organism of modified virulence the mean survival time of animals in each group was significantly less ($P < .01$) than the mean survival time of normal mice challenged previously with 10^8 fully virulent *D. pneumoniae* (*1*). Thus, the capacity to elaborate corticosterone appears to be an important factor in host survival, as suggested previously (*8*).

Rhythmicity of adrenocortical secretion has been documented in many animal species. The mouse adrenal cortex is most responsive to adrenocorticotrophic hormone at a time (0400 hours) when circulating corticosterone in lowest, and conversely, minimal response accompanies stimulation when circulating corticosterone is greatest (1600 hours) (*9*). As shown in Fig. 1, maximal corticosterone elaboration follows infectious challenge at 0400 hours, whereas challenge at 1600 hours evokes relatively little increase in corticosterone concentration compared to values in control mice sampled at the same time of day. Longest survival follows challenge at 0400 hours, whereas disease is most devastating after challenge at 1600 hours. These results support the observations of Beisel *et al.* (*10*) and suggest that the endogenous steroid responses to infectious challenge may be anabolic in type and an important factor in survival.

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References and Notes

1. R. D. Feigin, V. A. San Joaquin, M. W. Haymond, R. G. Wyatt, *Nature* **224**, 379 (1969); C. Triyanond, S. Surapanit, W. D. Sawyer, *Bact. Proc.* **69**, M4 68 (Abstr.) (1969).
2. R. D. Feigin, P. G. Shackelford, S. C. Choi, V. V. Weldon, *Exp. Med. Surg.*, in press.
3. I. Z. Beitins, M. H. Shaw, A. Kowarski, C. J. Migeon, *Steroids* **15**, 765 (1970).
4. B. E. P. Murphy, *J. Clin. Endocrinol.* **27**, 973 (1967).
5. A. W. Kimball, *Biometrics* **10**, 452 (1954).
6. E. Haus, D. Lakatua, F. Halberg, *Exp. Med. Surg.* **25**, 7 (1967).
7. M. Wongwiwat, S. Sukapanit, C. Triyanond, W. D. Sawyer, *Infect. Immun.* **5**, 442 (1972).
8. W. R. Beisel and M. I. Rapoport, *N. Engl. J. Med.* **280**, 541 (1964); *ibid.*, p. 596.
9. F. Ungar and F. Halberg, *Science* **137**, 1058 (1962).
10. W. R. Beisel, W. D. Sawyer, E. D. Ryll, D. Crozier, *Ann. Intern. Med.* **67**, 744 (1967).
11. We thank V. V. Weldon, K. Strobel, L. Pickering, D. Anderson, R. Lins, J. Campbell, S. Koslov, and D. Strobel for technical assistance; S. C. Choi for biostatistical consultation; P. R. Dodge for his critical appraisal of the manuscript; and J. Rodgers for help in its preparation. Supported by NIH grant 1R01HD04424, by PHS research career development award 1K04AI46206 to R.D.F., and by PHS postdoctoral research fellowship 1F04AI53004 to P.G.S.

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Complete Amino Acid Sequence of the Mu Heavy Chain of a Human IgM Immunoglobulin

Abstract. *The amino acid sequence of the μ chain of a human IgM immunoglobulin, including the location of all disulfide bridges and oligosaccharides, has been determined. The homology of the constant regions of immunoglobulin μ, γ, α, and ε heavy chains reveals evolutionary relationships and suggests that two genes code for each heavy chain.*

Because of the importance of immunoglobulin M (IgM) as the first antibody formed in the newborn animal and in the primary immune response, and because of its role in certain autoimmune diseases, we have determined the amino acid sequence and the carbohydrate composition of a human macroglobulin with a covalent molecular weight of 950,000 (*1*). The protein sequenced (Ou) is from a patient with macroglobulinemia and serves as a model for structural study of IgM antibodies just as Bence Jones proteins have been models for light chains and myeloma globulins have been models for IgG antibodies (*2*). Protein Ou is comprised of a pair of κ light chains disulfide-bonded to a pair of μ heavy chains to give a monomeric subunit of 190,000 molecular weight. Five such subunits are joined through an intersubunit bridge on each μ chain to form a pentamer (*3*). Because of this structural symmetry, the primary structure of the entire polymer could be established by amino acid sequence determination of the κ light chain and of the μ heavy chain and by identification of all of the disulfide bridges in each chain. We have reported the animo acid sequence of the κ light chain (*4*) and of the Fabμ fragment, which comprises the light chain and the Fd′ portion of the μ chain (*5*); we have also given extensive sequence data on parts of the remainder of the μ chain (*6*) and on the location and composition of the five oligosaccharides C1 to C5 (*7*). We now report the sequence of the 576 residues in this μ chain and the location of all disulfide bridges and oligosaccharides. This is the longest continuous sequence for a single polypeptide chain yet recorded.

Structural study of IgM is facilitated by limited cleavage of the polymer by incubation with trypsin for 4 hours at 60°C (*8*). This yields two fragments, Fabμ and Fcμ, and also some peptides excised from the portion of the μ chain between Fabμ and Fcμ (Fig. 1). Fabμ consists of the NH_2-terminal portion of the μ heavy chain (the first 213

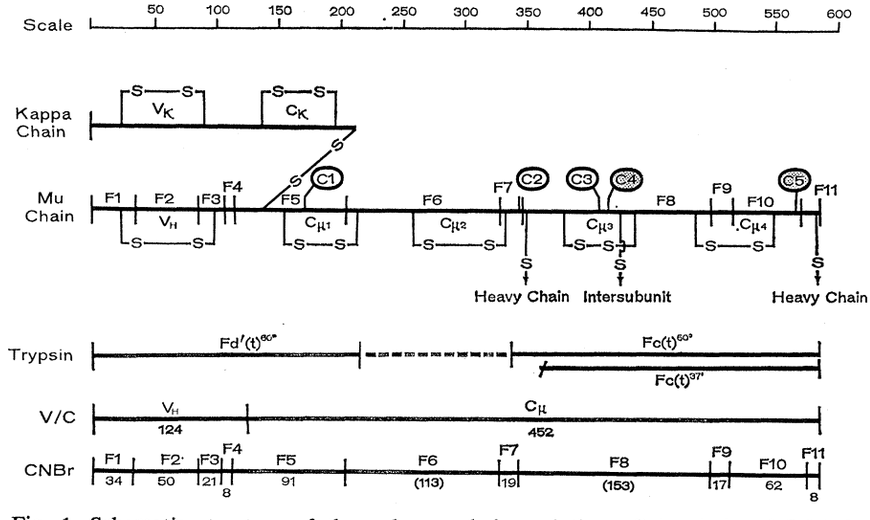

Fig. 1. Schematic structure of the μ heavy chain and the κ light chain of IgM Ou showing (i) the interchain and intrachain disulfide bridges, (ii) the two homology regions of the light chain ($V\kappa$ and $C\kappa$), and (iii) the five homology regions of the heavy chain (V_H and $C\mu 1$ to $C\mu 4$). The figure for the μ chain also shows the location of the five oligosaccharides (C1 to C5), the points of cleavage by trypsin with the respective fragments (Fd′ and Fc), the sites of cleavage by CNBr and the respective fragments (F1 to F11), and the point of division between the variable and constant regions (V_H and $C\mu$). The scale and the numbers indicate the number of amino acid residues in each chain and fragment.

residues are designated Fd′), which is linked to the intact light chain. Fcμ is the COOH-terminal segment comprising the last 251 residues of the μ chain. A pair of Fc segments is linked together through disulfide bonds to form the monomeric Fcμ fragment, and five pairs are linked to form the pentameric $(Fc)_5\mu$ fragment, which has a molecular weight of 340,000. The human μ chain has five homology regions or domains, each containing some 110 to 120 amino acid residues with one intrachain disulfide loop of about 60 residues (Fig. 1). Two of the domains (V_H and Cμ1) are in Fd′, one (Cμ2) is in the adjacent region that is degraded to peptides, and two (Cμ3 and Cμ4) are in Fc.

For determination of the sequence seven major procedures were required;

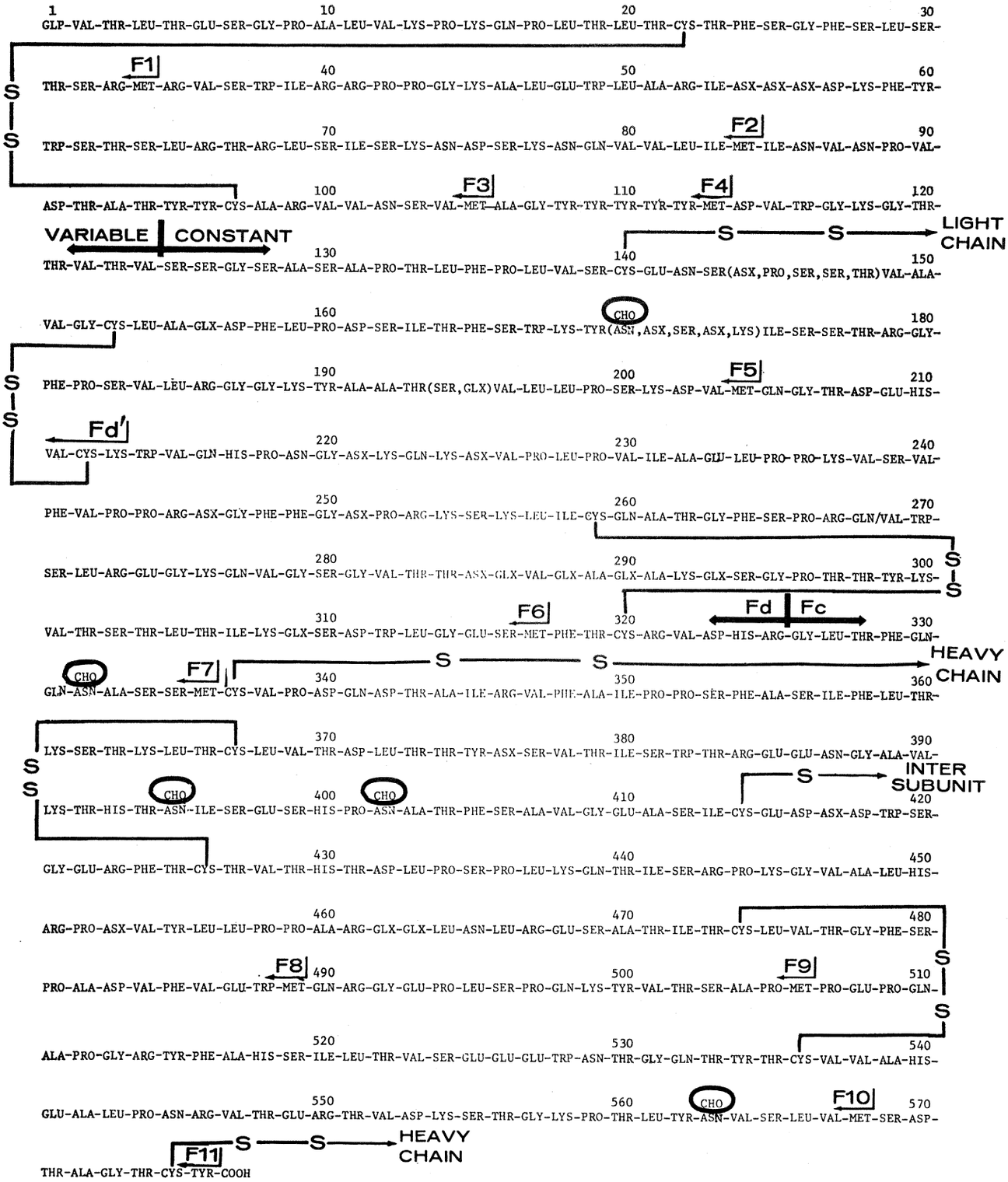

Fig. 2. Amino acid sequence of the μ heavy chain of IgM Ou. The position of the interchain and intrachain bridges and of the carbohydrate units is identified and also the sites of cleavage by CNBr and the 11 fragments obtained (F1 to F11).

these are (i) reduction and alkylation of the whole IgM followed by isolation of the light and heavy chains; (ii) limited tryptic digestion of IgM at 60°C and separation of the Fab and Fc fragments; (iii) cleavage in separate experiments of the μ chain and of the Fab and Fc fragments with CNBr and isolation of the resulting 11 fragments designated F1 to F11 (Fig. 1); (iv) digestion of the μ chain with trypsin and thermolysin in separate experiments followed by isolation and purification of some 60 tryptic peptides and more than 150 thermolytic peptides; (v) digestion with trypsin or chymotrypsin of some of the CNBr fragments and of Fab or Fc and purification of many of the resulting peptides; (vi) digestion with pepsin of the whole IgM molecule and purification of the peptic peptides; and (vii) special procedures such as the tryptic digestion of succinylated F5 and F6 fragments. Some of the peptides obtained by different procedures were identical; altogether nearly 600 different peptides were isolated or about one for each residue. Many of these peptides were completely sequenced; others were partially sequenced, and for some only the amino acid composition and terminal groups were determined. The procedures for peptide purification and sequence analysis by the manual Edman and dansyl-Edman methods have been fully described (*9*). In addition, the automatic protein sequencer (Beckman model 890) was used both for large fragments and for peptides with adherence to procedures described by Hermodson *et al.* (*10*).

The amino acid sequence of the μ chain is shown in Fig. 2 and is described by reference to the five homology regions beginning with V_H; this is the generic term for the variable (V) region of heavy chains regardless of class (μ, α, γ, δ, and ε).

The class character of heavy chains is expressed only in the constant (C) region, and idiotypic differences in sequence are restricted to the V region. This important principle was first recognized when we compared the NH_2-terminal sequence of the Ou μ chain with the sequences of a series of human γ1 chains (*11*). Complete sequence data from several laboratories (*6, 12*) have shown that the V regions of some heavy chains of different class may have twice as many identical residues in sequence as V regions of some chains of the same class. For example, the V_H sequence of the Ou μ chain has 65 to 70 percent homology with the V_H sequence of the Daw and Cor γ1 chains although the C regions of the μ and γ1 chains have only about 30 percent homology. Other human γ chains such as Nie and Eu (*12*) have identical sequence in the C region, but only 30 to 45 percent identity in the V_H region with Daw and Cor. Extensive partial sequence data on the NH_2-terminal sequences of many μ, γ, and α chains has led to the proposal (*13–15*) that at least three variable-sequence subgroups (V_{HI}, V_{HII}, and V_{HIII}) are common to all classes of heavy chains and that separate genes code for the V_H and C_H regions (*5, 6*). The Ou μ chain belongs to the V_{HII} subgroup which has a blocked NH_2-terminus.

The C region sequence of the human μ chain begins at or near Ser-125 in the Ou μ chain. This conclusion is based on our comparative sequence analysis of three other μ chains, Ga, Di, and Dau (*16*). These differ in sequence and in length of the V region; but they have identical sequence from Ser-125 (in the Ou numbering system) through Val-138, and in all of the many additional segments thereafter for which we have thus far completed the structure. Of course, as more μ chains are sequenced, the length of the V region may be extended by one or two more residues, as was the case for κ light chains (*2*). Thus, in the switch region, that is, the juncture of the V and C regions, human μ and γ chains share at most only the Ser-Ser sequence. This seems an insufficient recognition signal for the union of V and C genes.

The Cμ1 region includes the disulfide bridge to the light chain at Cys-140 and the first of five carbohydrate prosthetic groups (*7*). The Fd′ segment of Fabμ terminates at Lys-213 just after the second intrachain disulfide loop. Because the following portion, including most of Cμ2, is degraded to peptides by trypsin cleavage of IgM at 60°C we assume that it is a more exposed and flexible portion of the molecule. Technical problems including the cyclization of Gln-205 in CNBr fragment F6 and of Gln-268 have caused some difficulty in proof of the overlap and the sequence just after Gln-268. Our present evidence favors an overlap of Gln-Val, but based on the amino acid composition of the CNBr fragment F6 and the many peptides derived from this area not more than two residues are missing at this overlap. Except for several other short segments in parentheses where the exact order has not been ascertained, this is the only significant point of ambiguity in the entire sequence (*17*). Of course, in any protein this size there is the possibility of small errors in the assignment of sequence, and further checks are being made of additional peptides to verify the proposed structure.

We have earlier described the structural characteristics of the hinge region (*5*), the segment NH_2-terminal to and including the beginning of the Fc region. Completion of the sequence between Fd′ and Fc does not reveal any segment that is rich in proline and half-cystine like the hinge regions of the human γ and α chains. The human γ1 chain contains 3 half-cystines and 5 prolines in a sequence of 16 residues at the juncture of Fdγ and Fcγ (*12*), and γ2, γ3, and γ4 chains are even richer in half-cystine and proline (*18*). Human α1 chains have 4 half-cystines and 14 prolines in a 38-residue segment designated the hinge region (*19, 20*), which also has an oligosaccharide containing galactosamine. In contrast, only one interchain disulfide bond and few prolines are present at the beginning of the Fcμ region. The most proline-rich sequence of the μ chain is the sequence after Fd′, which contains 8 prolines in the 35-residue sequence from Pro-218 through Pro-252. Since this is a region of tryptic excision, it may correspond to the hinge regions of the γ and α chains although it lacks disulfide bridges.

The Cμ3 domain is rich in carbohydrate: within a space of 70 residues there are two complex oligosaccharides, C2 and C3, and a simple oligosaccharide, C4 (*7*). Both C3 and C4 are within an intrachain disulfide loop and are close to the intersubunit disulfide bridge that links the monomers to form a pentamer. Because the carbohydrate is bulky and hydrophilic we assume it to be at the surface of the IgM. This would greatly affect the conformation of the Cμ3 domain and the interaction of the ten such domains in the pentamer.

Unlike monomeric IgG antibody, a single pentameric IgM antibody can fix complement after interaction with specific antigen. In IgG the binding site for complement is supposed to be at the sequence just after the interchain disulfide bond at the beginning of the Fcγ region in the Cγ2 domain (*21, 22*). By analogy to IgG the complement-fixing site of IgM is assumed to

be on Fcμ. In the absence of antigen, the $(Fc)_5\mu$ fragment fixes complement up to 20 times more effectively than intact IgM on a molar basis, and the Fcμ monomer is equally effective (*21*). Plaut *et al.* (*21*) therefore conclude that the Fcμ portion of a single subunit of IgM antibody bears a suitable complement receptor site that is inaccessible in the whole IgM molecule. Because there is no close similarity in sequence at the beginning of the Fc regions of IgG and IgM, the binding site seems more dependent on molecular conformation than on primary structure. This fits with the usual requirements for complement fixation, namely, (i) an antigen-antibody interaction that may induce an allosteric rearrangement of the Fc region and (ii) a minimum of two monomeric IgG molecules or one pentameric IgM. Because C1q, the first component of the complement cascade, has a molecular weight comparable to that of intact IgM and has a six-stranded structure (*22*), it is unlikely that fixation involves a small site of peptide sequence.

The most unexpected finding of our sequence study of the μ chain is the relatively low degree of homology of the constant regions of μ and γ1 chains despite the high degree of homology of the V_H regions of μ and γ1 chains of the same subgroup. Although sequences of up to 13 consecutive residues are identical in the V_H region of some μ and γ1 chains (*6*), the longest single stretch of identity in comparing the 332 residues of Cγ with the 452 residues of Cμ is only the pentapeptide sequence Leu-Thr-Cys-Leu-Val. This involves Cys-367 in the fourth homology loop of the μ chain (Cμ3) and Cys-367 in the fourth homology loop of the γ1 chain. This reflects the fact that the greatest degree of homology centers around the intrachain half-cystine residues of all light and heavy chains. In contrast, the greatest disparity in structure exists in the segments between the domains especially in the hinge region and at the COOH-terminus of the chains. The intrachain disulfide bridges and the pattern of repeating homology regions are the most strongly conserved features of immunoglobulin chains, probably because they are essential for retention of the conformation of the chains and for mutual interactions.

When the three homology regions of the γ chain (Cγ1, Cγ2, and Cγ3) and the four of the μ chain (Cμ1 to Cμ4) are aligned by placing the intrachain half-cystine residues in register, their relationship can be compared either by visual inspection or by computer analysis of the nucleotide mutation distance. We have tried both approaches but cannot unambiguously order the relationship of the homology regions on the μ chain with those on the γ chain. The best fit seems to be between Cγ1 and Cμ1, between Cγ2 and Cμ3, and between Cγ3 and Cμ4. Excluding six gaps inserted to maximize the homology, the latter pair has 40 percent identity in amino acid sequence provided that one also omits the 19 additional residues that extend from the μ chain (Lys-558 to Tyr-576) for which there is no counterpart in the γ chain.

From the partial sequence data now available on the ε and α heavy chains, it appears that the most closely related pairs are ε and γ and μ and α. In the COOH-terminal domain (excluding the COOH-terminal nonadecapeptide "tail" of the μ chain), the differences between each pair of the sequences of human μ, γ, and ε are close in number, but the largest difference occurs between μ and ε: this led Bennich *et al.* (*23*) to suggest that the latter two genes diverged first. Less data are available for the human α chain. However, results of our laboratory (*20*) and of Kehoe *et al.* (*24*) indicate that the human μ and α chains have 55 to 60 percent identity in the last 50 residues, including the COOH-terminal nonadecapeptide tail that is missing in the γ and ε chains. Although this is almost twice as great as the homology between the other pairs of heavy chains described above, our data on the sequence of the rest of the α chain do not support such a high degree of homology throughout the entire lengths of the C regions of the μ and α chains.

Comparative sequence analysis of the heavy chains of different classes affords evidence of the evolutionary development of immunoglobulins M, G, A, and E and enables evaluation of the structural basis of their different functions. The finding that V region sequences of heavy chains of different classes, but of the same subgroup, can be twice as much alike as C region sequences of chains of different classes favors the hypothesis that two genes code for each immunoglobulin heavy chain, one for the V region and one for the C region.

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References and Notes

1. For the nomenclature of immunoglobulins and schematic diagrams of their polypeptide chain structure and of the Fab and Fc pieces see F. W. Putnam [(*2*) and *J. Human Evol.* **1**, 591 (1972)]. The variable and constant regions are referred to as the "V region" and the "C region," respectively. V_H and C_H are used to refer to the corresponding regions of heavy chains. Homology regions or domains consisting of segments of about 110 residues each containing one intrachain disulfide bridge are denoted Cμ1, Cμ2, and so on for μ chains [see *Bull. Wld. Hlth. Org.* **41**, 975 (1969) and *J. Immunol.* **108**, 1733 (1972)]. Standard three-letter abbreviations are used for amino acid residues.
2. F. W. Putnam, *Science* **163**, 633 (1969); ——— and H. Köhler, *Naturwissenschaften* **56**, 439 (1969).
3. A third polypeptide chain has been described in polymeric IgA and IgM [M. S. Halpern and M. E. Koshland, *Nature* **228**, 1276 (1970); J. Mestecky, J. Zikan, W. T. Butler, *Science* **171**, 1163 (1971)]. This chain, called J chain because of its supposed function of joining monomers, has been studied by many laboratories including our own. Only the composition and sparse data on the cysteine-containing peptides are available. The J chain does not correspond in composition or structure to any portion of the μ chain. Although one J chain per pentamer of IgM is thought to be attached to the intersubunit bridge at Cys-414, its exact location and function are unknown.
4. H. Köhler, A. Shimizu, C. Paul, F. W. Putnam, *Science* **169**, 56 (1970); F. W. Putnam, A. Shimizu, C. Paul, T. Shinoda, H. Köhler, *Ann. N.Y. Acad. Sci.* **190**, 83 (1971).
5. A. Shimizu, C. Paul, H. Köhler, T. Shinoda, F. W. Putnam, *Science* **173**, 629 (197→ C. Paul, A. Shimizu, H. Köhler, F. W. Putnam, *ibid.* **172**, 69 (1971).
6. F. W. Putnam, A. Shimizu, C. Paul, T. Shinoda, *Fed. Proc.* **31**, 193 (1972); F. W. Putnam, T. Shinoda, A. Shimizu, C. Paul, G. Florent, E. Raff, *Third International Convocation on Immunology* (Karger, Basel, in press).
7. A. Shimizu, F. W. Putnam, C. Paul, J. R. Clamp, I. Johnson, *Nature New Biol.* **231**, 73 (1971).
8. A. G. Plaut and T. B. Tomasi, Jr., *Proc. Nat. Acad. Sci. U.S.A.* **65**, 318 (1970).
9. T. Shinoda, K. Titani, F. W. Putnam, *J. Biol. Chem.* **245**, 4475 (1970); K. Titani, M. Wikler, T. Shinoda, F. W. Putnam, *ibid.*, p. 2171.
10. M. A. Hermodson, L. H. Ericsson, K. Titani, H. Neurath, K. A. Walsh, *Biochemistry* **11**, 4493 (1972).
11. M. Wikler, H. Köhler, T. Shinoda, F. W. Putnam, *Science* **163**, 75 (1969).
12. G. M. Edelman, B. A. Cunningham, W. E. Gall, P. D. Gottlieb, U. Rutishauser, M. J. Waxdal, *Proc. Nat. Acad. Sci. U.S.A.* **63**, 78 (196→ B. A. Cunningham, M. N. Pflumm, U. Rutishauser, G. M. Edelman, *ibid.* **64**, 997 (1969); E. M. Press and N. M. Hogg, *Biochem. J.* **117**, 641 (1970); H. Ponstingl and N. Hilschmann, *Hoppe-Seyler's Z. Physiol. Chem.* **353**, 1369 (1972).
13. H. Köhler, A. Shimizu, C. Paul, V. Moore, F. W. Putnam, *Nature* **227**, 1318 (1970).
14. A. C. Wang, J. R. L. Pink, H. H. Fudenberg, J. Ohms, *Proc. Nat. Acad. Sci. U.S.A.* **66**, 657 (197→ A. C. Wang, H. H. Fudenberg, J. R. L. Pink, *ibid.* **68**, 1143 (1971).
15. J. D. Capra, *Nature New Biol.* **230**, 61 (19→ J. M. Kehoe and J. D. Capra, *Proc. Nat. Acad. Sci. U.S.A.* **68**, 2019 (1971).
16. G. Florent, D. Lehman, F. W. Putnam, unpublished work.
17. This work was presented at the International Congress of Biochemistry, 1 to 6 July 1973 in Stockholm, Sweden. At this congress, N. Hilschmann and H. Ponstingl reported the sequence of the μ heavy chain of another human monoclonal IgM protein, but they have not yet published any sequence data. As is expected, there are many differences in sequence in the variable region of the two μ chains. However, in the constant region there appear to be only minor differences and these can be attributed to technical difficulties.
18. B. Frangione, C. Milstein, J. R. L. Pink, *Nature* **221**, 145 (1969).
19. C. Wolfenstein, B. Frangione, E. Mihaesco, E. C. Franklin, *Biochemistry* **10**, 4140 (1971);

E. Mendez, B. Frangione, E. C. Franklin, *ibid.*, in press.
20. V. Moore and F. W. Putnam, *ibid.* **12**, 2361 (1973); T. Low, V. Liu, F. W. Putnam, unpublished work.
21. A. G. Plaut, S. Cohen, T. B. Tomasi, Jr., *Science* **176**, 55 (1972).
22. K. B. M. Reid, D. M. Lowe, R. R. Porter, *Biochem. J.* **130**, 749 (1972).
23. H. Bennich, C. Milstein, D. S. Secher, *FEBS Fed. Eur. Biochem. Soc. Lett.*, in press.
24. J. M. Kehoe, C. Chuang, J. D. Capra, *Fed. Proc.* **32**, 968 (abstr.) (1973).
25. We thank J. Madison, P. Keim, J. Novotny, and G. East for technical assistance and Drs. J. L. Fahey and W. D. Terry of the NIH Clinical Center, Bethesda, Md., for plasma from patient Ou. Supported by grants CA-08497 from NIH, NP-10 from the American Cancer Society, and DRG-1134 from the Damon Runyon Fund for Cancer Research.

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Neuronal Properties of Nymphal and Adult Insect Neurosecretory Cells in vitro

Abstract. *Neurosecretory brain cells from late nymphal and adult cockroaches were grown for 1 to 3 months in a chemically defined medium in combined cultures with embryonic organs or oocytes from nymphal and adult specimens. Under these conditions, neurosecretory cells show vigorous axonal growth. Electron dense granules in cell perikarya and axons of 3-month-old cultures are suggestive of neurosecretory activity in vitro.*

The development in our laboratory of a chemically defined medium which supports long-term survival and nerve fiber outgrowth from intact embryonic brain ganglia (*1*), dissociated embryonic nerve cells (*2*), and nymphal ganglia of the stomatogastric system of the cockroach *Periplaneta americana* (*3*) suggested use of the same medium and techniques to explore the insect neurosecretory (NS) system. We have already reported successful results with nymphal and adult corpora cardiaca cultured in vitro for periods ranging between 4 and 8 weeks (*4*). It was the object of the present study to extend this analysis to the NS cells located in the brain. The selection of the two large paired protocerebral NS groups was dictated by considerations of the key role they play in the life of the insect as well as by technical reasons. They are an ideal object for experimentation because of their ready visualization in the intact living brain in the dorsomedial aspect of the protocerebrum and because of their fairly large size, which makes them easily distinguishable from adjacent nonneurosecretory cells. The extensive literature available on the structural, ultrastructural, and electrophysiological properties of these cells in vivo (*5*) was an additional reason for their selection for studies in vitro.

On removal of the head capsule under aseptic conditions, the two groups of medial NS cells were identified in the intact brain under a stereomicroscope by their position, as well as by the pale bluish color due to light scattering (Tyndall effect) by the NS granules which fill most of the perikarya. Histological and electron microscopic studies performed immediately after dissection of the two groups in several nymphal and adult specimens showed that each group consists of about 200 neurons and an undetermined number of glial cells. The three cell types, known as A, B, and C cells, were identified by their size, color affinity, and content of NS granules. The medium-size A cells are characterized by their high staining affinity for performic acid Victoria blue dye, which selectively stains cysteine- and cystine-rich peptides. The intense blue shade of the cells and of their axons in brain whole mounts, as well as in histological sections, makes it possible to identify these neurons under the light microscope (*6*). Cell counts performed in ten adult specimens stained with this technique give an average of 70 cells of type A per group. In the electron microscope, the perikarya and their axons are filled with electron dense granules. The B cells, of about the same size as the A cells, do not stain with Victoria blue but take a red color with Gomori's chrome hematoxylin phloxine technique, which is routinely used for staining NS material. In the electron microscope, they show a variable number of electron dense granules. They comprise about one-third of the entire cell population. The C cells are much larger than the A and B cells; only a few small electron dense granules are seen in the electron microscope in their perikarya. About ten of these large C cells are present in each group. Scattered between the A, B, and C cells are neurons with few or no electron dense granules. Whether they represent a nonneurosecretory cell type or NS cells in a quiescent stage is at present unsettled. Electron microscopic studies of a large number of other brain cells in short- and long-term cultures show no evidence of electron dense granules in the perikarya and axons.

Immediately after dissection from the brain, the two groups of NS cells were transferred into the culture medium consisting of four parts of Eagle solution and five parts of Schneider's insect medium in small glass vessels, according to the technique previously described (*1–4*). The explants were dissociated in situ into cell clusters of various sizes (Fig. 1a) or into individual cells with the help of microneedles; in a few instances the groups were explanted in toto. Intact groups, cell clusters, or individual cells were gently pressed on the surface of a cover slip laid on the bottom of the culture dish until they firmly adhered to it. The cultures were divided into three groups. In the first, NS explants were not combined with other tissues or organs; in the second, they were combined with brain, ganglia, and foregut segments from 16-day embryos of the same species; in the third, they were combined with ovariole segments dissected out from late nymphal or adult specimens. The several tissues were positioned at a distance of 0.5 to 1 mm from each other in a variety of geometrical arrangements. In the second and third groups, embryonic tissues or ovarioles were also pressed on the cover slip until they adhered to its surface. The culture vessels were then placed in desiccators and incubated at 29°C. The results reported below are based on observations of 400 cultures examined daily at the inverted microscope. Photomicrographs were taken by using the Nomarski microscope. When the cultures were discontinued between the end of the second week and the third month, they were fixed and stained in toto with performic acid Victoria blue or used for electron microscopic studies.

Up to the end of the first week, no nerve fiber outgrowth was evident from NS explants in any of the three groups. From the beginning of the second week, differences appeared between NS explants of the three groups and became more pronounced in subsequent periods. Small or large cell clusters or intact cell aggregates of the first experimental group showed signs of deterioration, and no nerve fiber outgrowth took