by passage of the complex through 'Sephadex G-100'. These results confirm our earlier conclusions and show that when thrombin is bound to the progressive antithrombin a_2-M some of the clotting activity of thrombin is retained, contradicting earlier reports[5,15]. The dissociable reaction of bovine a_2-M with thrombin and its clotting activity have been reported recently by Sugihara *et al.*[17].

We next investigated the potential clotting activity of a_2-M bound trypsin by incubating plasma a_2-M complexed with slightly less than 2, 1.5 and 1 molequivalent of trypsin, respectively, in our clotting system. No free trypsin was present in any of these experiments. Clotting was observed in all tubes containing a_2-M bound trypsin and clots appeared at 3.5, 4.5 and 20 h, respectively. It should, however, be mentioned that clot formation can be prevented or delayed by frequent shaking to determine the presence of fibrin fibres and we did not observe a clot in our initial experiment. None of the control tubes containing either fibrinogen, a mixture of fibrinogen and a_2-M or fibrinogen and trypsin formed clots or fibrin fibres. After further incubation, all clots gradually dissolved, again demonstrating the dual role of a_2-M bound trypsin as a clotting and clot-lysing enzyme indicated in our first experiment with trypsin bound to serum a_2-M.

The question of the physiological function of the thrombin- and trypsin-a_2-M complexes now arises. Astrup[18] emphasizing the state of dynamic equilibrium that often exists between opposing forces in complex biological systems suggested a "haemostatic balance" between continuous deposition of fibrin by coagulation and removal by the fibrinolytic system. A reservoir of plasmin for the physiological regulation of the clot-lysing process has been demonstrated. Ambrus *et al.*[19] found that "inactivated plasmin" in the form of plasmin-antiplasmin complex can lyse clots slowly. The results with thrombin-progressive antithrombin (a_2-M) complex reported here may form the counterpart to these findings, suggesting the presence of a small pool of reserve thrombin that is available for the clotting mechanism in the postulated "haemostatic balance".

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A Possible Precursor of Immunoglobulin Light Chains

IMMUNOGLOBULIN light chains are synthesized in heterologous cell-free systems from reticulocytes[1] and Krebs II ascites cells[2], and in reconstituted homologous systems from lymph nodes[3,4] and myeloma tumours[5,6]. Although the synthesis appeared to be essentially faithful, absolute identity with authentic light chains was not demonstrated and some small differences were observed. We report here a clear discrepancy between the product of certain cell-free reactions and authentic light chain. With the product of other cell-free reactions no such discrepancy is seen. We propose that light chains are initially synthesized as a precursor of slightly higher molecular weight and subsequently converted into the authentic product.

For these studies we used the MOPC 21 mouse myeloma, a tumour which produces IgG1 (κ) immunoglobulins[7], and a derived tissue culture line, P3K[8]. Protein synthesis was studied in rabbit reticulocyte lysates with added MOPC21 mRNA, and also in cell-free systems containing polysomes and microsomes from P3K cells.

Analysis of the cell-free product was based on fractionation of the entire reaction mixture by SDS polyacrylamide gel electrophoresis[9]. The use of this discontinuous gel system has allowed us to detect small amounts of radioactive light chain or precursor in the presence of the large excess of both carrier and radioactive haemoglobin invariably present in reticulocyte lysate incubations. We found this procedure simpler and more informative than an immunoprecipitation assay and it does not require specific antibody preparations.

Previous work has shown that active light chain mRNA is present in the 10–14S fraction of RNA from MOPC 21 myeloma microsomes[2]. Addition of this RNA to a cell-free system from

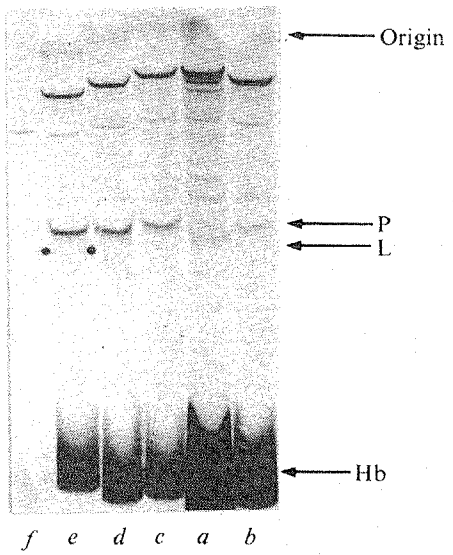

Fig. 1 SDS gel electrophoresis of products synthesized by the reticulocyte lysate. *a*, Autoradiograph of the control. *b–e*, The effect of the addition of 1 μl. (*b*), 2 μl. (*c*), 3 μl. (*d*) and 5 μl. (*e*) of MOPC 21 mRNA (80 A_{260} units/ml.). *f*, Repeat of *e* incubated at 0° C instead of 30° C. Hb shows the intense globin band and P the new component. L shows the position of the stained band of light chain added as marker, and is precisely indicated in gel *e* by two dots. Incubations (75 μl.) contained 40 μl. rabbit reticulocyte lysate (14), 1 mM ATP, 0.1 mM GTP, twenty amino-acids minus methionine (all at 20 μM, 15 mM creatine phosphate, a few crystals of creatine phosphokinase, 70 mM KCl, 1 mM Mg acetate, 10 μM ^{35}S-labelled methionine (10–30 Ci/mmol) and 20 μM haemin. After incubating for 1 h at 30° C, 1 μl. pancreatic RNAase (2 mg/ml.) was added and the incubation continued for a further 15 min. Samples (15 μl.) were boiled for 3 min with 2% SDS and 5% β-mercaptoethanol and electrophoresed on 16% SDS polyacrylamide gels[9]. After staining and destaining, longitudinal slices were dried and autoradiographed.

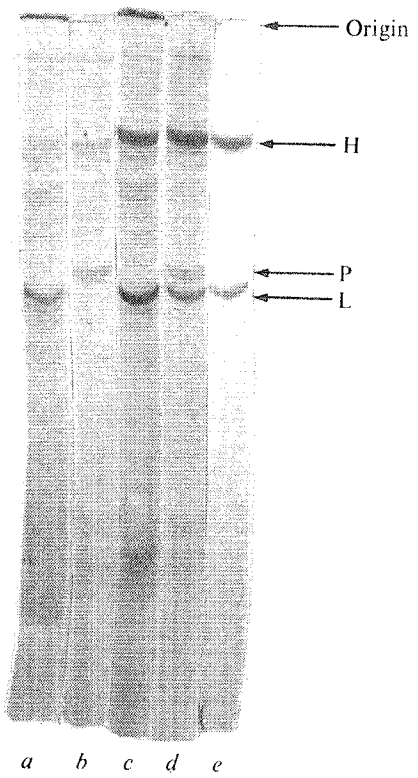

Fig. 2 SDS gel of cell-free products synthesized by microsomes and by microsome derived polysomes from P3K cells. Autoradiograph (*a*) microsomes, (*b*) microsome derived polysomes, (*c*) as (*a*) but with 400 c.p.m. ^{35}S-labelled MOPC 21 IgG added as marker, (*d*) as (*b*) with added radioactive marker, (*e*) radioactive marker alone. H and L are the heavy and light chains. Incubations (250 µl.) contained 75 µl. resuspended myeloma microsomes (15–20 A_{260} units/ml.) or microsome-derived polysomes (7–10 A_{260} units/ml.), 125 µl. ascites cell postribosomal supernatant (S-100), 25 mM Tris-HCl (*p*H 7.6), 75 mM KCl, 5 mM Mg^{2+}, 6 mM β-mercaptoethanol, 2 mM ATP, 0.2 mM GTP, 10 mM creatine phosphate, creatine phosphokinase (about 20 µg), 6 µCi/ml. ^{14}C-labelled amino-acid mixture (>45 mCi/m-atom from the Radiochemical Centre, Amersham), and the six missing amino-acids (20 µM each). Reactions were carried out at 37° C for 60 min and terminated by incubation with 20 µl. pancreatic RNAase (1 mg/ml. in 90 mM EDTA) at 37° C for 15 min. Samples (30 µl.) were boiled with 2% SDS and 5% β-mercaptoethanol and electrophoresed on 16% polyacrylamide gels as in Fig. 1. The preparation of subcellular fractions was as follows: 3–5 × 10^8 P3K cells from a spinner culture grown at a concentration 4–5 × 10^5/ml. were washed with 10 ml. cold 10 mM Tris-HCl (*p*H 7.6), 10 mM KCl, 1 mM $MgCl_2$ to allow swelling, and then homogenized in 3 ml. of the same buffer using 8–10 strokes in a tight-fitting Dounce homogenizer. Concentrated solutions were added to give a final composition of 50 mM Tris-HCl, *p*H 7.6, 25 mM KCl, 1 mM $MgCl_2$, 6 mM β-mercaptoethanol and 0.6 M sucrose. The nuclei were pelleted at 1,000*g* for 5 min and washed with the same buffer. Microsomes were pelleted from the post-nuclear supernatants at 30,000*g* for 7 min, resuspended in the same buffer using a loose homogenizer and repelleted. Microsome-derived polysomes were prepared by resuspending the microsomal pellet in 50 mM Tris-HCl (*p*H 7.6), 250 mM KCl, 2 mM $MgCl_2$, and 6 mM β-mercaptoethanol in a loose homogenizer. 'Nonidet P40' was added to a final concentration of 0.5% and the preparation was spun at 30,000*g* for 7 min. The polysomes were pelleted through a 2 ml. cushion of 1.5 M sucrose in the same buffer in the MSE 3 × 6 ml. swinging bucket rotor for 3 h at 60,000 r.p.m.

reticulocyte lysates led to the synthesis of a new ^{35}S-methionine labelled protein (component P, Fig. 1). Simultaneously it decreased the synthesis of globin and of minor endogenous products, including one in the light chain region. Increasing concentrations of mRNA progressively increased the synthesis of component P, which had a significantly slower electrophoretic mobility than MOPC 21 light chain (L in Fig. 1) from either mouse serum or cultured P3K cells. The presence of light chain peptides in digests of component P, shown below, suggests that it is a precursor of light chain.

Cell-free synthesis by P3K cell microsomes and by the polysomes derived from them, in the presence of supernatant factors obtained from Krebs II ascites cells, gave the products shown in Fig. 2. The major microsomal product was a component which had exactly the same electrophoretic mobility in SDS polyacrylamide gels as authentic light chain (L, Fig. 2). When polysomes derived from these microsomes were incubated under identical conditions, however, there was negligible radioactivity in the light chain position. Instead there was obvious incorporation of label into a component (P, Fig. 2) which had a lower electrophoretic mobility than authentic light chain. Its mobility was the same as that of component P synthesized in the reticulocyte system, and in both cases the gel band was frequently resolved into a closely spaced doublet. Since SDS polyacrylamide gels separate proteins primarily on the basis of molecular weight, it is possible to make an estimate of its size. Using eight proteins of known molecular weights ranging from 11,700 to 50,000 to calibrate the gels, we found that component P has an apparent molecular weight about 1,500 daltons greater than light chain.

The possibility that component P represents an artefact of

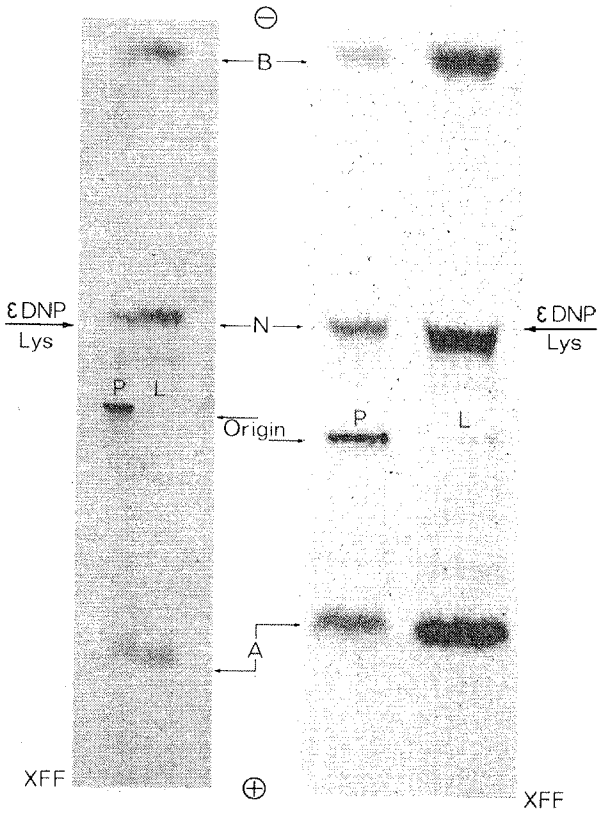

Fig. 3 Paper electrophoresis at *p*H 6.5 of the radioactive tryptic peptides from component P and light chain. Component P was eluted from the gel, oxidized with performic acid and digested with trypsin as described in ref. 2. L is a similar digest of MOPC 21 light chain labelled in tissue culture with ^{35}S-methionine[15]. P (left) is a digest of component P synthesized in the reticulocyte system (as described in Fig. 1). P (right) is a digest of component P synthesized by microsome-derived polysomes (as described in Fig. 2, except that the label was ^{35}S-methionine). εDNP and XFF (xylene cyanol FF) indicate the positions of external coloured markers. MOPC 21 light chain contains four methionine residues at positions 4, 11, 13 and 175. Met 4 occurs in the N-terminal tryptic peptide T1 present in band B (residues 1–9). Met 11 and Met 13 occur together in the tryptic peptide T2 which is present in band N (residues 10–18). Met 175 occurs in a tryptic peptide T16 also present in band N. The methionine containing peptide present in band A was not detected when the sequence of the chain was established[10]. We have termed it T2′ because its composition is identical to T2, indicating that it derives from it.

some kind has been considered. Preparation of the cell-free products for gel analysis involves incubation with pancreatic ribonuclease to digest any tRNA bound to the protein. Subsequently the samples are boiled with SDS and 2-mercaptoethanol to eliminate aggregation: the high temperature should also cleave the labile ester bond between amino-acids or peptides and tRNA or its terminal adenosine residue. To a certain extent the efficiency of this treatment is demonstrated by previous results[2], and by the syntheses of authentic globin in the reticulocyte system (Fig. 1) and authentic light chain from myeloma microsomes (Fig. 2). Further experiments have shown that the mobility of component P is not altered by incubation at 37° C for 45 min with acid (0.4 M HCl), alkali (0.4 M triethylamine bicarbonate, *p*H 10.0), or neuraminidase.

No carbohydrate has been detected in MOPC 21 light chain but theoretically the lower mobility of component P could be due to the addition of carbohydrate residues which are later removed during maturation of the protein. If this were so, it would imply that the reticulocyte lysate system contains the same polysaccharide synthesizing machinery as the mixed system containing myeloma polysomes. Although we cannot exclude this possibility, it seems unlikely.

Large scale incubations were carried out to label the presumed precursor with ^{35}S-methionine. After fractionation on SDS gels, component P was eluted, oxidized with performic acid and digested with trypsin[2]. Digests of component P from the mRNA-directed reticulocyte system and from microsome-derived P3K cell polysomes were compared with a digest of *in vivo* synthesized ^{35}S-methionine labelled MOPC 21 light chain. All three digests gave almost identical patterns of radioactive bands after fractionation by paper electrophoresis at *p*H 6.5 (Fig. 3). The insoluble radioactive material present at the origin of component P digests might be due to incomplete digestion, but this has not been proved. The radioactive bands A, N and B of Fig. 3 were analysed on other systems.

The main component of the neutral (N) and acidic (A) bands from the cell-free synthesized product were indistinguishable from the controls (peptides T2 and T2′) on chromatography and on paper electrophoresis at *p*H 3.5 (Fig. 4). The neutral band (N of Fig. 3) should include peptide T16[10] but this peptide occurs in low yields because of the unfavourable tryptic splits involving a Lys–Asp bond on each side. This peptide may in fact be T16 (?) (Fig. 4), but this is not certain because of the erratic behaviour of T16 in chromatography[10]. The nature of other minor components of bands N and A is obscure, but contaminating impurities are, of course, not

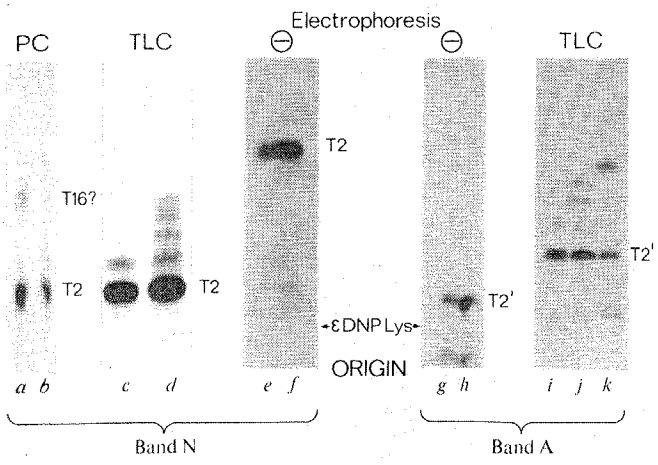

Fig. 4 Further identification of bands A and N from Fig. 3. Paper electrophoresis was at *p*H 3.5, paper chromatography (PC) and thin layer chromatography (TLC) were in butanol : acetic acid : water : pyridine (15 : 3 : 12 : 10 by volume). Peptides from products synthesized by microscome-derived polysomes are in *a* (band N) and *i* (band A), while those synthesized in the reticulocyte system are in *c* and *e* (band N), *g* and *k* (band A). Marker peptides are shown in *b*, *d*, *f*, *h* and *j*.

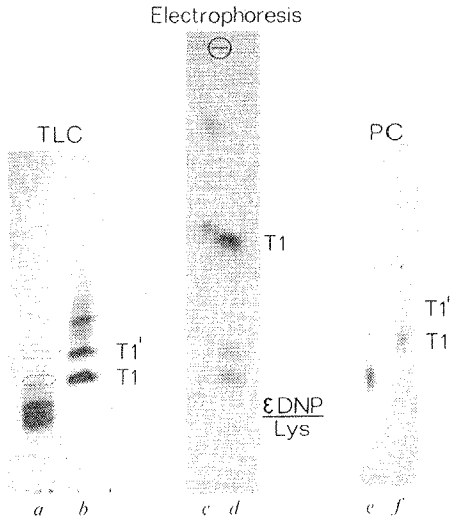

Fig. 5 Further identification of band B from Fig. 3. *a* and *c* are peptides from products obtained in the reticulocyte system and *e* is from microsome derived polysomes. *b*, *d* and *f* are the respective controls. The dotted lines indicate the ninhydrin staining of T1 and T1′ derived from the carrier non-radioactive MOPC 21 light chain added during the extraction of component P from the gels. Peptide T1 on chromatography gives rise to T1′ probably because of the lability of its N-terminal asparagine[10].

excluded. The unambiguous identification of T2 and its derivative T2′ in digests of component P proves that it is related to authentic light chain.

The basic band B from the cell-free product (P, Fig. 5) gave rise to one or two main components on chromatography, neither of which corresponded with the control (peptide T1). On paper electrophoresis at *p*H 3.5 it gave two components, one of which was clearly different from, and the other similar but not necessarily identical to, the control. The absence of the N-terminal peptide T1 from digests of component P shows that it has an altered N-terminus. The appearance of a new peptide(s) in the basic band suggests that this is a derivative of T1.

These results indicate that both cell-free systems synthesize a protein which differs from MOPC 21 light chain in its increased size and in its altered N-terminal tryptic peptide. This contrasts with our previous observation[2] that the same RNA fractions are able to direct the synthesis of authentic MOPC 21 light chain in a cell-free system derived from Krebs II ascites cells.

Further information about component P was obtained from reticulocyte lysate incubations containing ^{35}S formyl-methionine labelled $tRNA_F$ of *E. coli* as the sole source of label. Under these conditions the synthesized globin carries an extra N-terminal methionine residue which is not removed because of the presence of the formyl blocking group[11]. Addition of myeloma mRNA led to the appearance of a new band which coelectrophoresed with component P. This shows that synthesis of light chain is also initiated by Met $tRNA_F$ at the N-terminus of the precursor molecule.

The precise nature of the proposed precursor is still not clear. Its electrophoretic mobility in SDS gels indicates an increase in molecular weight, with respect to light chain, equivalent to about 15 extra amino-acids. However, it has not been finally established that this extra material consists exclusively of amino-acids.

Modifications have been shown to occur at the N-terminus, but we have not yet investigated the C-terminal peptide and therefore cannot exclude changes at this end also. Conversion of the precursor to light chain is likely to be due to enzymic activity in the microsomes. Attempts to convert precursor synthesized in either of the cell-free systems to light chain by subsequent incubation with microsomes have not been successful, however, suggesting that such enzymic activity is required

during, rather than after, synthesis. If this were so, intact component P should not be detectable *in vivo*, unlike the intracellular immunoglobulin precursors described by others[12].

We have shown previously that a cell-free system from Krebs II ascites cells (ascites S-30) directed by myeloma mRNA, produces light chain of the correct size. It would therefore appear that the enzymic activity is present in Krebs II ascites cells and is not restricted to plasma cells. Presumably the ascites S-30 (30,000*g* supernatant) contains a small and possibly variable amount of membraneous material which can convert the precursor into authentic light chain. The stability of the precursor synthesized by the reticulocyte system could then be due to the absence of such membraneous structures.

Our results could throw light on the mechanism of secretion. In contrast to intracellular proteins, which are made on free polysomes, secretory proteins are generally thought to be synthesized on microsomes. The signalling device whereby this segregation is achieved is unknown. It seems to us that a short amino-acid sequence at the N-terminus of a precursor protein would be a simple way to provide such a signal. Binding to a membrane would then occur after translation of the first few codons of the mRNA. This would be consistent with results which suggest that the binding of polysomes to the membrane occurs after protein synthesis has begun[6,13]. A prediction now under investigation is that heavy chains (as well as other proteins to be secreted) will also be synthesized as precursors, possibly with an analogous N-terminal sequence.

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Intercellular Transfer by Phage Receptor Site Lipopolysaccharide

TREATMENT of *Escherichia coli* cells with lysozyme and EDTA partially removes the outer layer of the cell wall containing lipopolysaccharide (LPS), leaving osmotically unstable spheroplasts[1]. These can be infected with phage nucleic acid[2] and can produce viable phage particles. Removal of LPS-containing phage receptor sites[3–5], however, leaves spheroplasts resistant to infection by intact phages[1]. We now show that LPS, obtained from phage-sensitive cells by aqueous phenol extraction, can provide functional phage receptor sites to spheroplasts prepared from cells lacking receptor sites.

Phage U3 adsorbs only to *E. coli* K-12 cells that have incorporated galactose into their cell wall[6]. If spheroplasts made from U3-resistant cells are mixed with LPS from U3-sensitive cells, they become sensitive to U3 infection. Typically, spheroplasts prepared from the U3-resistant *gal* deletion strain SA242 were incubated with an appropriately diluted LPS extract for 10 min then infected with U3 phage and plated with a U3-sensitive host (strain W3110) after remaining free phage had been inactivated by anti-U3 serum. The number of infective centres was corrected for the 0.01% of the free phage that survived antiserum. In some experiments a mixture of two complementing amber mutants of U3 (U3*am*7 and U3*am*14) was substituted for wild type U3. Since the spheroplast and plating strains were both non-permissive hosts, plaque formation required a double infection by U3*am*7 and U3*am*14 followed by complementation and formation of wild type U3 recombinants. This procedure showed no background plaques from the occasional free phages

Table 1 Requirements for U3 Receptor Site Attachment

Reaction mixture	P.f.u. *($\times 10^{-3}$) after infection with U3+ Experiment I	U3+ Experiment II	U3*am*7 U3*am*14 Experiment I	U3*am*7 U3*am*14 Experiment II
Complete (W3110 LPS + spheroplasts + phage)	3,000	790	1.3	10
− phage	0	0	0	0
− LPS†	15	20	0	0
− spheroplasts			0	0
Complete using SA242 LPS			0	
Complete using W4597 LPS			0	

The reaction mixture contained 0.1 ml. spheroplasts (about 1.6×10^8 treated cells) plus 0.1 ml. LPS in 0.05 M Tris-HCl (*p*H 7.4). The LPS concentration was optimal or near optimal for these data (see Fig. 2). After 10 min incubation at 37° C, 0.8 ml. of 3–4 $\times 10^8$ particles/ml. of phage U3+ or of an equal mixture of U3*am*7 and U3*am*14 (see text) suspended in PAM medium was added. (PAM was modified from Guthrie and Sinsheimer[9] and contained per litre: 10 g casamino acids, 8 g nutrient broth, 100 g sucrose and 2 g $MgSO_4$ added after autoclaving.) After another 20 min, 0.02 ml. anti-U3 serum was added and 20 min later samples were diluted in PAM and plated (in PAM plus 0.8% agar) with strain W3110. LPS was extracted by a method modified from Osborn[7] from cells grown in tryptone broth (1% tryptone, 0.5% NaCl) or in M9 minimal medium[10] containing 0.1% glycerol and 1 μg/ml. of thiamine. Frozen or fresh cell pellets were suspended in Tris buffer (0.05 M Tris-HCl, 0.3 M NaCl, *p*H 9.1) and the cells ruptured by passing the suspension twice through a French pressure cell. Cell débris was collected by centrifuging at 30,000*g* for 20 min, the pellets resuspended in 0.05 M Tris-HCl (*p*H 7.4), and shaken for 5 min at 60° C with an equal volume of redistilled phenol (saturated with 0.05 M Tris-HCl at 4° C). The resulting emulsion was rapidly cooled, centrifuged to separate the phenol and aqueous phases, and the aqueous extract removed and dialysed against 0.05 M Tris-HCl (*p*H 7.4) at 4° C to remove dissolved phenol. Extracts were stored at 4° C for short periods of time, or frozen at −20° C for longer periods. Spheroplasts were made from cells of strain SA242 after the method of Guthrie and Sinsheimer[9] as modified by Strauss[11]. Cells were harvested by centrifugation from broth (4×10^8 cells/ml.), resuspended in 0.35 ml. 1.5 M sucrose, and 0.17 ml. 35% bovine serum albumin, 0.02 ml. lysozyme (2 mg/ml. in 0.25 M Tris-HCl, *p*H 8.1) and 0.04 ml. 4% EDTA added in sequence. After 10 min incubation at room temperature, 10 ml. of PAM medium was added. The resulting suspension of spheroplasts could be stored in the refrigerator up to 24 h before use. Differences between duplicate experiments reflect variation from one spheroplast preparation to another.

* P.f.u. is the number of plaque forming units/ml. reaction mixture.

† P.f.u. in the absence of LPS is taken as background, and is subtracted from the number of p.f.u. observed for the complete reaction mixture.