binding to fragment 1 to 116, and its failure to bind fragment 44 to 88.

Our conclusion from the data in Table 1 is that there are at least three mutually exclusive sites on the myelin basic protein molecule capable of binding antibody induced by homologous protein. We cannot conclude that there are only three since some of the fragments may contain more than one site. That some serums bind one or two of the fragments and fail to bind others indicates that there is no cross-reactivity among the specific sequences detected in the three regions.

Fragment 1 to 20 was not bound significantly by any of the serums listed in Table 1, with the possible exception of serum A. This region may contain a weak site, but the fact that only one of several dozen serums tested bound this fragment led us to conclude it was of little importance. This nonantigenic region of the molecule is probably at least 43 residues in length. No serum was found to have significantly greater binding for the mixture of fragments from residues 1 to 89 than for fragment 44 to 88.

The immunization schedules yielding serum A and serum C differed only in the length of time between priming with IFA and challenge with CFA. During the 6-month delay, only those cells capable of recognizing the center site survived. This fact, coupled with the frequent occurrence of serums capable of binding the center site suggests that this region is the most antigenic portion of the molecule. This is also the most reactive region of the molecule in other respects: it includes part of the site that induces EAE in guinea pigs (residues 114 to 122) (*12*), it contains the single methylated arginine residue 107 (*13*), and the single threonine residue 98 reported to be the receptor for glycosylation (*14*), and it is part of the region that binds most readily to isolated myelin lipid fractions (*15*).

The technique used in our study to demonstrate multiple sites within the molecule capable of binding specific antibody differs somewhat from that used in other studies (*1, 2*). By manipulating the immunization schedule, we obtained unique antiserums that differed qualitatively in their ability to bind fragments derived from the whole molecule. The success of our experimental approach with myelin basic protein suggests that at least some of its specific antigenic regions do not depend upon conformational characteristics of the intact molecule as was the case with lysozyme (*2*).

Although the role of antibody to myelin basic protein in pathogenesis of EAE was not investigated, it should be noted that the ability of basic protein to induce antibody appears to be unrelated to its ability to induce disease. The amount of mycobacteria present in the immunization mixture is critical for antibody production but not for disease induction—a single injection of basic protein with 2.5 mg of mycobacteria induces high levels of antibody, whereas an equally encephalitogenic injection of basic protein with 0.1 mg mycobacteria induces almost no antibody. Antigenicity of the latter mixture can be enhanced, however, by chemical modification of the antigen (Table 1, serum F) or by priming of the host with repeated injections of antigen in IFA (serum A). In both instances, enhanced antibody production is accompanied by loss of ability to induce EAE.

BERNARD F. DRISCOLL
ALLAN J. KRAMER, MARIAN W. KIES
Section on Myelin Chemistry, Laboratory of Cerebral Metabolism, National Institute of Mental Health, Bethesda, Maryland 20014

References and Notes

1. S. Shinka, M. Imanishi, N. Miagawa, T. Amano, M. Inouye, T. Tsugita, *Biken J.* **10**, 89 (1967).
2. D. H. Sachs, A. N. Schechter, A. Eastlake, C. B. Anfinsen, *J. Immunol.* **109**, 1300 (1972).
3. B. F. Driscoll, M. W. Kies, E. C. Alvord, Jr., *ibid.* **112**, 392 (1974).
4. G. E. Deibler, R. E. Martenson, M. W. Kies, *Prep. Biochem.* **2**, 139 (1972); R. E. Martenson, G. E. Deibler, M. W. Kies, S. Levine, E. C. Alvord, Jr., *J. Immunol.* **109**, 262 (1972).
5. L. P. Chao and E. R. Einstein, *J. Biol. Chem.* **245**, 6397 (1970).
6. All fragments are identified by residue numbers reported for bovine myelin basic protein by E. H. Eylar, S. Brostoff, G. Hashim, J. Caccam, P. Burnett [*ibid.* **246**, 5770 (1971)].
7. P. R. Burnett and E. H. Eyler, *ibid.*, p. 3425.
8. P. R. Carnegie, *Biochem. J.* **111**, 240 (1969).
9. R. E. Martenson, G. E. Deibler, A. J. Kramer, in preparation.
10. E. C. Alvord, Jr., C. M. Shaw, S. Hruby, M. W. Kies, *Ann. N.Y. Acad. Sci.* **122**, 333 (1965).
11. R. E. Martenson, G. E. Deibler, M. W. Kies, S. S. McKneally, R. Shapira, R. F. Kibler, *Biochim. Biophys. Acta* **263**, 193 (1972).
12. E. H. Eylar, J. Caccam, J. J. Jackson, F. C. Westall, A. B. Robinson, *Science* **168**, 1220 (1970).
13. G. S. Baldwin and P. R. Carnegie, *ibid.* **171**, 579 (1971).
14. A. Hagopian, F. C. Westall, J. S. Whitehead, E. H. Eylar, *J. Biol. Chem.* **246**, 2519 (1971).
15. Y. London and F. G. A. Vossenberg, *Biochim. Biophys. Acta* **478**, 478 (1973).
16. We thank J. D. Stream for assistance in the preparation of antiserums and the cooperation of Dr. R. E. Martenson and G. Deibler in the preparation and characterization of the peptide fragments. B.F.D. and A.J.K. were supported by grant 828-A-4 from the National Multiple Sclerosis Society.

7 December 1973

Follicle-Stimulating Hormone Secretion in the Female Rat: Cyclic Release Is Dependent on Circulating Androgen

Abstract. *In adult cyclic female rats, intravenous injections of an antiserum to testosterone prevented the continued increase in serum follicle-stimulating hormone (FSH) which normally occurs during the early morning hours of estrus. This treatment did not prevent the initial increases in serum FSH (and luteinizing hormone) which occur during the critical period (1400 to 2000 of proestrus), nor did it interfere with subsequent ovulation. These observations indicate the existence of two separate mechanisms for preovulatory FSH release in the rat and implicate circulating testosterone as the stimulus for continued secretion of FSH during estrus.*

In the rat, the preovulatory increases in serum follicle-stimulating hormone (FSH) may be separated into two phases. The initial period of release occurs on the afternoon of proestrus (during the "critical period") and is characterized by rapid increases in serum concentrations of FSH as well as of luteinizing hormone (LH). The second phase of FSH release follows the first and occurs between midnight on the day of proestrus and 0600 of the following morning (day of estrus). During this second phase, serum LH has returned to baseline values while serum FSH is maintained at concentrations equal to, or greater than, those observed during the critical period (*1*). Thus, the greater part of FSH secretion in the female rat occurs in the absence of LH release. In this respect gonadotropin secretion in the rat differs from that of the human and the monkey in which both gonadotropins are released concurrently at midcycle (*2*).

The essential role of circulating estrogen in the stimulation of preovulatory LH release has been clearly established in several species (*3*). In has been tacitly assumed, in the absence of evidence to the contrary, that the "positive feedback" action of estrogen pro-

vides the essential stimulus for both phases of preovulatory FSH release in the rat (*4*). This report indicates that androgens are required for the second phase of FSH release during the morning of estrus in the rat.

Adult female rats, obtained from the Holtzman Company, Madison, Wisconsin, were maintained under conditions of controlled lighting (lights on 0500 to 1900). Vaginal smears were obtained each morning for a period of at least 2 weeks and served as the criterion for establishing the length of the estrous cycle (4 or 5 days) and the day of anticipated preovulatory LH release (proestrus). At approximately 1700 on the afternoon of the day preceding proestrus, each rat was lightly anesthetized with ether and received an intravenous injection (via the femoral vein) of either 0.3 ml of a sheep antiserum to testosterone (*5*) or an equal volume of 0.9 percent saline. All animals were killed by decapitation at intervals ranging from 23 to 42 hours after injection. Animals killed at 1500 on days of the cycle other than proestrus or estrus (estrus +1, +2, or +3 in Fig. 1) received no treatment prior to decapitation. Serum concentrations of LH, FSH, and testosterone were determined by appropriate radioimmunoassay systems (*6*).

As indicated in Fig. 1, serum testosterone concentrations are maximal on the afternoon of proestrus during the preovulatory release of LH and decline rapidly thereafter. The injection of an antiserum against testosterone produced no significant changes in the circulating levels of LH or FSH during the interval of expected LH release (1600 to 2000) but virtually abolished the second phase of FSH which normally occurs during the early morning hours of estrus. All animals killed at 1100 on the day of estrus had ovulated, as indicated by the presence of tubal ova.

Although it has been reported that injections of testosterone stimulate follicular development in the rat (*7*), the existence and possible physiological significance of a preovulatory increase in serum testosterone has received but little attention (*8*). The present report suggests that the increased levels of testosterone found during proestrus in the female rat stimulate the continued secretion of FSH during estrus. Although it is possible that the injected antiserum may have had effects other than neutralization of testosterone and dihydrotestosterone (*5*), it is unlikely that the effects of the antiserum can be attributed to the neutralization of circulating estrogens. It has been clearly demonstrated that antiserums which are effective in neutralizing estrogen in the rat are also effective in preventing preovulatory LH release (*3*), an effect which was clearly absent in our treated animals. In light of the suggestion that androgen treatment may alter the molecular characteristics and biological half-life of FSH (*9*), we have considered the possibility that the secondary phase of increased serum FSH may result from a transient decrease in the rate at which FSH is removed from the circulation. However, an assessment of the rate of disappearance of circulating FSH following hypophysectomy at 0200 of estrus provides no indication of a decreased rate of FSH clearance (*10*). In addition, it seems unlikely that the testosterone acts directly on the pituitary gland to produce a selective release of FSH (*11*).

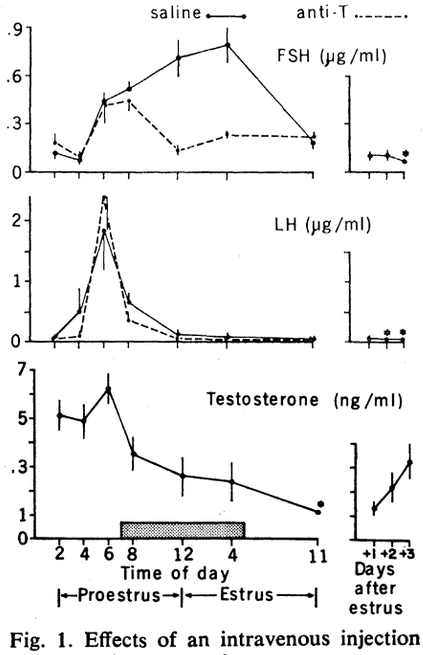

Fig. 1. Effects of an intravenous injection of an antiserum against testosterone on serum FSH in the cyclic rat. Serum concentrations (mean ± standard error) of LH and FSH are expressed in terms of the appropriate NIAMD reference preparations. Serums from antiserum-injected animals were not assayed for testosterone. Points indicated by an asterisk were below the sensitivity of the assay system. Indicated times encompass the interval from 2 p.m. proestrus to 11 a.m. of estrus; hours of darkness (1900 to 0500) are indicated by the stippled bar on the abscissa. Each group contained four or five rats, except the estrus-0400 groups, which contained seven rats. Points on dashed lines: values following an injection of sheep antiserum to testosterone (*anti-T*); points on solid lines: values following a saline injection.

In the absence of circulating testosterone the preovulatory pattern of gonadotropin secretion in the rat is altered to resemble that of other species in which release of LH and FSH are coincident (*2*). While it is not yet clear whether the initial period of FSH release in the rat is accomplished by mechanisms comparable to those which operate in other species, it is now clear that the second phase of FSH release is dependent on androgens. The mechanisms by which circulating androgen promotes this relatively unique second phase of FSH release remain to be determined.

VERNON L. GAY*
R. L. TOMACARI
Department of Physiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

References and Notes

1. V. L. Gay, A. R. Midgley, Jr., G. D. Niswender, *Fed. Proc.* **29**, 1880 (1970); T. A. Daane and A. F. Parlow, *Endocrinology* **88**, 653 (1971).
2. R. F. Weick, D. J. Dierschke, F. J. Karsch, W. R. Butler, J. Hotchkiss, E. Knobil, *Endocrinology* **93**, 1140 (1973); A. R. Midgley, Jr., and R. B. Jaffe, *J. Clin. Endocrinol. Metab.* **28**, 1699 (1968).
3. M. Ferin, A. Tempone, P. E. Zimmering, R. L. Vande Wiele, *Endocrinology* **85**, 1070 (1969); T. Yamaji, D. J. Dierschke, J. Hotchkiss, A. N. Bhattacharya, A. H. Surve, E. Knobil, *ibid.* **89**, 1034 (1971); S. E. Monroe, R. B. Jaffe, A. R. Midgley, Jr., *J. Clin. Endocrinol. Metab.* **34**, 342 (1972).
4. N. B. Schwartz, *Recent Prog. Horm. Res.* **25**, 1 (1969).
5. The antiserum, generously supplied by Dr. G. D. Niswender, was raised against testosterone-11α-succinyl-BSA and has been characterized in two systems. In a radioimmunoassay system which quantitates the ability of a given antiserum to bind various steroid molecules, testosterone and dihydrotestosterone were more than 100 times as active as other tested molecules, including estradiol-17β and androstendione. In a biological system, the selected volume of antiserum was capable of neutralizing the inhibitory effects of circulating testosterone on the pituitary of castrated male rats. Following orchidectomy, serum testosterone concentrations comparable to those observed in intact male rats were maintained by a subcutaneous Silastic implant containing crystalline testosterone or dihydrotestosterone. Within 24 hours after intravenous injection of the antiserum into implant-bearing animals, serum LH levels had increased to levels normally seen after removal of the testes. The lack of unbound circulating testosterone in antiserum-injected females is indicated by the ability of a 50-μl sample of pooled serum from such animals (collected at 1800 of proestrus; approximately 21 hours after intravenous injection of the antiserum) to bind 83 percent of the tritiated testosterone in a 2-ml solution containing approximately 10 pg of testosterone (incubation at 20°C for 2 hours). Under comparable conditions, serum from saline-injected females bound only 8 percent. While these characterizations indicate that the antiserum effectively neutralized circulating testosterone, they do not define the steroid required for optimal FSH secretion, nor do they distinguish between testosterone and dihydrotestosterone as the probable active agent.
6. LH concentrations were determined by the method of G. D. Niswender, A. R. Midgley, Jr., S. E. Monroe, and L. E. Reichert, Jr. [*Proc. Soc. Exp. Biol. Med.* **128**, 807 (1968)]. The antiserum was supplied by Dr. Niswender while the ovine LH for iodination was supplied by Dr. Reichert. Rat FSH was quantitated

with the materials and instructions supplied with the NIAMD assay kit distributed by NIH. Serum testosterone concentrations were determined by the unpublished method of R. L. Goodman, J. Hotchkiss, F. J. Karsch, and E. Knobil, with an antiserum supplied by Dr. I. H. Thorneycroft.
7. R. H. Naqvi and D. C. Johnson, *J. Endocrinol.* **45**, 29 (1969).
8. V. L. Gay, *Physiologist* **16**, 318 (1973); R. E. Flavo, A. Buhl, B. Cook, Program of the 55th Annual Meeting of the Endocrine Society, A-110 (1973).
9. N. D. Diebel, M. Yamamoto, E. M. Bogdanove, *Endocrinology* **92**, 1065 (1973).
10. In five cyclic female rats, removal of the pituitary gland at approximately 0200 of estrus, followed by collection of sequential blood samples (heart puncture at 3 and 45 minutes, and decapitation at 90 minutes after injection) indicated an average "half-life" of 96 minutes for serum FSH at that stage of the cycle. The half-life of serum FSH as determined by similar methods on the afternoon of proestrus has been estimated at 113 minutes [see (*1*)].
11. L. Debeljuk, A. Arimura, A. V. Schally, *Endocrinology* **90**, 1578 (1972).
12. This research was supported by a grant from the Population Council and by PHS research grant HD06664.
* Career development awardee of the National Institute of Child Health and Human Development.

23 November 1973

Electron Diffraction of Wet Biological Membranes

Abstract. *Electron diffraction patterns were obtained for the first time from single wet phospholipid bilayers and from wet human erythrocyte membranes by using a temperature-controlled electron microscope hydration stage. Selective area diffraction showed the existence of semicrystalline domains. A structural transition was observed at the transition temperature of the wet dipalmitoyl lecithin bilayer.*

Electron diffraction has not been as popular as x-ray diffraction in the study of the structure of biological membranes. The main problem is that the membrane specimen to be studied by electron diffraction has to be dried. This problem has been overcome by the development of hydration stages for electron microscopes (*1*). A membrane specimen can now be studied in an electron microscope under more physiological conditions by both imaging and diffraction. Artifacts introduced during drying, fixation, and staining are thus eliminated, and it is only necessary to limit radiation damage from the electron beam by use of sensitive emulsions (*2*).

The advantages of the electron diffraction technique over other diffraction methods are the following: (i) Specimens of only one or several membrane thicknesses (25 to several hundred angstroms) can be examined because of the strong interaction between the specimen and the incident electron beam. (ii) The data collection time is very short (of the order of seconds). (iii) Very small areas can be observed by use of the selective area diffraction technique. The signal-to-noise ratio can also be improved by this method. (iv) A very small amount of specimen is required and impurities can be screened. (v) The shape and morphology of the specimen may be observed by imaging.

We have used the electron diffraction technique to study both phospholipid bilayers and human erythrocyte membranes. Dipalmitoylphophatidylcholine was spread on a Langmuir trough at a surface pressure of 40 dyne/cm, and a bilayer film was formed over micromesh squares by slowly dipping 1000-mesh grids through the surface. The grids were immediately inserted into the hydration stage in the electron microscope. The entire operation was performed under a water-saturated atmosphere. Human erythrocyte ghosts were prepared by a modified Dodge method (*3*). The sample was placed on a grid coated with carbon and Formvar. Excess solution on the grids was removed in a water-saturated atmosphere before they were inserted into the hydration stage.

A hydration stage has been constructed for a Siemens Elmiskop IA electron microscope. The stage is so designed that the temperature of the specimen can be controlled in the range −10° to 50°C under 100 percent humidity. The degree of hydration of the specimen can also be varied if an external water reservoir is used. The structure and function of this stage are described elsewhere (*4, 5*).

To minimize the radiation damage to the specimen, the electron beam dose was kept below 10^{-5} coulomb/cm^2 (measured by a Faraday cage) for each diffraction pattern recording. This was achieved by using a 10-μm second-condenser aperture, and by recording the patterns with Kodak No-Screen x-ray films, which were 17 times more sensitive than electron image plates (*2*). The patterns deteriorated if the radiation dose was increased 50-fold. All patterns were taken at 100 kv, the specimen being under saturated water vapor pressure for the corresponding temperatures.

Typical diffraction patterns of a single wet unsupported dipalmitoylphosphatidylcholine bilayer taken below the transition temperature are shown in Fig. 1, A and B. A streaked hexagonal pattern (Fig. 1A), or in some cases several similar patterns misoriented from one another (Fig. 1B), were usually seen when the observed area was about 5 μm in diameter. The streaking does not diminish if the observed area is further reduced. When the observed area is widened the pattern becomes a typical continuous ring powder pattern, although the ring always remains sharp. Outer rings of $1/3^{1/2}$ and $1/2$ times the spacing of the inner pattern can also be seen. These spacings are characteristics of the α-phase of lipid hydrocarbon chain packing (*6*). The streaking suggests imperfections in the hexagonal packing.

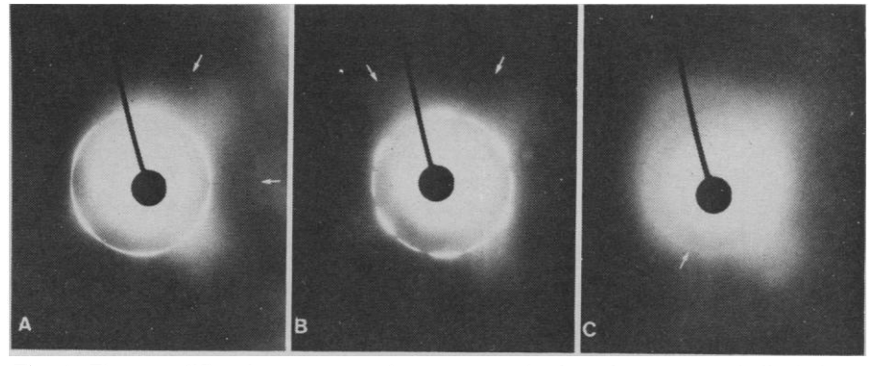

Fig. 1. Electron diffraction patterns of a wet, unstained, and unsupported dipalmitoyl lecithin bilayer. The observation area is 5 μm in diameter. (A) Pattern taken at 30°C showing a streaked hexagonal pattern at spacings of 4.25 and 2.45 Å (arrows). (B) Pattern taken at 39°C showing two misoriented hexagonal lattices. Both show spacings of 4.30 and 2.48 Å (arrows). (C) Pattern taken at 44.0°C showing a diffused ring (arrow) at 44 Å.