al., *Virology* **113**, 465 (1981); D. Steffen and R. A. Weinberg, *Cell* **15**, 1003 (1978); D. Steffen *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4554 (1979); N. A. Jenkins *et al.*, *J. Virol.* **43**, 26 (1982).
6. I. M. Verma, personal communication.
7. M. P. Goldfarb, K. Shimizu, M. Perucho, M. Wigler, *Nature (London)* **296**, 404 (1982); K. Shimizu, M. Goldfarb, M. Perucho, M. Wigler, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 383 (1983).
8. D. L. Rimm, D. Horness, J. Kucera, F. R. Blattner, *Gene* **12**, 301 (1980); B. G. Williams and F. R. Blattner, *J. Virol.* **29**, 555 (1979).
9. L. I. Lobel and S. P. Goff, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4149 (1984).
10. B. Seed and T. Maniatis, personal communication [noted in *Molecular Cloning*, T. Maniatis, E. F. Fritsch, J. Sambrook, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1980)].
11. B. Levinson, G. Khoury, G. Vande Woude, P. Gruss, *Nature (London)* **295**, 568 (1982).
12. J. H. McCutchan and J. S. Pagano, *J. Natl. Cancer Inst.* **41**, 351 (1968).
13. P. Schwartzberg, J. Colicelli, S. P. Goff, *J. Virol.* **46**, 538 (1983); P. Schwartzberg, J. Colicelli, M. L. Gordon, S. P. Goff, *ibid.* **49**, 918 (1984); P. Schwartzberg, J. Colicelli, S. P. Goff, *Cell* **37**, 1043 (1984).
14. S. P. Goff, P. Traktman, D. Baltimore, *J. Virol.* **38**, 239 (1981).
15. B. Hirt, *J. Mol. Biol.* **26**, 365 (1967).
16. E. M. Southern, *ibid.* **98**, 503 (1975).
17. E. G. Bernstine *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3899 (1973).
18. C. L. Stewart *et al.*, *ibid.* **79**, 4098 (1982).
19. W. King and M. C. Nguyen-Huu, unpublished data.
20. P. S. Sarma *et al.*, *Virology* **33**, 180 (1967).
21. J. H. Miller, in *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1972).
22. W. King, M. D. Patel, L. I. Lobel, S. P. Goff, C. C. Nguyen-Huu, *Science*, in press.
23. C. Van Beveren, E. Rands, S. K. Chattopadhyay, D. R. Lowy, I. M. Verma, *J. Virol.* **41**, 542 (1982); J. G. Sutcliffe *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3302 (1980); C. Van Beveren *et al.*, *ibid.*, p. 3307.
24. We thank J. Van Oostrum for gifts of DNA ligase. Supported by PHS grant 2 P30 CA 23767 awarded by the NCI to S.P.G. and R01 CA 37176 to C.N.-H. Additional support was provided to S.P.G. by the Hirschl Trust and by the Searle Foundation and to M.C.N.-H. by the National Cancer Cytology Center.

22 October 1984; accepted 9 January 1985

Silver-Intensified Gold and Peroxidase as Dual Ultrastructural Immunolabels for Pre- and Postsynaptic Neurotransmitters

Abstract. *An ultrastructural immunostaining method that uses silver-intensified gold was combined with another procedure that uses biotin peroxidase conjugates to allow simultaneous identification of two neurotransmitter-related antigens in the central nervous system. Tyrosine hydroxylase–immunoreactive neurons labeled with silver-intensified gold could be differentiated at both light and electron microscopic levels from glutamate decarboxylase–immunoreactive neurons labeled with peroxidase. Cross reactivity of the second group of immunoreagents with the first group was reduced by the heavy metal silver shell formed around the colloidal gold immunoglobulin complex. With this dual pre-embedding method, peroxidase-stained axons containing the inhibitory neurotransmitter γ-aminobutyric acid were found to synapse directly on silver-stained dopamine neurons in the rat dorsomedial hypothalamus. This approach can be used in combination with a post-embedding immunocytochemical colloidal gold procedure, allowing ultrastructural identification of three neurotransmitter-related antigens in the same tissue section.*

The primary function of neurons may be considered to be one of intercellular electrochemical communication and information processing, generally occurring through the release of one (or more) of 30 to 50 different neuroactive substances, loosely termed neurotransmitters. The electrical response of a postsynaptic cell is determined both by its own receptors and by the type of neurotransmitter released from the presynaptic cell. The complexity of connections in the central nervous system is bewildering, particularly in nonlaminated areas like the hypothalamus, where obvious morphological clues to cell type may be absent. Studies based on immunocytochemical identification of a single primary antiserum against a neurotransmitter-related antigen have proved useful in delineating previously unrecognized neuronal systems. The possibility of identifying and differentially labeling both the presynaptic and postsynaptic elements in the same histological section would allow a more specific characterization of both the biochemistry and the physical circuitry in any area of the brain. Such an analysis of neuronal relations must be based on ultrastructural confirmation of synaptic interaction.

Three objectives may be considered in dual ultrastructural identification of two antigens. (i) The final markers must be readily seen and discriminated from each other with both light and electron microscopy. (ii) Antibody cross-reaction between the series of reagents used to identify two different primary antiserums must be minimized. (iii) Both methods should be sensitive enough to detect low antigen concentrations. Pre-embedding immunostaining with peroxidase followed by post-embedding staining with colloidal gold has been successfully used for dual ultrastructural immunocytochemistry with combinations of cytoplasmic antigens, such as tyrosine hydroxylase (TH), the synthesizing enzyme for catecholamine neurotransmitters, or glutamate decarboxylase (GAD), the synthesizing enzyme for the inhibitory transmitter γ-aminobutyric acid (GABA), together with vesicular antigens such as neurophysin (*1–3*); as this pre-embedding/post-embedding method may work poorly with two cytoplasmic antigens, a different approach was needed for dual identification of GAD and TH.

Although gold particles can be used without intensification for immunocytochemical staining for light microscopy (*4*), significant membrane damage must occur to achieve penetration of gold particles large enough to see (20 to 40 nm); even then the resultant pinkish immunostaining can be faint. One purpose of this study was to find a simple intensification procedure compatible with reasonable ultrastructural preservation. Ideally, the technique should work with the smallest gold particles available, thus reducing the severity of membrane damage necessary for tissue penetration. Finally, the procedure should be compatible with, yet differentiable from, other ultrastructural immunostaining markers, particularly horseradish peroxidase, to allow for identification of two antigens on the same thin electron microspic section. Various methods of intensification of many different metals including silver, cobalt, osmium, and gold have been described (*5, 6*).

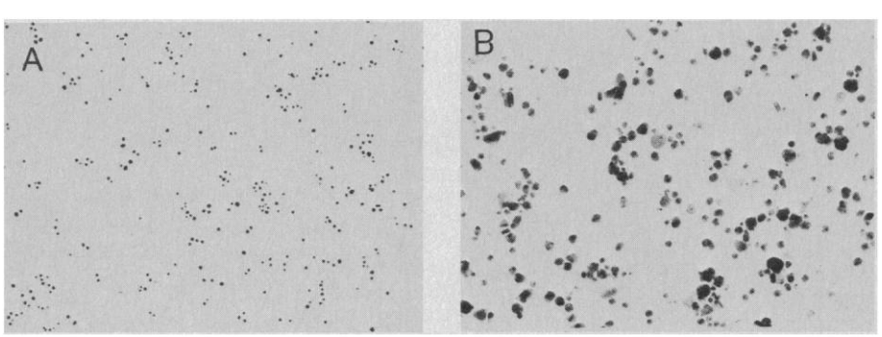
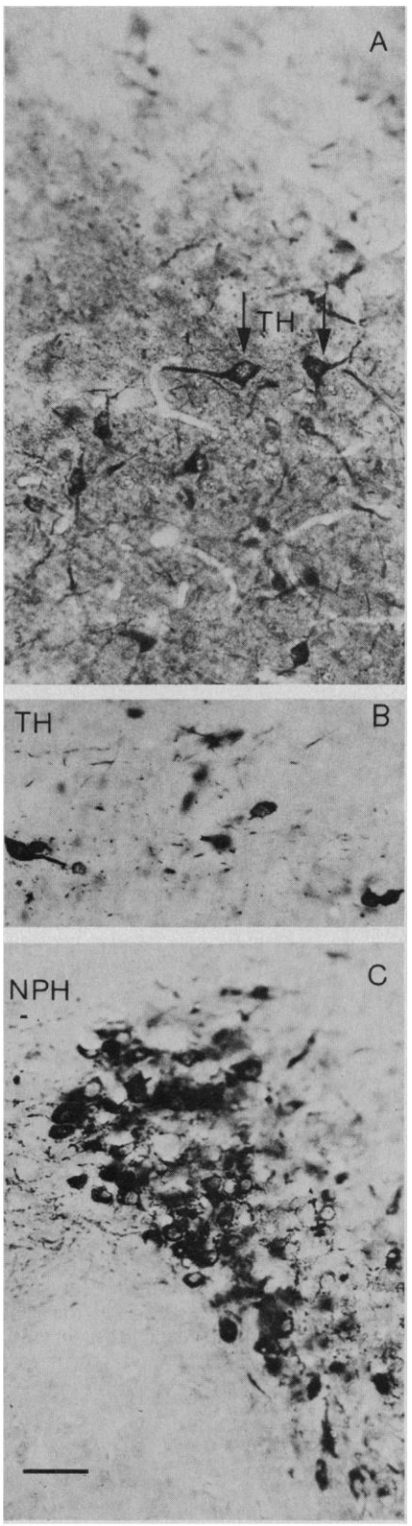


Fig. 1. Effect of intensification on colloidal gold size. (A) Colloidal gold particles (5 nm). (B) Gold (5 nm) intensified for 20 minutes on a Formvar-coated slot grid. Intensification is influenced by the intensity of ambient light, reagent concentration, agitation, and incubation time in the silver solution.

A number of permutations of different sized colloidal gold markers adsorbed to a number of different protein carriers including protein A, immunoglobulin G (IgG), and biotin and intensified with different chemical treatments were tried. One method, based on classical methods of intensification of heavy metals (*5*), worked well (Fig. 1): After being fixed in 4 percent paraformaldehyde and 0.05 percent glutaraldehyde, hypothalamuses were cut in 30- to 50-μm sections. An extensively characterized rabbit antiserum against TH (*7*) was used at a dilution of 1:1000 overnight. After repeated washing, sections were immersed in tris-buffered saline (*p*H 8.2) with goat antiserum to rabbit IgG adsorbed to 5-nm gold particles. Preparation and adsorption of colloidal gold to proteins has been described (*1, 4, 8*). After 2 hours of incubation, sections were washed and intensified in a silver solution (*5, 9*). Initially, the colloidal gold, and later the newly precipitated silver, catalyzed the reduction of silver nitrate to metallic silver by hydroquinone. Between 15 minutes and 2 hours later sections were washed and placed in 10 percent normal rabbit serum to block vacant antigen binding sites of the goat antiserum to rabbit IgG. Sections were incubated overnight in sheep antiserum to GAD (*10, 11*), followed by biotinylated rabbit antiserum to sheep IgG, and then in an avidin biotinylated peroxidase complex (Vector) (*12*). After reaction with diaminobenzidine and hydrogen peroxide, sections were osmicated and embedded in Epon.

Although nonintensified sections stained with 5-nm colloidal gold show no apparent cellular staining, silver-intensified gold particles were easily found with light microscopy. In addition to cells in the dorsomedial hypothalamus (Fig. 2A), TH-immunoreactive neurons labeled with silver-intensified gold (SIG) (*13*) were seen in the hypothalamic arcuate (Fig. 2B) and paraventricular nuclei, periventricular area, preoptic area, posterior hypothalamus, and in the substantia nigra and ventral tegmental area. Depending on the degree of intensification, labeled cells had a mottled gray to dense black appearance. The background remained clear. Cell bodies, axons, and proximal dendrites could be found stained with the SIG method. With electron microscopy the SIG label appeared as somewhat circular patches throughout the cytoplasm of labeled neuron profiles (Fig. 3). The original size of the 5-nm colloidal gold particles was increased greatly by the silver intensification (Fig. 1).

As an additional test for immunostaining specificity and versatility, and to test the method with other antigens, the SIG immunocytochemical method was used with primary antiserum against other putative neurotransmitters and related substances including somatostatin, neurophysin (Fig. 2C), prolactin, and glutamate decarboxylase; in all hypothalamic regions examined, control immunostaining with peroxidase gave similar results to the SIG method. The basic SIG method can be used with both pre-embedding and post-embedding immunocytochemistry, and with colloidal gold adsorbed to immunoglobulins, biotin or avidin, and protein A.



GAD-immunoreactive boutons and axons labeled with peroxidase were found throughout the hypothalamus (*11, 14*). With light microscopy, GAD-immunoreactive boutons stained a diffuse brown could be seen in contact with TH-immunoreactive cells with a particulate gray or black appearance. After osmication, the brown HRP reaction product darkened; depending on the degree of silver intensification of the gold, diffuse peroxidase label and particulate silver label could still be distinguished. At the ultrastructural level GAD-immunoreactive boutons were found in synaptic contact with SIG-labeled TH-immunoreactive cell bodies and dendrites in the dorsomedial hypothalamus (Fig. 3). GAD-immunoreactive axons also synapsed on many unlabeled somata and dendrites of unidentified neurotransmitter content. Synaptic contacts were symmetrical; presynaptic peroxidase-labeled axons contained small clear vesicles, with an occasional larger dense-core vesicle. Peroxidase reaction product was found diffusely throughout the cytoplasm of the presynaptic GABA axon. Hypothalamic dopaminergic neurons may participate in a wide variety of functions, including direct or indirect regulation of pituitary hormones. The finding of GABA-ergic terminals in synaptic contact with dopaminergic neurons suggests that the inhibitory neurotransmitter GABA may reduce electrical activity and the release of dopamine from postsynaptic cells, thereby exerting the opposite effect of dopamine itself [for a review, see (*7*)]. The functional implications of direct GABA inhibition of dopamine-containing neurons in the medial hypothalamus will be discussed (*3*).

Colloidal gold can be used as a pre-embedding ultrastructural marker without intensification (*1*), but even large 10-

Fig. 2. (A) Light photomicrograph of SIG-TH-immunoreactive neurons (arrows) in the dorsomedial hypothalamus from an Epon block. Peroxidase stained GAD-immunoreactive axons cover the field, except at the top of the block where thin sections have already been cut. (B) SIG-stained TH-immunoreactive neurons in the hypothalamic arcuate nucleus. (C) SIG-labeled neurophysin (NPH)-immunoreactive neurons in the paraventricular nucleus show a similar localization and morphology to those cells stained with peroxidase (*19*). Note the absence of staining in the nuclear region of cells. No staining was seen with deletion of the primary antisera. Bar, 50 μm.

or 20-nm dispersed particles are difficult to see, and therefore can escape recognition. With appropriate intensification, single particles can be seen even at the lowest electron microscopic magnification (×1500); unlike unintensified small colloidal gold or ferritin, intensified gold can easily be recognized in Epon sections counterstained with lead citrate and uranyl acetate. SIG-immunostained TH profiles were found deeper in tissue sections than peroxidase-stained GAD profiles; this difference may relate either to better penetration of the 5-nm gold adsorbed IgG molecule compared to peroxidase reagents, or to anatomical differences between GAD and TH immunoreactive neurons. Under the assumption of one IgG per 5-nm gold particle of this size (*15*), the adsorbed IgG gold complex, which would represent the largest complex in the SIG immunostaining procedure, would theoretically be on the order of 10 nm in diameter, smaller than either peroxidase-antiperoxidase, avidin-biotin-peroxidase, or immunoglobulin-ferritin complexes (*16*). The silver intensification procedure forms a sphere of heavy metal around the colloidal gold particle, encompassing not only the colloidal gold particle, but also the primary rabbit TH antibody as well as the goat antiserum to rabbit IgG adsorbed to the gold particle. This heavy metal sphere would prevent further cross-reaction of the enclosed immunoglobulins with the second set of immunoglobulins used to detect the second antigen, GAD. Unadsorbed goat antiserum to rabbit IgG had been removed by discarding the supernatant after repeated centrifugations during preparation of the IgG-gold complex (*1, 4*). The intensification procedure as outlined here also works to intensify other metallic immunocytochemical markers, including colloidal silver.

This particular combination of silver-intensified colloidal gold and peroxidase used as dual ultrastructural markers for pre-embedding immunocytochemical staining works well with two cytoplasmic antigens (*17*). This procedure may also be used to identify antigens concentrated in vesicles (neurophysin, somatostatin, and prolactin) if the vesicle membrane is made permeable with freeze-thawing, sonication, or detergents. Two neurotransmitter antigens can also be identified by a different method with pre-embedding peroxidase (or SIG) staining, followed by post-embedding immunogold staining (*1–3*); as the two double-staining procedures are compatible, immunostaining of three antigens in the same thin section has been possible (Fig. 3D), indicating that the SIG procedure combined with the peroxidase procedure is compatible with antigen retention for further immunocytochemical characterization. Simultaneous identification of even more antigens might be possible with the use of different sizes of gold particles or radioactively labeled immunoglobulins (*18*).

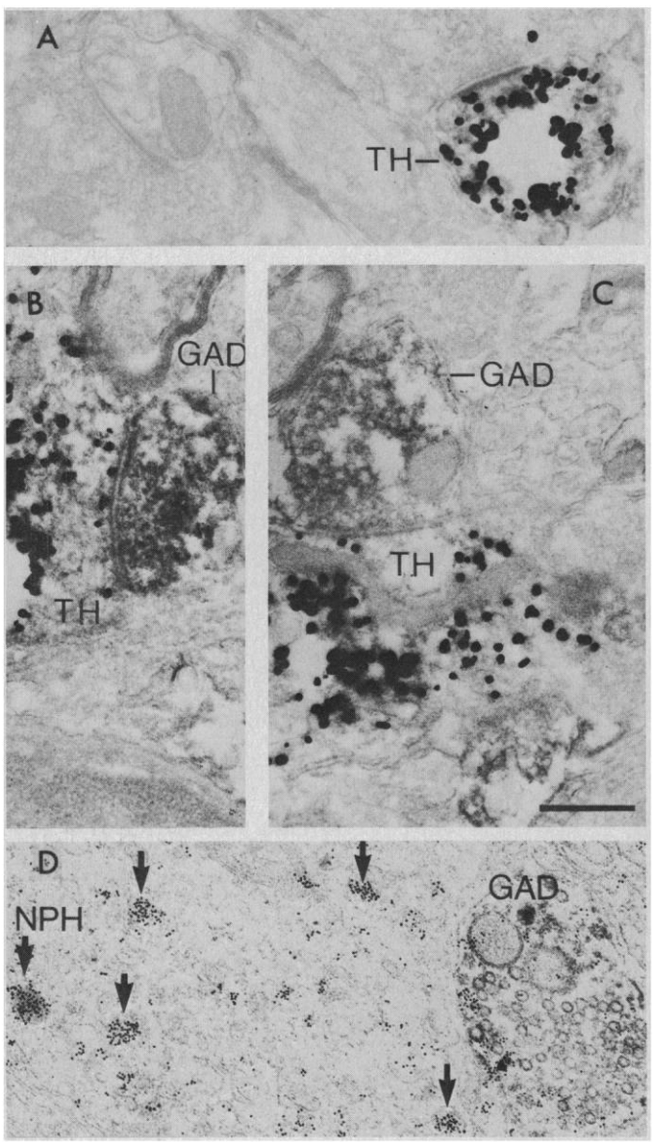


Fig. 3. (A) SIG-labeled TH-immunoreactive dendrite from the dorsomedial hypothalamus, taken from the block shown in Fig. 2A. (B and C) Peroxidase-immunoreactive GAD terminal synapses on a SIG-labeled TH-immunoreactive dendrite. Bar, 0.5 μm. (D) From tissue already double-stained for TH and GAD before being embedded in plastic, post-embedding immunostaining with 10-nm colloidal gold shows the location of a third antigen, neurophysin (NPH), in neurosecretory vesicles (arrows) in the paraventricular nucleus. A GAD peroxidase-immunoreactive bouton is seen in apposition to a proximal dendrite of the paraventricular nucleus containing neurosecretory vesicles stained by the post-embedding procedure (*1, 2*). SIG-stained TH profiles, found in the same section, are not shown.

ANTHONY N. VAN DEN POL
Section of Neurosurgery,
Yale University Medical School,
New Haven, Connecticut 06510

References and Notes

1. A. N. van den Pol, *Q. J. Exp. Physiol.* **69**, 1 (1984).
2. ———, *Soc. Neurosci. Abstr.* **9**, 84 (1983).
3. ———, in preparation.
4. J. DeMey *et al.*, *Cell Biol. Int. Rep.* **5**, 889 (1981).
5. A. A. Pearson and S. L. O'Neill, *Anat. Rec.* **95**, 297 (1946); F. Timm, *Dtsch. Z. Gesamte Gerichtl. Med.* **46**, 706 (1958); G. Danscher, *Histochemistry* **71**, 1 (1981); U. Brunk, A. Brun, G. Skold, *Acta Histochem.* **31**, 345 (1966); F. Haug, *Histochemie* **8**, 355 (1967); N. M. Tyrer and E. M. Bell, *Brain Res.* **73**, 151 (1974).
6. G. Danscher and J. O. R. Norgaard, *J. Histochem. Cytochem.* **31**, 1394 (1983); L. Sternberger *et al.*, *ibid.* **14**, 711 (1966); C. S. Holgate *et al.*, *ibid.* **31**, 938 (1983).
7. A. N. van den Pol, R. Herbst, J. P. Powell, *Neuroscience* **13**, 1117 (1984).
8. M. Horisberger, *Biol. Cell.* **36**, 253 (1979); J. Roth, M. Bendayan, L. Orci, *J. Histochem. Cytochem.* **26**, 1074 (1978).
9. The intensifying solution consisted of 6.5 m*M* silver nitrate and 76 m*M* hydroquinone in an acidic citrate buffer (*p*H 4.0) in the presence of 20 percent gum arabic colloid.
10. W. H. Oertel, D. E. Schmechel, M. L. Tappaz, I. Kopin, *Neuroscience* **6**, 2689 (1981).
11. M. L. Tappaz, W. H. Oertel, M. Wassef, E. Mugnaini, *Prog. Brain Res.* **55**, 77 (1982).
12. S. Hsu, L. Raine, H. Fanger, *J. Histochem. Cytochem.* **29**, 577 (1981).
13. Although TH is present in neurons containing dopamine, norepinephrine, and epinephrine, immunoreactive perikarya and dendrites in the hypothalamus are probably dopaminergic, whereas axons, possibly arising from other neural loci, could contain any of the three neurotransmitters [for a review, see (*7*)].
14. S. R. Vincent, T. Hokfelt, J. Y. Wu, *Neuroendocrinology* **34**, 117 (1982).
15. E. L. Romano, C. Stolinski, N. C. Hugh-Jones, *Immunocytochemistry* **11**, 521 (1974); J. Roth, *Histochem. J.* **14**, 791 (1982); M. Horisberger, *Experientia* **34**, 721 (1978).
16. J. A. Madri and K. W. Barwick, *Lab. Invest.* **48**, 98 (1983); L. Sternberger, *Immunocytochemistry* (Wiley, New York, 1979).
17. A. N. van den Pol, *Soc. Neurosci. Abstr.* **10**, 440 (1984).
18. C. Cuello, J. V. Priestley, C. Milstein, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 665 (1982); J. J.

Geuze, J. W. Slot, P. A. van der Ley, R. C. Sheffer, *J. Cell Biol.* **89**, 653 (1981); J. Roth, *Histochem. J.* **14**, 791 (1982).
19. A. N. van den Pol, *J. Comp. Neurol.* **206**, 317 (1982).
20. I thank W. Oertel, D. Schmechel, I. Kopin, and M. Tappaz for the antiserum to GAD, J. Powell for the antiserum to TH, and K. Szigetti and G. Collins for assistance with the electron microscopy. Support was provided by NIH grants NS 16296 and NS 10174 and by the American Parkinson Disease Association.

18 June 1984; accepted 6 December 1984

Homology of β-Lactoglobulin, Serum Retinol-Binding Protein, and Protein HC

Abstract. *The milk protein β-lactoglobulin has been extensively studied but its function has not been identified. A clue regarding the function of a protein can be obtained by discovering a genetic relationship with a protein of known function through comparisons of amino acid sequence. Such comparisons revealed that β-lactoglobulin is similar to human serum retinol-binding protein and to another human protein of unknown function known as complex-forming glycoprotein heterogeneous in charge (protein HC). β-Lactoglobulins from several species have been found to bind retinol, while the absorption and fluorescence properties reported for the unidentified heterogeneous prosthetic group of protein HC are retinoid-like. The role of serum retinol-binding protein in vitamin A transport in the circulation suggests that the other two homologous proteins may function in the binding and transport of retinoids; β-lactoglobulin may facilitate the absorption of vitamin A from milk and protein HC may mediate the excretion of retinol-derived metabolites.*

β-Lactoglobulin (BLG) is the major globular protein component of bovine milk whey. It exists as an equilibrium mixture of monomer (molecular weight of 18,500) and dimer under physiological conditions (*1*). BLG has only been found in the ruminants (as the dimeric form) and in the pig (as the monomer). Milks from rodents, primates, and lagomorphs are devoid of BLG (*2*). The milk of a member of each of two species of aquatic mammals, the dolphin (*Tursiops truncatus*) and the manatee (*Trichechus manatus*), contains high concentrations (up to 2 percent weight to volume) of monomeric BLG's. A partial sequence has been obtained for one dolphin BLG (Fig. 1). While comparing the sequences of this protein and bovine BLG (BBLG) to those of other proteins, we observed the similarity of both BLG's to the published sequence (*3*) of human serum retinol-binding protein (RBP). The similarity became apparent when residue 1 of the BLG's was aligned with residue 6 of RBP (Fig. 1) and three gaps were placed in each sequence. RBP is also longer and extends six residues beyond the end of the alignment. Dolphin BLG (in regions where the sequence is known) was identical to RBP at six sites that are not among the 33 residues that were identified in BBLG and RBP.

The strength of the case for homology of a pair of proteins can be enhanced by the addition of a third divergent homolog to the alignment; the probability of the similarity between all three proteins being coincidental is very low (*4*). The addition of a protein known as the complex-forming glycoprotein heterogeneous in charge (protein HC) to the alignment (Fig. 1) provided further links between the sequences of RBP and BLG's. Protein HC is an α_1-glycoprotein that was originally isolated from the urine of patients with chronic cadmium poisoning (*5*). It is also found in normal urine, plasma, and cerebrospinal fluid in relatively low amounts but is present in high concentrations in the urine of patients with tubular proteinuria and in both blood and urine of patients on renal dialysis (*6*). The sequence of HC (*7*) was added to the alignment (Fig. 1) so that residue 1 of RBP corresponded with residue 2 of HC, and residue 1 of BBLG with residue 7 of HC, with gaps inserted to optimize the homology. Although the number of identical residues between HC and RBP and between HC and BBLG were less than between RBP and BBLG, the pattern of identities provided evidence of a familial relationship. In comparing all three proteins, 64 positions were identical between one or another pair of proteins, while 15 positions had identical residues in all three complete sequences. A large proportion of these residues were amino acids that occur infrequently in proteins (*4*), which decreases the possibility of a coincidental similarity. Comparison of the codons for the amino acids in corresponding positions shows that the percentage of amino acids that are identical or differ by one base in their codons was 62 percent for BBLG and RBP, 62 percent for HC and BBLG, and 58 percent for HC and RBP. The percentages of identical residues and the normalized alignment scores (NAS) of computer aligned sequences (Table 1), in comparison with the corresponding scores for randomized sequences of the same composition, indicate a relationship between the three proteins (*4*).

Although there are differences between the numbers of disulfide bonds in RBP, BBLG, and HC, the arrangements of these bonds based on reported data (*1, 3, 7*) are consistent with the view that the

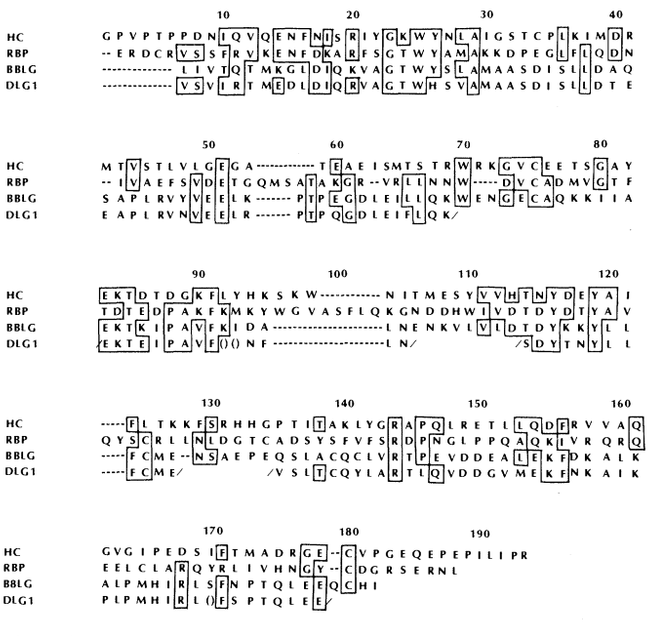


Fig. 1. A comparison of the amino acid sequences of protein HC (HC), human serum retinol-binding protein (RBP), bovine β-lactoglobulin (BBLG), and dolphin β-lactoglobulin-1 (DLG-1). The sources of the sequences are given in the text, apart from DLG-1 (*17*). The alignments are slightly adjusted from that given by the pairwise computer alignments of sequences, so as to give an optimal alignment of the four sequences. Boxed residues are identical in pairs of nonisologous sequences. D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; and Y, tyrosine.