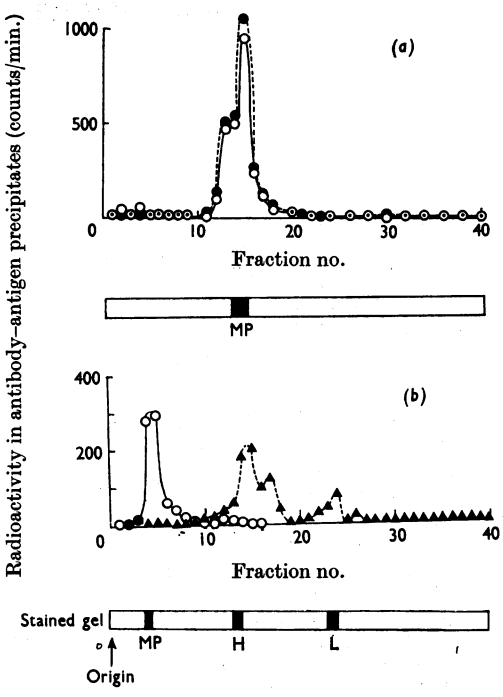


Fig. 2. Immunological identification of radioactive myeloma protein and its sub-units after polyacrylamide-gel electrophoresis. Polyacrylamide electrophoresis was carried out in SDS–phosphate–urea (see the text), the gel was cut into 1 mm. fractions, protein was eluted, myeloma determinants were precipitated with antiserum to light chains and heavy chains, and the radioactivity of the antibody–antigen precipitates was determined. (*a*) Extracellular ^{3}H-labelled G myeloma protein 5563 from a 3 hr. incubation with [^{3}H]valine and [^{3}H]leucine. The position of the stained-myeloma-proteins band in a duplicate gel is indicated below the Figure. (○) Antibody–antigen precipitate obtained with antiserum to myeloma protein; (●) protein precipitated with 7% (w/v) trichloroacetic acid. (*b*) ^{3}H-labelled extracellular protein before (○) and after (▲) reduction with 0·1 M-2-mercaptoethanol into light chains and heavy chains, precipitated with antiserum to myeloma protein. A duplicate gel stained for protein, shown below the Figure, indicates the positions of protein bands. (*a*) and (*b*) represent electrophoresis runs on two different occasions. Although the duration of the electrophoresis runs was similar, the migration rate of a given protein varied with different sets of gels. H, Heavy chains; L, light chains; MP, myeloma protein.

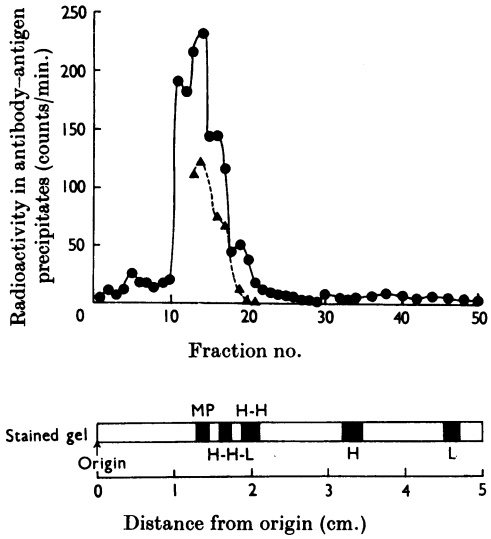


Fig. 3. Immunological reactivity of radioactive myeloma-protein intermediates from a 4min. pulse after polyacrylamide-gel electrophoresis. Ascitic 5563 plasma-cell-tumour cells (2×10^8) were incubated with [^{3}H]valine and [^{3}H]-leucine for 4min. The cells were disrupted in the presence of alkylating agent and carrier proteins, and the myeloma-protein determinants were purified and loaded on to polyacrylamide gel for electrophoresis in SDS–urea–phosphate, pH7·2. The gel was cut into 50 1mm. pieces. Radioactivity in antibody–antigen precipitates was measured with (●) anti-Fc fragment or (▲) anti-light chain. A duplicate gel stained for protein, shown below the Figure, indicates the positions of protein bands. H, Heavy chains; L, light chains; MP, myeloma protein.

H–H (Fig. 3). Peak protein fractions were chosen for treatment with antiserum specific for light chains. This antiserum absorbed with excess Fc fragment had a high antibody–antigen ratio for G myeloma protein, and the quantity used (50μl.) did not precipitate all the myeloma protein. However, it precipitated 50–56% of the radioactivity in the peak coinciding with myeloma protein and H–H–L, which contain light chains, but only 20% of radioactivity in the H–H peak region, where some overlap with H–H–L is expected, and no radioactive protein on the far side of this peak. This provided further confirmation of the identity of the three protein bands. No detectable radioactivity in the position of free light chains or the position of half-molecules was evident with either antiserum.

DISCUSSION

The search for heavy-chain intermediates in the assembly of IgG stemmed from our study of the time-course of the labelling of the chains of IgG (Williamson & Askonas, 1968*a*). A lag in the appearance of label in heavy chains of intracellular IgG suggested that newly completed heavy chains had a short existence as intermediate structures. We had earlier shown that a pool of free light chains turns over rapidly as an intermediate in IgG assembly (Askonas & Williamson, 1967*a*), and this led to a proposal that light chains might attach to heavy chains thus facilitating the release of the latter from their site of synthesis on the polyribosomes. This hypothesis would predict that half-molecules (L–H) would be likely intermediates, and the methods used in the present study were therefore chosen with this in mind. These experiments led to the detection of label in two heavy chain-containing fragments of myeloma protein, H–H and H–H–L, in pulse-labelled intracellular fractions, behaving as intermediates in IgG assembly. No evidence for the existence of half-molecules was found, and we conclude that half-molecules are not significant intermediates in the assembly of IgG in this tumour.

In these studies only the order of formation of the interchain disulphide bonds was revealed, and this does not necessarily reflect the sequence of association of the chains. Non-covalent interaction of light chains with heavy chains on heavy-chain-synthesizing polyribosomes cannot be excluded, but formation of the inter-heavy-chain disulphide bonds appears to precede the formation of light-chain–heavy-chain disulphide bonds. The presence of light chains on heavy-chain-synthesizing polyribosomes was reported by Shapiro, Scharff, Maizel & Uhr (1966) from studies on the murine plasma-cell tumour MPC 11. However, in immunological studies of the nascent chains of polyribosomes from tumour 5563 cells with antisera specific for either light chains or heavy chains the evidence suggested that few if any light chains were attached to nascent heavy chains (Williamson & Askonas, 1967). It is of course conceivable that non-covalently attached light chains would not have been found associated after centrifugation through sucrose. Scharff (1967) showed that the MPC 11 tumour synthesizes and secretes not only 7s IgG but also half-molecules, light-chain dimers and free light chains. Despite the presence of half-molecules in the MPC 11 tumour cells, the kinetics of labelling did not support the idea of a precursor relationship between half-molecules and completed IgG. In addition, the secretion of some half-molecules by plasmacytoma MPC 11, rather than the final IgG molecules only, as found for lymph nodes and the 5563 tumour (Askonas & Williamson, 1967*a*), suggests a partial block in formation of inter-heavy-chain disulphide bonds in some molecules in the MPC 11 tumour line. Two plasma-cell tumour lines have been

described (Potter & Kuff, 1964) that secrete only half-molecules (S_{20} 3·9s), in which disulphide-bond formation between the heavy chains appears to be completely blocked. Thus preliminary inter-heavy-chain bond formation is not essential for interaction between light chains and heavy chains, but may be the usual pathway in normal assembly of IgG. Light-chain dimers, which have been detected by Cioli & Baglioni (1967) in a human plasmacytoma, are unlikely to be intermediates in IgG assembly, and are probably merely by-products in tumours where excess light-chain production occurs.

The intermediates, H–H and H–H–L, were found in pulse-labelled myeloma protein isolated by ion-exchange chromatography. Their presence in this fraction suggests that they may have been in the form of four-chain molecules with one or both of the light chains non-covalently attached to the heavy chains. The failure to detect a radioactive band in the position of free light chains when the material was analysed by electrophoresis may only reflect the much lower amount of radioactivity that could be expected on the basis of the smaller size of light chains and the fact that the specific radioactivity of light chains in assembled myeloma protein labelled during a pulse of only 4min. is much lower than that of heavy chains (Williamson & Askonas, 1968*a*).

The formation of inter-heavy-chain disulphide bonds before the disulphide bonds joining light to heavy chains raises the possibility that heavy-chain-dimer formation precedes heavy-chain release; thus intrapolyribosomal interaction of heavy chains could occur, permitting inter-heavy-chain disulphide-bond formation even before one of the two heavy chains is completed. It seems reasonable that association of light chains with heavy-chain dimers occurs in the vicinity of heavy-chain-synthesizing polyribosomes; it is also feasible that light chain might mediate release of heavy-chain dimer, but we have no evidence either for or against this possibility. It is equally likely that heavy-chain dimer may exist briefly as a separate entity.

It needs to be stressed that the order of chain interaction and inter-chain disulphide bond formation may not be coincident. Irrespective of the order of non-covalent assembly, the order of the formation of interchain disulphide bonds may vary with the relative stability of the two types of interchain disulphide bonds under the reducing conditions of the microenvironment in which assembly takes place; their stability to reduction has been found to vary for IgG from different species (Williamson & Askonas, 1968*b*).

The elucidation of the order of non-covalent interaction of the chains of IgG will depend either on the demonstration that the order of interchain disulphide bonds does, in fact, reflect the order of non-covalent assembly of a given species of IgG, or upon a direct demonstration of the order of assembly in a cell-free system. Such cell-free experiments on the assembly of haemoglobin have lent support to the idea that free α-chains could mediate the release of β-chains from their site of synthesis (Shaeffer, 1967). The development of a cell-free system from 5563 tumour tissue is briefly reported by Williamson (1967).

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