Characterization of a Mouse Ascites Cell-Free System†

M. Jacobs-Lorena and C. Baglioni*

ABSTRACT: A cell-free system prepared from mouse ascites cells and used in the translation of rabbit globin and histone mRNA has been studied. The Mg^{2+} optimum for the translation of rabbit globin and HeLa cells mRNA has been found to be 2–3 mM. The optimal concentration of different labeled amino acids used in the cell-free system has been determined. The concentration of globin mRNA that is optimally translated has been determined and the conditions under which the cell-free system responds linearly to the addition of mRNA have been defined. The effect of the presence of rRNA has been studied. Small amounts of rRNA, in the range that may contaminate mRNA preparations, have little or no effect on protein synthesis; large amounts of rRNA stimulate translation of mRNA, possibly by protecting it from nucleases.

A cell-free system prepared from Krebs ascites mouse cells has been successfully used for the translation of viral (Kerr *et al.*, 1966; Mathews and Korner, 1970; Smith *et al.*, 1970; Aviv *et al.*, 1971) and mRNA (Housman *et al.*, 1971; Mathews *et al.*, 1971, 1972). The most useful property of this cell-free system is the very low level of endogenous protein synthesis, due to a preincubation step, and hence the large stimulation obtained with added mRNA. Moreover, since free amino acids are eliminated by gel filtration, proteins of very high specific activity are synthesized.

The ascites cell-free system was first developed approximately 10 years ago (Kerr *et al.*, 1962) and has since been greatly improved by several authors. We have used this cell-free system for the translation of rabbit globin mRNA (Jacobs-Lorena and Baglioni, 1972) and of HeLa cells histone mRNA (Jacobs-Lorena *et al.*, 1972). Here we report some observations on the conditions that are optimal for translation of rabbit globin mRNA. The addition of different labeled amino acids and of rRNA have been studied and the conditions to obtain maximal incorporation and stimulation have been determined. Stimulation is here defined as the ratio of incorporation with added mRNA over that without mRNA.

Experimental Procedures

Materials. All labeled amino acids were obtained from New England Nuclear, except for [^{3}H]phenylalanine purchased from Schwarz-Mann. Dithiothreitol and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes)[1] were purchased from Sigma. The scintillant butyl-PBD was purchased from C.I.B.A. Rabbit globin mRNA was prepared according to the procedure described by Evans and Lingrel (1969), with the only exception that linear rather than exponential sucrose gradients were used.

Methods. Krebs II ascites cells were obtained from Dr. A. Burness (Sloan Kettering Institute, New York) and maintained by intraperitoneal injection of 0.2 ml of ascitic fluid every 7 days. The ascitic fluid of five mice was collected 7 days after transplanting the cells and an extract was prepared following the procedure of Mathews and Korner (1970), with minor modifications. The cells were washed five times with 10–20 volumes of 0.9 M NaCl buffered with 35 mM Tris-HCl at pH 7.6, and broken by 50 strokes of a tight-fitting Dounce homogenizer. A tenfold concentrated solution was immediately added to the homogenate to obtain the final salt concentration of extract buffer (120 mM KCl, 5 mM magnesium acetate, 6 mM mercaptoethanol, and 20 mM Hepes–HCl, pH 7.6).

PREPARATION OF CELL-FREE SYSTEM. The homogenate obtained from ascites cells was centrifuged 10 min at 30,000*g* and the supernatant was incubated 40 min at 37°, after addition of 2 mg/ml of creatine phosphate, 0.2 mg/ml of creatine kinase, GTP to 0.2 mM, and ATP to 1 mM. The incubation mixture was then centrifuged for 5 min at 30,000*g* and the supernatant was passed through a Sephadex G-25 column (2.5 × 36 cm) equilibrated with extract buffer. The fractions eluted with the excluded volume were combined, distributed in 0.5-ml aliquots, and stored at −75°. The protein concentration of these fractions was about 8 mg/ml. We have used our preparations for over a year without noticing any loss of activity.

COMPOSITION OF THE CELL-FREE SYSTEM. Each incubation contained 6 volumes of the ascites extract passed through Sephadex G-25, 1 volume of master mix, and 3 volumes of RNA or amino acid solutions in water. The composition of the master mix was: 0.24 M KCl, 0.1 M dithiothreitol, 80 mM Tris-HCl (pH 7.6), 10 mM ATP, 2 mM GTP, 20 mg/ml of creatine phosphate, and 6.7 mg/ml (500 U/ml) of creatine phosphokinase. The final concentration of these components in the incubation was one-tenth, except for KCl which was 96 mM because it was present in the buffer used to equilibrate the G-25 column. Other components as well were already present in this buffer. Unlabeled amino acids were added to a final concentration of 0.1 mM; fivefold change in the concentration of unlabeled amino acids had no effect on protein synthesis. All the labeled amino acids were purchased from New England Nuclear. When [^{3}H]amino acid labeling mixture was used, those amino acids which are absent in this mixture were added separately unlabeled.

The master mix was kept frozen in small aliquots at −75° and used for 6 months. It was often found convenient to dry the labeled and unlabeled amino acids in the small test tubes used to run the incubations. Typical reaction mixtures were run in final volumes of 10–25 μl and duplicate aliquots

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[1] Abbreviation used is: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

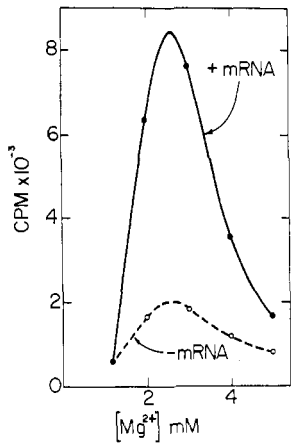

FIGURE 1: Dependence on Mg^{2+} concentration of translation of globin mRNA by the ascites cell-free system. For each point 1.3 μg of globin mRNA was incubated 60 min at 30° with different concentrations of Mg^{2+} and 2.9 μCi of [^{3}H]phenylalanine in a final volume of 20 μl. The mRNA was omitted from the reaction mixtures used to determine the endogenous incorporation (−mRNA). The cpm corresponding to the average of duplicate 8-μl samplings are indicated. The ascites extract used in this experiment was passed through a Sephadex G-25 column equilibrated with extract buffer containing 2 mM Mg^{2+} (see Methods).

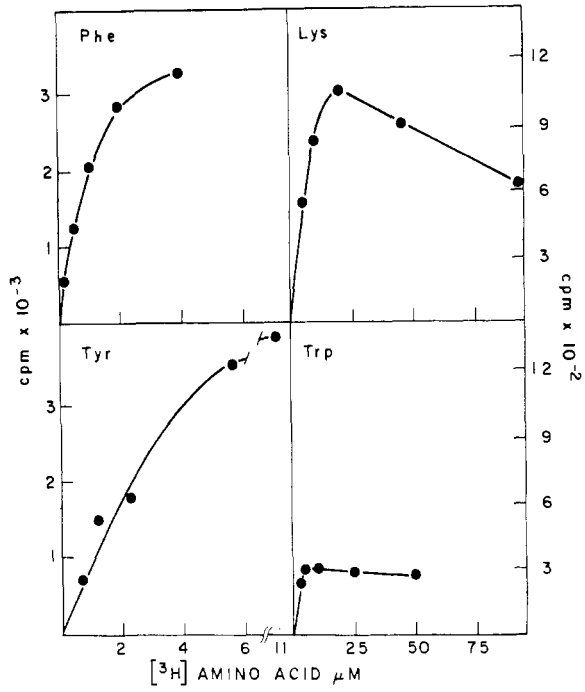

FIGURE 2: Protein synthesis by the ascites cell-free system with different amounts of labeled amino acids. For each point 0.2 μg of globin mRNA were incubated 60 min at 30° with the indicated amount of [^{3}H]amino acid in a final volume of 10 μl. The cpm corresponding to the average of duplicate 2-μl samplings are indicated.

of 4–10 μl were sampled and counted as described previously (Jacobs-Lorena and Baglioni, 1972).

Results

Mg^{2+} Concentration. Different Mg^{2+} concentrations have previously been used in the ascites cell-free system with different viral or mRNAs. Encephalomyocarditis virus RNA has been translated at 5 mM Mg^{2+} (Mathews and Korner, 1970; Smith *et al.*, 1970; Aviv *et al.*, 1971), whereas rabbit globin mRNA was at 5 mM (Housman *et al.*, 1971) and mouse globin (Mathews *et al.*, 1971) and calf lens mRNA (Mathews *et al.*, 1972) were at 2.5 mM Mg^{2+}. Only one Mg^{2+} concentration curve has been reported (Mathews and Korner, 1970). We have thus determined the optimal Mg^{2+} concentration for the translation of rabbit globin mRNA (Figure 1). The optimum is between 2 and 3 mM and for this reason 3 mM Mg^{2+} has been used in all the following experiments. The optimum for the endogenous incorporation (without added mRNA) is also between 2 and 3 mM. In experiments with histone mRNA (Jacobs-Lorena *et al.*, 1972) a similar Mg^{2+} optimum was observed (not shown).

Use of Different Labeled Amino Acids. It is convenient to label the cell-free product of different mRNAs with specific amino acids, if one wants to identify it by peptide mapping after tryptic digestion. We have thus determined the optimal concentration of labeled amino acids that we have used in different experiments (Figure 2).

The results obtained with the four amino acids tested (tyrosine, phenylalanine, lysine, and tryptophan) indicate that by increasing the concentration of labeled amino acid in an incubation mixture, the radioactivity in the product increases until a plateau is reached. Since the ascites extract has been passed through a Sephadex G-25 column, the only unlabeled amino acids present are the ones added and those in aminoacyl-tRNA. If the amount of this is negligible, labeled amino acids with an equal specific activity should give an incorporation dependent on the amino acid composition of the cell-free product coded for by the mRNA employed. However, the results obtained are not fully in agreement with these expectations.

Phenylalanine and tyrosine appear to plateau at approximately the same concentration and to give a similar total incorporation, even though there are 2.7 times as many phenylalanine residues in rabbit globin as tyrosine residues (von Ehrenstein, 1966; Braunitzer *et al.*, 1966). With tryptophan a plateau is reached at half the concentration of lysine, although tryptophan has twice the specific activity of lysine. The ratio of maximum incorporation obtained with these two amino acids is however within the expected range (Figure 2) since there are eight times as many lysine residues in rabbit globin as tryptophan residues (von Ehrenstein, 1966; Braunitzer *et al.*, 1966).

The results obtained are consistent with the hypothesis that there is a small amount of aminoacyl-tRNA for some amino acids, whereas there is almost none for other amino acids. They also point out something unexpected in the case of lysine; above a certain concentration of this amino acid the incorporation declines rapidly (Figure 2) as if some inhibitory substance were present in the [^{3}H]lysine solution. Each amino acid seems thus to behave in a slightly different way in the cell-free system and a concentration–response curve should be run to find the conditions for optimal incorporation. Among the amino acids that we tested the best incorporation was obtained with tyrosine.

Effect of mRNA Concentration on Incorporation and Stimulation. When RNA is translated in a cell-free system, it can either be tested for stimulatory activity or used to obtain a large amount of the cell-free product, in order to characterize it. We have thus tried to determine how much mRNA should be added to the ascites cell-free system to obtain maximal stimulation or the highest yield of cell-free product. These experiments have been devised with the rationale of finding

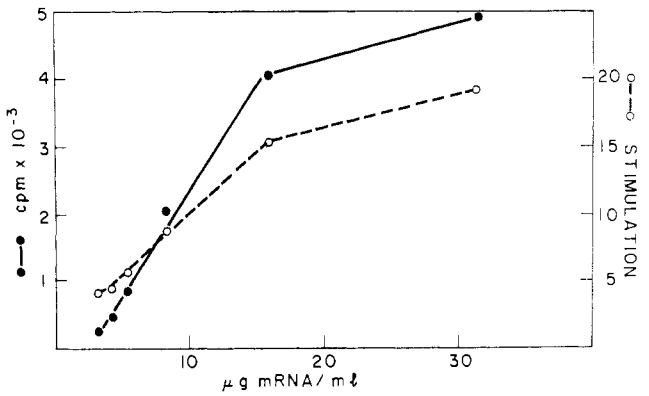

FIGURE 3: Relationship between globin mRNA concentration and stimulation of protein synthesis in the ascites cell-free system. For each point the indicated amount of globin mRNA was incubated 60 min at 30° with 120 μCi/ml of [³H]tyrosine and all the components of the cell-free system. A series of control incubations was carried out without addition of globin mRNA. The cpm corresponding to the average of identical samplings of each incubation mixture were determined; the difference between the cpm obtained in the incubations with added mRNA minus the cpm of the corresponding incubations without added mRNA is reported (solid line). The ratio between the incorporation with added mRNA over that obtained without added mRNA (stimulation) is also indicated (dotted line). See text for further explanations.

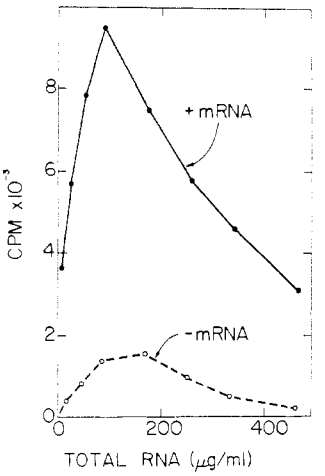

FIGURE 4: Effect of the addition of *E. coli* rRNA on protein synthesis by the ascites cell-free system. For each point 0.22 μg of globin mRNA were incubated 60 min at 30° with different amounts of *E. coli* rRNA and 2.7 μCi of [³H]tyrosine in a final volume of 25 μl. A series of control incubations was carried out without addition of globin mRNA (−mRNA). The cpm corresponding to the average of duplicate samplings of 7 μl are indicated. The first point shows incorporation obtained with mRNA alone or with no addition of RNA.

the conditions that would allow the most economical use of mRNA, since rather small amounts of mRNA are usually available.

The results of these experiments are shown in Figure 3. An increasing amount of globin mRNA has been added to cell-free incubations of identical volume; all the components of the cell-free system are present in exactly the same final concentration, except for globin mRNA. The range of mRNA concentrations used per milliliter of incubation mixture is reported in the bottom scale of Figure 3.

The results obtained indicate that maximal stimulation (*i.e.*, highest ratio of incorporation with added mRNA over endogenous incorporation) is achieved at high concentrations of mRNA, whereas the maximum yield of cell-free product for a given amount of mRNA is obtained with a somewhat lower input of mRNA. The optimal mRNA concentration to obtain the highest yield of cell-free product is, under the conditions of our experiments, 1 μg of mRNA/40 μl of ascites extract.

These experiments allow us to establish the globin mRNA concentration that is optimal for translation by the ascites cell-free system. Within a range of mRNA concentrations (Figure 3) the incorporation is linearly related to the input of mRNA. The cell-free system can thus be used within this range to determine the amount of mRNA present in a given preparation. At concentrations higher than the optimal the response of the cell-free system is not linear. This indicates that mRNA is less efficiently translated and may suggest that, if more than one species of mRNA is present within a preparation, there may be competition among the different species for translation by the cell-free system.

Effect of Addition of rRNA on Translation of mRNA. When mRNA is fractionated on the basis of its size only, it may often be contaminated with small amounts of rRNA or of degradation products of rRNA. We have thus investigated the effect that the presence of rRNA has on the translation of globin mRNA. For this purpose we have used 16S rRNA of *Escherichia coli* purified by repeated sucrose gradient fractionation (a kind gift of K. Gross, Mass. Institute of Technology).

In the experiment shown in Figure 4 all the components of the cell-free system have been kept constant and an increasing amount of 16S rRNA has been added to different incubation mixtures. Although in each incubation globin mRNA is present in the same concentration (8.7 μg/ml), twice as much incorporation is observed in the presence of approximately 100 μg/ml of 16S rRNA, as compared with the incubation with no added rRNA. Also the endogenous incorporation is stimulated by the addition of rRNA, although the absolute increase observed is much less than that observed with globin mRNA. Similar results have been obtained when 18S rabbit reticulocyte rRNA has been added to the ascites cell-free system (not shown).

At concentrations higher than 100 μg/ml, rRNA inhibits protein synthesis (Figure 4). This inhibition is possibly due to changes in the ionic composition of the incubation medium or to other effects of adding such large amounts of RNA. The stimulation of translation observed with lower concentrations of rRNA suggests that "noninformational RNA" may allow better translation of mRNA, possibly by protecting it from degradation by nucleases. It should be pointed out that an appreciable stimulation by rRNA of translation of mRNA is observed with concentrations of rRNA that are twice as large as that of mRNA (Figure 4). Contamination of mRNA with small amounts of rRNA would thus have no effect on the translation of the mRNA in the cell-free system. On the other hand, a twofold stimulation of the ascites cell-free system can be obtained by the addition of rRNA only. Therefore, a stimulation of protein synthesis in this range by an RNA preparation should not be taken as an indication of presence of mRNA.

We have tested the effect of added rRNA on the time course of translation of globin mRNA. The results shown in Figure 5 indicate that both in the presence or in the absence of rRNA, translation of globin mRNA proceeds at a rate almost linear for almost 45 min and then at a slower rate; in the presence

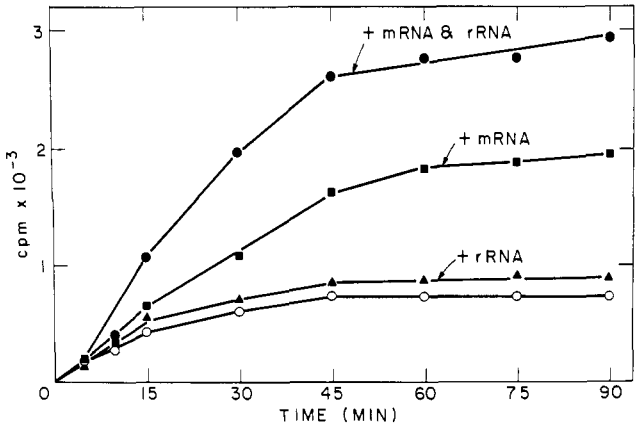

FIGURE 5: Kinetics of protein synthesis with globin mRNA in the presence of rRNA. For each experiment 3 μg of globin mRNA was incubated with 7.5 μCi of [^{3}H]amino acids in a final volume of 70 μl. Where indicated 16S rRNA of *E. coli* was added to a final concentration of 0.1 mg/ml. Globin mRNA was omitted from the controls (○, ▲). Aliquots of 5 μl were taken at the times indicated.

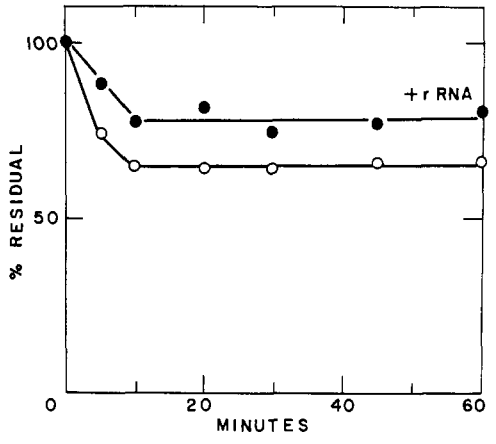

FIGURE 6: Protection of labeled mRNA by rRNA. For each experiment 5000 cpm (about 0.01 μg) of [^{3}H]uridine-labeled histone mRNA obtained from cleaving sea urchin embryos and purified by sucrose gradient centrifugation (a gift of Dr. Arthur Skoultchi) were incubated in a final volume of 0.15 ml with 50 μM cold amino acids and 100 μg/ml of *E. coli* 16S RNA where indicated. Aliquots of 20 μl were taken at the times indicated and counted after precipitation with 5% trichloroacetic acid.

of rRNA the initial rate of translation is increased, but in both cases 80–85% of the translation is over by 45 min.

The possibility that rRNA protects mRNA from degradation by nucleases has been tested by adding [^{3}H]uridine-labeled sea urchin histone mRNA (a kind gift of Dr. Arthur Skoultchi) to the ascites cell-free system. The results obtained (Figure 6) indicate that part of the labeled RNA is digested to trichloroacetic acid soluble fragments in the first 10 min of incubation; in the presence of rRNA there is a slight protection of the labeled RNA (Figure 6). This method of analysis is very insensitive, however, since it allows one to measure only extensive degradation of RNA and not single nucleotide breaks, which from the point of view of translation of an RNA species, are equally important.

Discussion

We have shown that the Mg^{2+} concentration that is optimal for translation of rabbit globin mRNA is quite narrow. The same Mg^{2+} optimum has been observed with HeLa cells histone mRNA (Jacobs-Lorena *et al.*, 1972) and it seems quite possible that other mRNAs as well will best be translated at 2–3 mM Mg^{2+}. The preincubation step is however carried out at 5 mM Mg^{2+}. When the Mg^{2+} optimum of a cell-free system made with ascites extract not preincubated was determined, the endogenous incorporation showed little dependence on Mg^{2+} concentration between 1 and 5 mM; the stimulation with globin mRNA in this cell-free system showed a maximum at 3 mM Mg^{2+} but was severalfold lower than that obtained with the preincubated extract (M. Jacobs-Lorena, unpublished results).

Our results indicate that when a particular amino acid is used to label the cell-free product, it is convenient to determine in advance the optimal amount to be added to the cell-free system. We have observed that maximal incorporation is obtained at a concentration of about 10 μM of the labeled amino acid.

The amount of mRNA that is added to the cell-free system is also quite important. We have thus determined the concentration of globin mRNA that is translated optimally, giving at the same time a high stimulation and good yield of cell-free product. We have established that the presence of relatively small amounts of rRNA has little or no effect on the translation of globin mRNA by the ascites cell-free system. Larger amounts of rRNA may even favour translation of mRNA, possibly by protecting it from nucleases.

There has been one report that translation of viral RNA is dependent on the addition of tRNA to the ascites cell-free system (Aviv *et al.*, 1971) and a recent report that also translation of globin mRNA is dependent on added tRNA (Metafora *et al.*, 1972). We have tried to supplement our cell-free system with tRNA from rabbit liver (a kind gift of Dr. David Housman, Massachusetts Institute of Technology) or tRNA from HeLa cells obtained during the preparation of histone mRNA (Jacobs-Lorena *et al.*,1972). We have failed to observe any stimulation of the incorporation obtained with globin mRNA. Our cell-free system is thus not dependent on tRNA in translating this mRNA. The major difference in the preparation of the ascites extract is the substitution of the rather fast gel filtration step in the procedure followed by us, with a 12-hr dialysis step (Metafora *et al.*, 1972). It seems possible that during this dialysis step the tRNA present in the ascites extract is partially inactivated, since addition of exogenous tRNA prior to the dialysis fails to produce a fully active cell-free system (Metafora *et al.*, 1972).

In conclusion, the ascites cell-free system provides an almost ideal tool for the translation of RNA. It is likely that this cell-free system will be increasingly used by several investigators and we have thus studied some parameters that will be useful to them.

Acknowledgments

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Specific Cleavage between Variable and Constant Domains of Rabbit Antibody Light Chains by Dilute Acid Hydrolysis†

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ABSTRACT: Rabbit antibody light chains were subjected to limited acid hydrolysis and the degree of cleavage assessed by gel filtration and quantitative Edman degradation. Hydrolysis was carried out in the presence of 10% acetic acid–pyridine (pH 2.5) or in this solution in the presence of 25% 1-propanol or 7 M guanidine·HCl, for periods up to 5 days. The range of cleavage achieved under these conditions was 15–90%, optimal cleavage occurring after 4 days in the guanidine solution. The appearance of half-molecules was demonstrated by gel filtration on Sephadex G-75 in acid–urea following full reduction and alkylation. There was no evidence for cleavage at other sites since smaller peptides were not demonstrated. A unique site of cleavage was shown to occur near the beginning of the constant region by Edman degradation. In order to demonstrate a unique sequence beginning at the cleavage point without separation of the two halves of the chain, the amino terminus of the light chain was selectively blocked. After removal of the amino-terminal alanine on one light chain (3315) by Edman degradation, the second residue, glutamine, was cyclized to pyrrolidonecarboxylic acid and the chain simultaneously cleaved in acid in the presence of guanidine. A single sequence of 26 residues was obtained which, on the basis of homology with human and other rabbit light chains was placed at position 109–135, close to the beginning of the constant region. This indicated that the cleavage probably occurred between Asp-109 and Pro-110, a bond previously shown to be acid labile. This approach should greatly aid not only in the sequencing of rabbit antibody light chains but also in defining the roles of the constant and variable domains in the structure and function of antibody.

Cleavage at the switch region between variable and constant domains of immunoglobulin light chains has been described by use of enzymes (Solomon and McLaughlin, 1969; Karlsson *et al.*, 1969). However, the yield of half-molecules produced by these methods is low, of the order of 10–22% and there is great variability in conditions required among various light chains. The rabbit light chain appears to be more resistant to the action of endopeptidases and the more vigorous conditions required result in cleavage at other sites (Poulsen *et al.*, 1972). The separation of variable and constant region segments (V_L and C_L) for structural studies, activity determinations and studies of the distribution of allotypic determinants requires that the cleavage be specific in the switch region and occur in reasonable yield.

Attention has recently been drawn to cleavage of aspartylproline peptide bonds in various proteins exposed to low pH and temperatures in the range of 25–40° during enzymic and chemical hydrolysis (Piszkiewicz *et al.*, 1970). The suggestion (Freedlender and Haber, 1972) that such a cleavage occurred during demaleylation after tryptic digestion of pneumococcal antibody light chains and the placement of the labile Asp–Pro bond in the constant part of the switch region (Strosberg *et al.*, 1972) led us to investigate dilute acid hydrolysis as a means for specific cleavage of the rabbit antibody light chain into variable and constant halves.

Materials and Methods

Preparation of Light Chains. Homogeneous antibodies to the type VIII pneumococcal polysaccharide were obtained using established procedures (Pincus *et al.*, 1970; Cheng and Haber, 1971). The antibodies were subjected to mild reduction and alkylation (Fleischman *et al.*, 1962) and heavy and light chains were separated by gel filtration on Sephadex G-100 using 1 M propionic acid as the eluent.

Aliquots of one light-chain preparation (2388) were di-

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