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13. Fluosol-DA 20 percent (Green Cross Corporation) was obtained from Alpha Therapeutics Corporation. The emulsion consists of 20 percent (weight to volume) perfluorochemicals—that is, seven parts of perfluoro-Decalin and three parts of perfluorotripropylamine, Pluronic F-68 (2.7 percent, weight to volume), and yolk phospholipids (0.4 percent) as the emulsifier and glycerol (0.8 percent) as a cryoprotecting agent. Krebs-Ringer bicarbonate solution and hydroxyethyl starch (3.0 percent, weight to volume) were added to give the preparation physiological osmolarity and oncotic pressure. The combined surface area of the emulsion particles available for oxygen diffusion is 1.82×10^8 cm^2/liter (about 100 times the surface area of whole blood). The half-life of Fluosol-DA in vivo is about 12 hours.
14. Data on the delay of tumor growth were analyzed with a Basic program for the Apple II+ microcomputer. The program derives the best fit curve for each set of data, then calculates the median, mean, and standard error of the mean for individual tumor volumes and the day on which each tumor reached 500 mm^3. Statistical comparisons were carried out with the Dunn multiple comparisons test after a very significant effect was found by analysis of variance.
15. For the tumor excision assay, all reagents were sterilized with 0.22-μm Millipore membranes and were added aseptically to the tumors. Mice were killed and soaked in 70 percent ethanol. Their leg tumors were excised under sterile conditions in a laminar flow hood and were minced to a fine brei with small curved scissors. Four tumors were pooled to make each treatment group. Approximately 1 g of tumor brei was used to make each single-cell suspension. Each sample was suspended in 20 ml of Dulbecco's phosphate-buffered saline (Gibco) containing deoxyribonuclease (93 μg/ml; Sigma) and trypsin (1.85 mg/ml; Gibco) in a 50-ml plastic centrifuge tube. The samples were incubated for 10 minutes at 37°C, after which the liquid was gently decanted and discarded. Tumor homogenates were resuspended in the enzyme-containing phosphate-buffered saline, mixed (Vortex mixer), incubated and rocked for 10 minutes, and mixed again. The deoxyribonuclease concentration was then increased to 2.5 mg/ml in each tube. After being thoroughly mixed, each sample was filtered through a 135-μm stainless steel mesh in a Nuclepore Swin-Lok holder into a 50-ml plastic centrifuge tube. The samples were centrifuged (E-C model PR-5) at 1000 rev/min and 4°C for 10 minutes, at which time the supernatant was decanted and the pellet resuspended in Eagle's MEM containing 10 percent fetal bovine serum and antibiotics (Gibco). The samples were centrifuged again, the supernatants decanted, and the pellets resuspended in Eagle's MEM containing 10 percent fetal bovine serum and antibiotics. These single-cell suspensions were counted and plated for the colony-forming assay.
16. The influence of oxygen on radiosensitivity appears to be largely quantitative rather than qualitative, since it affects mainly the dose required to produce a given end point. When the effect of oxygen is strictly quantitative, the degree of its influence can be specified by a constant DMF. Thus, dose-effect curves at different oxygen concentrations are related by a constant multiplier, and their shape is independent of local oxygen concentration. We assumed zero growth delay with 0 rad and calculated the factor that related the control dose to the same growth delay achieved with 1000 rads, Fluosol-DA, and 95 percent oxygen.
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Partial Characterization of 21.5K Myelin Basic Protein from Sheep Brain

Abstract. *The 21,500 molecular weight (21.5K) variant of myelin basic protein (MBP) was isolated from sheep brain and partially characterized. Digestion with cyanogen bromide and trypsin yielded peptides which showed that approximately 30 additional amino acids were inserted at the equivalent of the amino acid at position 57 in the bovine 18.5K MBP sequence. An unusually hydrophobic peptide Pro, Val, Leu, Trp, Lys was present in this region. Ornithine was present in hydrolyzates of 21.5K MBP, but it was not detected in any of the peptides.*

In 1977, Barbarese *et al.* (*1*) showed that mouse brain contained four myelin basic proteins (MBP) with apparent molecular weights of 21.5K (21,500), 18.5K, 17K, and 14K. Subsequently, these variants were found in myelin from other species (*2*). Originally it was thought that 21.5K and 17K MBP's were precursors of 18.5K and 14K MBP's, respectively (*1*). However, studies on the synthesis of the MBP's indicated that they were more likely to be the products of separate messenger RNA's (*3, 4*).

Sheep brain was chosen for the isolation of 21.5K MBP for our study. An established method (*5*) for the preparation of MBP was followed, except that Sephadex G-75 (superfine) with 0.2*M* KCl-HCl buffer, *p*H 2.0, was used. Separation was monitored by gel electrophoresis and by an immunoblotting technique (*6*). A monoclonal antibody (*6, 7*) that reacts with the amino acid sequence Ala-Ser-Asp-Tyr-Lys-Ser in MBP was used to locate 18.5K and 21.5K sheep MBP in the fractions. From repeated chromatography on Sephadex G-75 (superfine), approximately 18 mg of 21.5K MBP was isolated from 200 g of brain.

Duplicate samples of 21.5K MBP were hydrolyzed and analyzed (Table 1). Surprisingly, ornithine was found in all hydrolyzates of 21.5K MBP but not in any of the peptides (*8*) nor in hydrolyzates of sheep 18.5K MBP. The association of ornithine with 21.5K MBP requires further study.

As 21.5K MBP contained three methionine residues, digestion with cyanogen bromide (CNBr) was used to ascertain their positions. The digest was fractionated by gel filtration (*9*). Electrophoresis of the fractions and transfer of the peptides to nitrocellulose revealed three peptides (as seen by staining with amido black) with apparent molecular weights of approximately 14K, 6K, and 2K; these values were determined from a comparison with a CNBr digest of myoglobin. Only the 14K peptide reacted

with the monoclonal antibody, a property indicating that it contained the amino acid sequence located between residues 130 and 135 in bovine MBP. The CNBr peptides were hydrolyzed, and their amino acid compositions are given in Table 1. Amino acid analysis of the 14K peptide indicated a molecular weight closer to 13K.

Tryptic digestion and preparative peptide mapping (*5*) of the 2K CNBr peptide gave peptides (Fig. 1) identical to those reported to be present in the first 19 residues of bovine 18.5K MBP (*10*).

In that the 6K CNBr peptide gave definitive evidence for the position of most of the nonhomologous peptides found in 21.5K MBP, the positions of these are shown in Fig. 2. Peptides CNBr 6–1 to 6–7 were homologous with those in bovine 18.5K MBP (*10*). No evidence was obtained for Arg-[52] which is present in bovine MBP. The overlap of the 2K CNBr and 6K CNBr peptides was given by a tryptic peptide, 21.5-4, from 21.5K MBP (Fig. 1).

Peptides CNBr 6–8a, 6–8b, and 6–8c were not found in tryptic digests of 18.5K sheep MBP. On peptide maps, 6–8c migrated to a position closely similar to the tryptophan peptide observed by Barbarese *et al.* (*1*) in digests of mouse 21.5K MBP and mouse 17K MBP; it was unusual in being so hydrophobic, with the composition Pro, Leu, Val, Trp, Lys. In that homoserine was detected in the hydrolyzate of the 6K CNBr peptide and not in any of the peptides isolated and, in addition, because a glutamic acid residue was unaccounted for in the peptides, it is likely that the methionine occurs in the sequence Glx-Met. A methionine residue was found in tryptic peptide 21.5–14. This peptide was neutral and was located just above peptide CNBr (6–5) on a peptide map of 21.5K MBP.

In a tryptic digest of CNBr 13K, the balance of peptide 21.5–14 has not yet been located; however, from the amino acid composition of CNBr 13K, its presence was indicated. Peptide 13–1b had a composition and electrophoretic mobility that would indicate a homology with

Table 1. Amino acid composition of sheep 21.5K MBP and peptides.

	21.5K MBP*		CNBr peptides†			Tryptic peptides‡ not homologous with those in 18.5K bovine MBP				
	Mole percent	Residues	2K	6K	13K	21.5–14	13–1b	6–8a	6–8b	6–8c
Asp	7.7	15		4.1	8.5	1.9	1.1			
Thr	3.9	8	1.0	1.2	5.1					
Ser	9.4	19	3.7	5.1	12.3	1.2		0.8	1.0	
Glu	8.5	17	2.0	2.0	10.0	1.2	1.2	0.9		
Pro	7.0	14	1.2	5.0	9.1				1.7	1.0
Gly	12.5	25		8.5	20.3	1.3	1.3	1.2	1.1	
Ala	8.0	16	3.7	2.9	8.3	1.2	1.8		0.9	
Val	4.7	9		1.1	2.1	1.0				1.0
Met	1.3	3	0.5§	0.5§	0.6§	0.5				
Ile	1.7	3		1.0	1.9					
Leu	7.3	14	1.0	4.7	7.0	1.0			1.0	1.2
Tyr	2.4	5	1.0		4.1		0.8			
Phe	4.2	8		2.8	5.5	1.0				
His	4.8	10		3.0	6.3		1.6		0.7	
Lys	7.6	15	2.3	3.6	8.8	1.0		1.0		1.0
Arg	7.9	16	2.3	5.6	10.3		1.0		1.0	
Trp‖	1.1	2		1	1					1

*The composition of MBP was determined from average and extrapolated values obtained from duplicate hydrolysis for 24, 48, 96, and 120 hours, with 6*M* HCl at 105°C. Ornithine was present in a molar ratio of 1:1. †Duplicate analyses of 24-hour hydrolyzate; the results represent residues per mole of Leu for 2K, per mole of Ile for 6K, and per 7 mole of Leu for 13K. ‡Twenty-four-hour hydrolyzates of peptides obtained from tryptic digests; residues per mole of Lys or Arg. §From homoserine. ‖From presence of peptides staining with Ehrlich reagent.

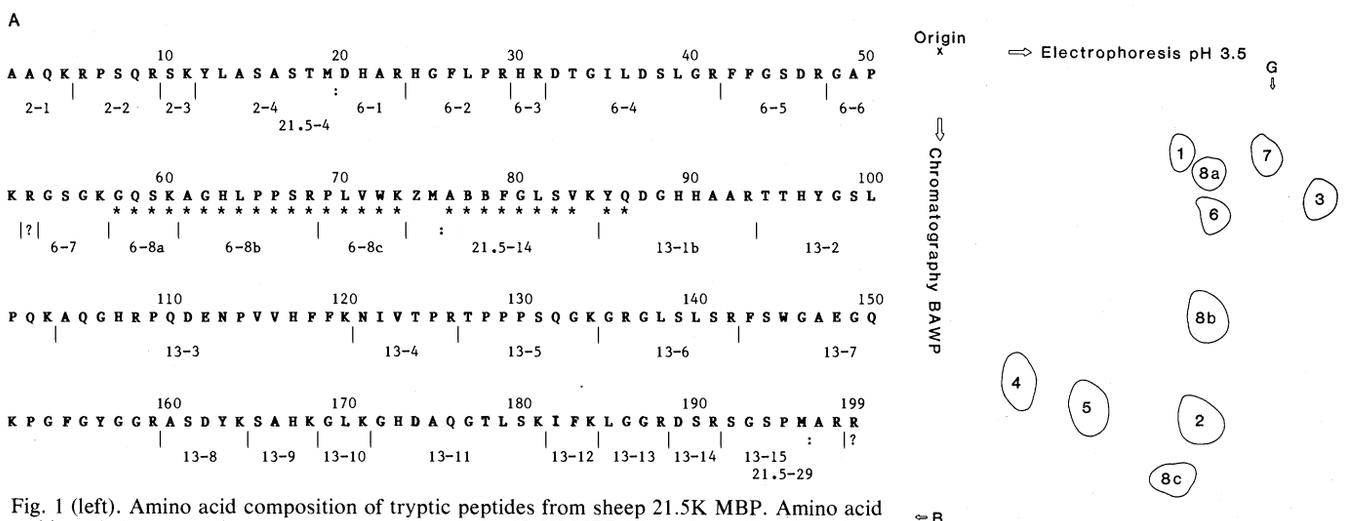

Fig. 1 (left). Amino acid composition of tryptic peptides from sheep 21.5K MBP. Amino acid residues that were not homologous to those in bovine 18.5K MBP (*9*) are from 57 to 86. Where the sequence of amino acids cannot be inferred is indicated by *. Peptides were isolated from 2K CNBr (2-), 6K CNBr (6-), and 13K CNBr (13-) fragments of 21.5K MBP, and also from 21.5K MBP. Only the overlap peptides from 21.5K MBP (21.5-) are shown. The yields of the peptides were similar except for 13–6, which was lower because of microheterogeneity due to methylation of Arg[136]. The ratio of amino acids in the hydrolyzates was close to 1.0 except where two hydrophobic amino acids were expected to be adjacent in the peptides (for example, in 13–4). No evidence was obtained for the presence of Arg[52] and the COOH-terminal Arg which are present in bovine MBP. Sites of digestion with CNBr are shown by : and trypsin by |. Single letter abbreviations used to indicate the amino acid residues are as follows: A, alanine; R, arginine; N, asparagine; D, aspartic acid; B, aspartic acid or asparagine; C, cysteine; E, glutamic acid; Z, glutamic acid or glutamine; Q, glutamine; G, glycine; H, histidine; I, isoleucine; L, leucine; K, lysine; M, methionine; F, phenylalanine; P, proline; S, serine; T, threonine; W, tryptophan; Y, tyrosine; and V, valine. Fig. 2 (right). Peptide map of tryptic digest of 6K CNBr peptide from 21.5K MBP. The solvent system consisted of butanol, pyridine acetic acid, and water (*BAWP*) (*5*); *G*, methyl green marker; *B*, xylene cyanol marker. Peptides were located with fluorescamine, eluted, and analyzed (*5*).

peptide T11 in bovine MBP (*10*) with the addition of Tyr and Gln. It was located just to the left of peptides CNBr 6–8a and 6–6 (Fig. 2). The remaining tryptic peptides in the CNBr 13K peptide were apparently homologous with those in bovine MBP; however, peptides 13–5 and 13–11 were not completely separated.

Therefore, our data show that 21.5K MBP differs from 18.5K MBP by the addition of approximately 30 amino acid residues at the equivalent of residue 57 in bovine 18.5K MBP. As the peptides were hydrolyzed for only 24 hours and as prolonged hydrolysis of 21.5K MBP indicated additional Val residues, it is possible that there could be Val-Val sequences in peptides 6–8c and 21.5–14 because such sequences are resistant to hydrolysis (*5*).

It is likely that the difference in structure between 17K MBP and 14K MBP is due to a similar insert of approximately 30 amino acids as peptide maps of 17 and 21.5K MBP (*1*) showed peptides clearly similar in location to peptides 21.5–14, 6–8a, 6–8b, and 6–8c. Moreover, results on the incorporation of ^{35}S-labeled methionine into mouse 21.5K and 17K MBP's indicated that an additional methionine was present in these proteins (*4*).

Originally, Barbarese *et al.* (*1*) proposed that the additional 30 amino acids were attached at the NH_2-terminal end of 21.5K MBP and that these were removed during metabolic processing to produce 18.5K MBP. The evidence presented for the position of the insert supports the conclusion, from studies on the synthesis of MBP (*3*), that 21.5K MBP cannot be a metabolic precursor of 18.5K MBP.

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8. Analyses were performed on two different Dionex amino acid analyzers and the peak in the hydrolyzate was compared with authentic ornithine. Approximately 1 mole of ornithine was found per mole of 21.5K MBP. No ornithine was found in hydrolyzates of any of the peptides. Free ornithine was released from 21.5K MBP by digestion with trypsin, clostripain, pepsin, and *Staphylococcus aureus* V8 protease. Ornithine has been reported in hydrolyzates of two other proteins [K. Sletten, I. Aakesson, J. O. Alvsakar, *Nature (London) New Biol.* **231**, 118 (1971); A. K. Allen and A. Neuberger, *Biochem. J.* **135**, 307 (1973)].
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Estradiol Is Concentrated in Tyrosine Hydroxylase–Containing Neurons of the Hypothalamus

Abstract. *Localization of [^{3}H]estradiol in tyrosine hydroxylase–containing neurons of rat brain was shown by a combined technique of autoradiography and immunohistochemistry. [^{3}H]Estradiol was concentrated in the nuclei of tyrosine hydroxylase–containing neurons in the nucleus arcuatus, nucleus periventricularis hypothalami, and the zona incerta. These results suggest that estradiol acts directly on dopamine-producing neurons of the tuberoinfundibular system and incertohypothalamic system.*

Tuberoinfundibular dopamine-producing neurons are involved in the regulation of prolactin secretion and gonadotropin secretion (*1*). Dopamine has an inhibitory effect on prolactin release (*2*), but its effect on gonadotropin secretion is not clear (*3*). The association of dopamine terminals with luteinizing hormone–releasing hormone (LHRH) terminals in the palisade zone of the median eminence suggests an interaction between dopamine and LHRH (*4*). The activity of the dopamine neurons changes during the estrous cycle of the rat, being highest during diestrus and lowest during proestrus (*5*). Prolactin stimulates dopamine turnover in the median eminence (*6*). Autoradiographic techniques have revealed that estradiol-concentrating neurons are localized in arcuate and periventricular nuclei of the hypothalamus (*7*), where dopamine neurons and terminals exist (*8*). Applying a combined technique of autoradiography and immunohistochemistry (*9*), Sar and Stumpf showed that [^{3}H]estradiol is concentrated by the central noradrenergic neurons in the lower brainstem of the rat (*10*). I used the same technique to locate both [^{3}H]estradiol and antibodies to the enzyme tyrosine hydroxylase in the same brain section. The results indicate that dopamine neurons of the tuberoinfundibular system as well as of the incertohypothalamic system are target cells for estrogen.

Seven adult female Holtzman Sprague-Dawley rats were ovariectomized and 48 hours later were given intravenous injection of 17β-[2,4,6,7-^{3}H]estradiol (specific activity 105 Ci/mmole) at 0.5 μg per 100 g of body weight. Fifteen minutes before the administration of [^{3}H]estradiol, two of the ovariectomized rats each received unlabeled 17β-estradiol at 100 times the amount of the labeled substance. Rats were decapitated 1 hour after injection of [^{3}H]estradiol. The forebrain and midbrain were dissected, placed on a tissue holder, and frozen in liquefied propane (−180°C). Serial frozen sections (4 μm) were thaw-mounted on slides coated with photographic emulsion (Kodak NTB3) and stored at −15°C for autoradiographic exposure (*11*). After photographic exposure, the slides were fixed in 4 percent paraformaldehyde solution for 30 seconds at 4°C; rinsed briefly with phosphate-buffered saline (PBS), *p*H 7.5; and developed (Kodak D-19 developer, fixation with Kodak fixer). After being rinsed with PBS, the autoradiographic slides were processed for immunoperoxidase stain-