Cerebral Protein Metabolism in Relation to Neuroendocrine Function

By M. B. TER HAAR and P. C. B. MACKINNON
(*Department of Human Anatomy, University of Oxford, Oxford OX1 3QX, U.K.*)

An investigation has been made into the incorporation of [^{35}S]methionine into protein in various discrete areas of the brain that are considered to be involved in neuroendocrine function. Marked changes in incorporation in the preoptic area were seen in the rat and mouse during puberty and in the rat during the oestrous cycle. Marked changes during the oestrous cycle were also seen in the median eminence and anterior pituitary concomitant with the surge of luteinizing-hormone secretion that occurs before ovulation. Further, a circadian rhythm in the incorporation of [^{35}S]methionine into protein was observed in the rat, with the fluctuation in the male being almost 180° out of phase when compared with that of the female.

Effects of some Adrenocorticotrophin Analogues on Protein Synthesis in Brain

By H. W. READING (*Department of Pharmacology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, U.K.*)

Certain peptide fragments of the adrenocorticotrophin molecule delay loss of conditioned avoidance response in rats. The effects of the D- and L-isomers

of adrenocorticotrophin-(4-10)-heptapeptide on cerebral RNA and protein metabolism in rats were compared. Neither isomer had any effect on incorporation of labelled orotate or on brain and pancreatic ribonuclease activity. The L-isomer, however, showed a marked and specific stimulatory effect on [^{14}C]leucine incorporation into brain protein.

The Effect of Inhibitors of Protein Synthesis to Inhibit Brain Protein Synthesis and Prevent the Hyperactivity and Hyperpyrexia in Rats Produced by Inhibition of Monoamine Oxidase and the Administration of L-Tryptophan or 5-Methoxy-*NN*-dimethyltryptamine

By D. G. GRAHAME-SMITH (*Department of Chemical Pathology, St. Mary's Hospital Medical School, London W.2, U.K.*)

Parenteral cycloheximide, acetoxycycloheximide and emetine all inhibit brain protein synthesis and prevent the hyperactivity and pyrexia normally produced in mice by a monoamine oxidase inhibitor and either L-tryptophan or 5-methoxy-*NN*-dimethyltryptamine. It is suggested that the brain protein may be involved in mediating the effects of brain 5-hydroxytryptamine and administered 5-methoxy-*NN*-dimethyltryptamine.

MOLECULAR ENZYMOLOGY GROUP COLLOQUIUM ON 'ENZYMOLOGICAL ASPECTS OF AFFINITY CHROMATOGRAPHY'

Affinity Chromatography: a Tool in Protein Chemistry

By MEIR WILCHEK (*Department of Biophysics, The Weizmann Institute of Science, Rehovot, Israel*)

Conventional procedures for the purification of proteins are based on differences in the physical and chemical properties that exist between the proteins to be purified and all other substances present in the mixture. Such separations are usually laborious and the yields are frequently low, primarily because of the many steps required for purification and the low concentrations of many of the proteins to be purified. Many macromolecules of biological importance, such as enzymes, repressors, permeases, receptors and hormones, fall into this group. One way to overcome these difficulties is through the use of 'affinity chromatography' (Cuatrecasas *et al.*, 1968).

Affinity chromatography exploits the reversible and

specific interactions of ligands with macromolecules for their purification. Purification is affected by chromatography of the mixture, containing the protein to be purified, on a column of an insoluble matrix to which a specific ligand was covalently attached. Proteins and other molecules not exhibiting appreciable affinity for the ligand will pass unretarded through the column, whereas those that recognize the ligand will be retarded to an extent related to their affinity to the ligand. Elution of the bound protein is readily achieved by changing the medium to conditions unfavourable for combination, e.g., change in pH or salt concentration.

Workers in several laboratories have attempted such a technique for the isolation of antibodies, nucleotides, complementary strands of nucleic acids and enzymes by utilization of polystyrene or cellulose for the insoluble supporting matrix. Until recently only limited success has been encountered in the

application of these methods to the purification of enzymes (Silman & Katchalski, 1966).

Successful application of the method requires that the adsorbent will possess several favourable characteristics. It should have minimal non-specific interaction with proteins. The insoluble matrix should form a loose porous network that permits easy entry and exit of soluble macromolecules and that retains good flow rate. The chemical structure of the supporting material must permit the convenient and extensive attachment of the specific ligand under relatively mild conditions and through chemical bonds that are stable during adsorption and elution.

Beaded agarose (Sephacrose) has many of the desired properties listed above, and has been used successfully in numerous purifications after activation with CNBr (Porath *et al.*, 1967).

The general principles and potential application of affinity chromatography were illustrated by its application to the purification of staphylococcal nuclease directly from the crude extracts, α -chymotrypsin and carboxypeptidase A (Cuatrecasas *et al.*, 1968). The dramatic efficiency of this method was illustrated by the purification of avidin from egg-white (a protein that binds biotin). A 4000-fold purification of this protein was obtained in one step by chromatography of egg-white on a biocytin-Sepharose column (Cuatrecasas & Wilchek, 1968). This method has been used in several published studies on the purification of enzymes, antibodies, antigens and cells. The ligands used in these studies were competitive inhibitors (Wilchek & Gorecki, 1969), allosteric effectors (Chan & Takahashi, 1969), coenzymes (Collier & Kohlhaw, 1971) and antigens (Wofsy & Burr, 1969).

Until recently all the applications of affinity chromatography used a ligand-Sepharose column to purify the macromolecules. We have taken the reverse approach. An active protein with affinity for a certain ligand was bound to Sepharose and used to purify these ligands or ligand-bound peptides (Givol *et al.*, 1970). This approach is very important in studies of protein structure. Selective chemical modification of proteins is an important tool in studying their structure-function relationship (Vallee & Riordan, 1969). The major difficulty in these studies is the localization of the modified amino acid residue. Therefore it is desirable to have a method that will enable a one-step isolation of the modified peptide from the peptide mixture derived from a modified protein.

Site-specific reagents are used extensively for affinity labelling of the active site of proteins. In these studies analogues of the substrate possessing a chemically active group are made to react with the protein to form a covalent bond with an amino acid residue in the active site. A general method was developed for the isolation of such peptides by

affinity chromatography where the labelled peptide could be isolated in essentially one step. The method is based on the fact that the native protein can be used to bind the ligand specifically. When the native protein is covalently attached to Sepharose, the affinity-labelled peptide is the only one in the proteolytic digest that recognizes the enzyme and is bound to the protein-Sepharose column. This method was used for the isolation of labelled peptides from affinity-labelled ribonuclease on a ribonuclease-Sepharose column (Gorecki *et al.*, 1970), for labelled peptide of staphylococcal nuclease on a nuclease-Sepharose column (Wilchek, 1970) and for affinity-labelled peptides from anti-2,4-dinitrophenyl antibodies on an anti-2,4-dinitrophenyl-Sepharose column (Givol *et al.*, 1970).

The method described above, which is limited to cases of affinity labelling, was extended to many other chemical modifications of proteins by using antibodies specific to the modified moiety. In fact, any chemical modification creates a new antigenic determinant (hapten) that, on injection into animals, can elicit antibodies specific to this hapten. Only peptides that possess such a ligand will be specifically sequestered by the antibody-matrix columns and thereby removed from the entire peptide mixture. This approach was applied to the isolation of nitrotyrosyl-containing peptides from nitrotyrosyl-lysozyme on anti-nitrotyrosyl-Sepharose and for the isolation of arsenylphenylazotyrosine-containing peptides from carboxypeptidase A modified with azobenzene-*p*-arsonate on an anti-azobenzene-*p*-arsonate-Sepharose column (Wilchek *et al.*, 1971). An anti-2,4-dinitrophenyl-Sepharose column is now being used for determination of the sequences of proteins containing tryptophan, tyrosine, methionine and cysteine residues after covalent attachment of 2,4-dinitrophenyl groups to these residues by various means.

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Affinity-Chromatography Studies on the A Protein of Lactose Synthetase

By IAN P. TRAYER and K. W. OLSEN (*Department of Biochemistry, University of Birmingham, Birmingham B15 2TT, U.K., and Department of Biochemistry, Duke University Medical Center, Durham, N.C. 27706, U.S.A.*)

The terminal step in the biosynthesis of lactose in the mammary gland is catalysed by the enzyme lactose synthetase according to the following reaction:

The synthetase has been resolved into two protein components, the A protein and the milk protein, α -lactalbumin (Brodbeck *et al.*, 1967). In the absence of α -lactalbumin, the A protein has been shown to be a galactosyltransferase that catalyses the incorporation of galactose from UDP-galactose in a β -(1 $\rightarrow$ 4)-linkage with free or protein-bound *N*-acetylglucosamine, and is found in many mammalian tissues (Brew *et al.*, 1968; Hill *et al.*, 1968):

Under these conditions glucose is a very poor acceptor in the transferase reaction and has K_m about 1M, which is decreased about 1000-fold in the presence of α -lactalbumin, effectively allowing the A protein to catalyse reaction (1) (Morrison & Ebner, 1971*a,b,c*). α -Lactalbumin has no effect on the V_{max} of this reaction. Thus the unique ability of mammary gland to synthesize the lactose is dependent on it being the only tissue to synthesize α -lactalbumin.

Although it is membrane-bound in the mammary gland this galactosyltransferase (the A protein) is found soluble in milk, and this material is usually the starting point for its preparation. It is our experience, and apparently that of others (Fitzgerald *et al.*, 1970), that purification of the A protein by 'conventional' protein-separation techniques is both tedious and time-consuming and results in low yields of the final product. The introduction of affinity-chromatography methods has greatly simplified the purification of the A protein. Initial experiments were made by attaching α -lactalbumin covalently by diazotization to amino-cellulose and polyaminostyrene. Better separations were achieved, however, on columns of α -lactalbumin covalently linked to

Sephacrose 4B by the CNBr procedure of Axén *et al.* (1967). The bovine A protein was bound to this column in buffers containing glucose, and was specifically eluted from the column in a homogeneous form by omitting glucose from the developing buffer (Trayer *et al.*, 1970; Trayer & Hill, 1971). Andrews (1970) used *N*-acetylglucosamine instead of glucose to effect the binding of human A protein to the α -lactalbumin-Sephacrose conjugate. The binding of the A protein to these affinity columns may be very similar to that occurring between the A protein and α -lactalbumin in solution, since the conjugate can replace soluble α -lactalbumin in lactose synthesis. Indeed, the fact that the A protein binds to this conjugate provided the first direct evidence that a complex is formed between the two proteins. More recently (Shaper *et al.*, 1971; R. L. Hill, R. Barker, K. W. Olsen, J. H. Shaper & I. P. Trayer, unpublished work) UDP and *N*-acetylglucosamine have been covalently linked to Sepharose and used as affinity columns in the purification of the A protein. The conjugates were prepared by reaction of the hexanolamine derivatives of UDP and *N*-acetylglucosamine with CNBr-treated Sepharose.

With the UDP-Sephacrose columns Mn^{2+} can be used in a manner similar to glucose with the α -lactalbumin-Sephacrose columns, i.e. the A protein can be adsorbed on the column in the presence of Mn^{2+} and eluted from it by the removal of this bivalent cation. Elution from the *N*-acetylglucosamine-Sephacrose conjugate can be achieved by the aid of borate buffer or 1.5M-urea. When milk whey solutions were passed first down a UDP-Sephacrose column and then down an *N*-acetylglucosamine-Sephacrose column, preparations of the A protein close to homogeneous form were obtained.

These techniques have also been used in the purification of glycogen synthetase (Shaper *et al.*, 1971), but the general applicability of these methods with other nucleotide diphosphate- and glycosyl-substituted Sepharose derivatives extends their use to the purification of a wide range of enzymes. Preliminary results with an ADP-Sephacrose derivative to bind the muscle protein myosin and its biologically active subfragments confirm this.

The galactosyltransferase purified by these techniques is a glycoprotein, and appears to be heterogeneous in size depending on the carbohydrate content. One species containing 12% carbohydrate and having a molecular weight of 42000 has been well characterized (Trayer & Hill, 1971), but species with molecular weights close to 50000 are readily observed by electrophoresis in gels containing sodium dodecyl sulphate. Our understanding of the function of the polysaccharide component of the enzyme is incomplete and awaits further study, but it is noteworthy that the A protein is normally bound to the membranous structures of the Golgi apparatus in