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ISOLATION OF HORMONAL PROTEINS AND ANTIBODIES BY AFFINITY CHROMATOGRAPHY*

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SUMMARY

The versatile technique of biospecific affinity chromatography has found application in the purification as well as isolation of many hormones and their antibodies. Hormones can be effectively concentrated from very dilute solutions such as urine or other fluids including culture media. The technique may be expected to have a big impact in the isolation of hormone receptors. Its limitations are not unique to the above macromolecules but extend to the general method itself, the most important being the development of stable immobilized ligands and efficient but still mild solvent systems for elution. The use of dilute ammonia solutions at low temperatures has been quite successful for displacing either the hormone or the antibody.

INTRODUCTION

Development of suitable and simple methods of isolation of biologically active substances has been one of the major preoccupations of biochemists ever since the recognition that highly specific interactions between molecules irrespective of their size form an integral part of the regulation of biological processes. The addition of the powerful technique of affinity chromatography to the list of separation techniques available represents a notable advance. This technique, with its versatility of being applicable to both small, large and very big macromolecules, is unique in that it is dependent on the highly stereospecific interactions between substances. Thus, in order to effect a separation of a substance x, for example a hormone present in circulation or tissue extracts, an interacting specific ligand such as antibody y immobilized on an insoluble support is required. After appropriate equilibration in which binding of x to immobilized y takes place to the exclusion of other unreactive substances, x can be eluted by a suitable solvent. In theory the ligand may be immobilized by either physical adsorption or by chemical linkage to the support. In practice the second approach is favored because the process can be repeated without significant loss of the ligand.

* The work referred to in this article was done in close collaboration with Dr. Jerker Porath during his brief visit to the Hormone Research Laboratory in San Francisco, California and to our own Institute in Montreal.

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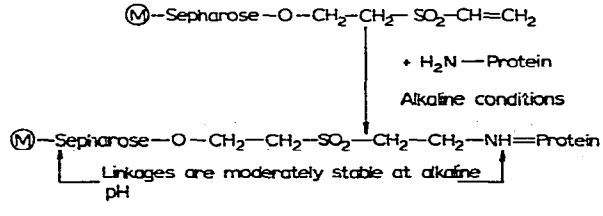
The report by Axén *et al.*[1] in 1967, that molecules could be linked through their amino groups to insoluble polysaccharide matrices which form gels, represented the beginning of modern affinity chromatography. Since then, efforts have been devoted to the development or improvement of coupling methods, variations in solid matrices, introduction of spacers for overcoming hindrances in stereospecific interactions and last but not least the methods for elution which may determine the success or failure of this technique.

To the endocrinologist engaged in research or clinical work, affinity chromatography provides a convenient means of isolating or concentrating hormones, antibodies and receptors. The technique can be used for purifying or concentrating hormones from complex tissue extracts and fluids such as blood or urine, sorting heterogeneous antibodies into various categories dependent on their affinity to closely related structures and even separating native hormone from the inactive denatured molecules. During the last ten years affinity chromatography has found application in the isolation of peptide and protein hormones, hormonal antibodies, steroid binding proteins and receptors for steroid as well as protein hormones. The present article is limited to a discussion of the isolation of protein hormones and their antibodies by the use of affinity chromatography (Table I). For a more general discussion of the principles and practice of the technique the reader is referred to other more exhaustive literature[2] and to articles elsewhere in this volume.

THE MATRIX

Successful application of affinity chromatography depends on the selection of a suitable matrix on which the ligand is anchored[3]. Sepharose, which is a bead form of agarose gel, has useful properties which meet many if not all the criteria required of an ideal matrix. This material as well as some polyacrylamide gel supports are commercially available and have most commonly been employed in investigations concerned with hormones or antibodies. In general, the matrices are activated in a form through which the ligand (hormone or antibody) can be linked via one of its side chains. In most of the literature surveyed to date, CNBr-activated Sepharose, which is also available in a ready-to-use form from Pharmacia (Uppsala, Sweden), has been the matrix of choice for general use in this field.

Sepharose activated by divinyl sulfone (DVS) according to the method of Porath[3] has been used to isolate antibodies to several hormones in a single step procedure[4,5] after immobilization of the purified hormone in the following manner:



This activated gel is apparently not yet commercially available. The procedure used for coupling in this laboratory can best be illustrated with ovine lutropin, with which we have had the most experience. Four millilitres of the activated gel are washed and

TABLE I

PROTEIN HORMONES AND ANTIBODIES PURIFIED BY AFFINITY CHROMATOGRAPHY

Unless otherwise indicated, CNBr-activated agarose has been employed as the matrix for immobilizing the ligand.

Hormones

Hormone	*Source*	*Ligand*	*Elution*	*Ref.*
Growth hormone	Monkey pituitary	Antibody to human chorio-mammotropin	Not done	18
Prolactin	Human amniotic fluid	Rabbit antibody to human pituitary prolactin	4 *M* NaSCN, pH 7.4	21
Human chorio-mammotropin	Plasma/ tumor extracts	Guinea pig antibody	6 *M* Guanidine HCl, pH 3.1	22
Human chorio-gonadotropin	Crude extracts	Concanavalin A	Methyl-α-D-glucoside	8
Human chorio-gonadotropin	Hydatidiform mole	Rabbit antibody to urinary chorionic gonadotropin	2 *M* sodium trichloroacetate	23
Human chorio-gonadotropin	Human term placenta	Rabbit antibody to urinary hormone	4 *M* $MgCl_2$, 6 *M* Guanidine HCl	24
Human chorio-gonadotropin like factor	Extracts of microorganism	Concanavalin A	Methyl-α-D-glucoside	25
Thyrotropin	Bovine pituitary	Concanavalin A	Methyl-α-D-glucoside	9
Thyrotropin	Whale pituitary	Concanavalin A	Methyl-α-D-glucoside	10
Thyrotropin	Bovine pituitary	Concanavalin A	Methyl-α-D-glucoside/ methyl-α-D-mannoside	10
Thyrotropin	Human pituitary	Rabbit antibody to human thyrotropin/ α-subunit of human chorio-gonadotropin	Guanidine HCl gradient	19
Lutropin	Ovine pituitary	Rabbit antibody	6 *M* guanidine HCl, pH 1.5	6
Lutropin	Ovine pituitary	Purified rabbit IgG	Dilute NH_4OH	14
Lutropin	Bovine pituitary	Concanavalin A	Methyl-α-D-glucoside/ methyl-α-D-mannoside	9
Gonadotropin	Salmon pituitary	Concanavalin A	Methyl-α-D-glucoside	11, 12

(Continued on p. 146)

TABLE I *(continued)*

Hormones

Hormone	*Source*	*Ligand*	*Elution*	*Ref.*
Follitropin	Ovine pituitary	Concanavalin A	Methyl-α-D-glucoside	13
Gonadotropins	Bull seminal plasma	Concanavalin A	Methyl-α-D-glycoside	26
Insulin	Porcine/ dog plasma	Guinea pig antibody to bovine insulin	1 *M* Acetic acid	27
Glucagon	Gut	Antibody to glucagon		28

Hormone antibodies

Antibody against	*Ligand*	*Elution*	*Ref.*
Human prolactin	CNBr-agarose prolactin	4 *M* NaSNC, pH 7.4	21
Human growth hormone	Divinyl sulfonyl Sepharose–hormone	1 *M* Sodium trifluoroacetate	4
Ovine lutropin	Divinyl sulfonyl Sepharose–lutropin	2 *M* Sodium trichloroacetate	4
Ovine lutropin	Divinyl sulfonyl Sepharose–lutropin	Dilute NH_4OH	5
Ovine lutropin β-subunit	Divinyl sulfonyl Sepharose–lutropin	Dilute NH_4OH	5
Human lutropin	Divinyl sulfonyl Sepharose–human choriogonadotropin	Dilute NH_4OH	5
Rat prolactin	Divinyl sulfonyl Sepharose–rat prolactin	Dilute NH_4OH	5

suspended in 10 ml of 0.25 *M* $NaHCO_3$, pH 9.0, in a conical centrifuge tube. After removal of the supernatant, 6–8 mg of purified lutropin dissolved in 6 ml of the same buffer are added. An aliquot of the supernatant is saved for measurement of its UV absorbance to estimate the extent of coupling. The tube is covered with a screw cap and shaken gently in an end-over-end shaker at room temperature. After 2–5 h, the absorbance of the supernatant may be determined. The entire supernatant is carefully removed and 10 ml of 0.25 *M* $NaHCO_3$, pH 9.0, containing 500 mg glycine are then added and shaken for 90 min to block the excess of reactive sites. The gel is then washed twice with Tris–HCl, pH 7.6, and stored at 4°C. The gel is quite stable between pH 3.5 and 8[3] but it is preferable to store these subunit-containing hormones at around neutrality to prevent any dissociation. Either DVS-Sephasrose 4B or 6B may be used for this coupling. In routine experiments very good coupling of sheep lutropin to DVS-Sepharose was obtained, ranging from 75 to 95% in about 5–6 h at room temperature. The degree of coupling depends upon the hormone and is thus variable. For example, the lowest degree of coupling among the several hormones

tested was found with purified human growth hormone; only about 30% of the added hormone was coupled in 2 h as compared to about 75% for ovine lutropin. Other hormones or substances yielded the following results under similar conditions: human choriogonadotropin, 66%; NIH rat prolactin, 73%; IgG to ovine lutropin, 78%; Sigma concanavalin A, 80%.

According to Porath[3], a definite advantage gained by cross-linking of Sepharose with divinyl sulfone is that the gels become more rigid and afford good flow properties. This has been found to be the case in our own experience with prolonged and repeated use of the same column over 25 times[5]. The occasional reduction in flow-rates is mostly attributable to small amounts of lipid materials that may be present in some serum samples or to extracts that occlude the top surface of the gel bed. This can be avoided by centrifugation at high speed and careful handling.

ELUTION METHODS IN HORMONAL AFFINITY CHROMATOGRAPHY

After effective adsorption of the hormone or antibody to the matrix and washing off of the unrelated substances, the next crucial step in affinity chromatography is the successful elution of the desired component and its recovery in a biologically active form. There are no standard procedures for this and establishing successful conditions is an empirical process. In general, any technique or condition that will render the hormone–antibody complex unstable or disrupt it will be suitable for elution. The methods that are currently available include lowering or raising the pH, inclusion of high concentrations of chaotropic agents such as perchlorate, iodide, thiocyanate, trifluoroacetate, trichloroacetate, etc. and the use of the classical denaturing agents such as high concentrations of urea or guanidine hydrochloride. Many of these agents have been applied for the hormones or antibodies listed in Table I with varying degrees of success.

A major drawback of many elution methods is that they are rather drastic and require additional manipulation to recover the substance under study in a form that retains biological activity. This is best illustrated for example with lutropin, a hormone which has a quaternary structure. Drastic conditions such as 6 *M* guanidine hydrochloride at pH 1.5 during affinity chromatography on Sepharose-linked antibody[6] may lead to total disruption of the oligomeric hormone, rendering it inactive. Although subsequent manipulation may restore some activity it is nevertheless incomplete. However, the chemical composition of the isolated hormone is apparently similar to a preparation isolated by conventional methods. Therefore, in bioaffinity chromatography it is essential to use efficient but milder conditions to effect the dissociation of antigen–antibody complexes.

In searching for alternatives to the cumbersome use of high concentrations of chaotropic ions or denaturants, we have found that dilute NH_4OH is an effective but mild reagent[5]. In some instances even as dilute a solution as 0.01 *M* NH_4OH can displace a significant proportion of the total immunoglobulin adsorbed on the affinity column[5]. These components were presumably of a low affinity. Those of a higher affinity require the use of more concentrated solutions such as 0.1 *M* or 0.25 *M* NH_4OH. It may even be possible to fractionate antibodies of presumably different affinities by carefully introducing a gradient of NH_4OH. As NH_4OH is volatile it may be completely removed from the eluted solution by immediate lyophilization. In

practice, the elution with NH_4OH may be carried out in a manner illustrated by the following example. Two millilitres of rabbit antiserum to ovine lutropin were passed through the immobilized columns containing the hormone. The antiserum was circulated continuously through the column at room temperature to ensure maximum formation of antigen–antibody complex. The unwanted serum proteins were then washed out with 0.1 *M* Tris–HCl–NaCl, pH 7.6, at the same temperature. This was then followed by a wash at 4°C with 0.05 *M* NH_4HCO_3, which removed all the non-volatile salts. The pH of this solution, 8.5, does not induce dissociation of the complex and thus no antibodies are lost. The next step consists of percolating the column with various concentrations of NH_4OH at 4°C (pH 10–11.5). The displacement of antibody from the column was very rapid and usually complete in less than 2 h. The eluted antibody has been recovered in a lyophilized form. In several experiments, we have recovered about 70% or more of the lutropin antibody originally present in the antiserum. After elution the column may be regenerated by passage of Tris–HCl–NaCl, pH 7.6, buffer and stored at 4°C. The use of NH_4OH does not appear to affect the performance of the affinity column during subsequent repeated runs. Thus it may find application to other systems as well. An additional advantage of the use of NH_4OH is that the high pH is not detrimental even to the glycoprotein hormones which, as pointed out earlier, possess a quaternary structure. Unlike the low pH which rapidly induced dissociation[6], the hormone is relatively stable to the rather high pH conditions.

GROUP SPECIFIC ADSORBENTS FOR HORMONAL PROTEINS

Separation of a certain class of compounds becomes feasible when reagents are available which have the capability to interact specifically with certain special features (groupings) of the molecules. In endocrine research two methods based upon group separation may be discussed briefly: (a) use of lectins for hormone purification and (b) application of protein A to antibody purification.

Isolation of glycoprotein hormones by concanavalin A–Sepharose chromatography

Among the hormones of the pituitary gland and placental tissue that are normally secreted into the circulation in the glycosylated form, lutropin, follitropin, thyrotropin and choriogonadotropin (both from human and mare) have most extensively been studied. Since they contain carbohydrate contents ranging from 15 to 45% these hormones are amenable to purification or concentration by means of affinity chromatography on concanavalin A–Sepharose. Concanavalin A, generally abbreviated as con-A, belongs to the class of lectins, which are defined as proteins of animal or plant origin[7] exhibiting a selected affinity for carbohydrate groupings. Different lectins vary in their affinity to different sugars and by an appropriate choice of affinity columns, separation methods could be devised.

Application of con-A immobilized on Sepharose for the purification of glycoprotein hormones was first described by Dufau *et al.*[8]. Human choriogonadotropin, lutropin and follitropin were strongly bound by con-A–Sepharose columns. After washing away the unadsorbed materials, the hormones were eluted by the use of competing glycosides. The technique is applicable for use with very small amounts of glycoprotein hormones, *e.g.*, nanogram to microgram amounts of hormones as en-

countered in clean up of radioactively labeled products, or to very large scale batch work involving several kilograms of pituitary tissue[9]. Con-A–Sepharose is also available in ready-to-use form from Pharmacia and is relatively stable for long periods at 4°C. Bloomfield *et al.*[9] were able to fractionate glycoprotein fractions equivalent to 0.4–8 kg of bovine pituitary tissue on single large columns of con-A–Sepharose and thus concentrate thyrotropin and lutropin acivities. Subsequent purification required only one or two additional steps to obtain high quality hormone. Con-A–Sepharose chromatography has been successfully used by others also to concentrate whale and bovine thyrotropins[10] present in relatively crude extracts and in the isolation of salmon gonadotropins[11,12]. In our own experience, con-A–Sepharose chromatography was found to be essential for eliminating a non-glycoprotein fraction which co-chromatographed with ovine follitropin[13] in several purification steps, and thus was of great value in the eventual isolation of the hormone in a highly purified active form.

The general method of recovery of the glycoprotein hormones from the column has usually been stepwise elution using differing concentrations (usually 0.2 *M* and above) of methyl-α-D-glucoside or mannoside, both of which are commercially available*. This method generally results[8,9,10,13] in tailing of the glycoprotein hormone fraction, which has generally been attributed to the presence of forms of the hormone with various degrees of glycosylation. For example, the partially desialylated species appears to be more strongly adsorbed on con-A–Sepharose and is thus leached out more slowly by the high concentration of the competing glycoside[8]. This may not be the sole reason for the tailing phenomenon, as we have found it to occur in the chromatography of ovine pituitary lutropin, a hormone which has negligible amounts of sialic acid. Attempts to elute the hormone in discrete peaks using gradient elution have not been successful in our hands[14]. Bloomfield *et al.*[9] have speculated that differences in mannose contents, which are presumably the major sites of interaction of glycoproteins with con-A–Sepharose, may be responsible for the different affinity of bovine lutropin to the lectin. In this respect, it may be of particular interest that native ovine lutropin, which is normally adsorbed on con-A–Sepharose, is completely excluded from this column when it is chemically deglycosylated[15]. This treatment has resulted in removal of nearly all the hexoses in the molecule and thus favors the speculation that mannose residues are required for specific retention on con-A–Sepharose.

As the conditions of the elution from con-A–Sepharose columns are mild, excellent recoveries of hormone activity have been recorded in all the reports discussed above.

Many other lectins isolated from lentils or wheat germ have been isolated and show different specificity for other carbohydrates, and it may be expected that additional sources of materials will be added to the growing list. Many of these are also commercially available. However, at this time, no studies of the glycoprotein hormones have been reported.

Use of protein A for affinity chromatography of hormone antibodies

Protein A is a component of the cell wall of the bacteria *Staphylococcus aureus*

* Some batches of methyl glucosides obtained from commercial suppliers have significant UV absorbance and thus should be screened before use to avoid interference in monitoring of the column effluents.

strain that interacts specifically with IgG from most mammalian species[16,17]. Immobilized protein A on Sepharose provides a high capacity adsorbent for the purification of IgG-type antibodies. The operation of the technique would be very much similar to that described for immobilized hormones, including methods of elution. Specific reports related to its use in the isolation of hormone antibodies have not yet appeared. As compared to affinity chromatography using immobilized hormone, separations obtained from the protein A–Sepharose column would be less specific.

CONCLUSIONS

The selective isolation and purification of biologically active macromolecules by the technique of biospecific affinity chromatography exploits their unique property of binding to ligands in a specific and reversible manner. The technique has found application in the isolation of enzymes, antibodies, hormones and other macromolecules of biological interest. Leaching of the ligand during the run or instability during elution is a problem that should be considered[20]. With improvements in the art of coupling of ligands to suitable matrices and the development of milder elution methods for recovery of products in an active form, we may expect further applications of affinity chromatography in endocrine research. Because of the presence of hormone receptors, usually in very small quantities, in target tissues, this technique may be the only feasible approach to the isolation and characterization of these macromolecules. Affinity chromatography played a key rôle in demonstrating and providing for the first time a reliable method for separating primate prolactin and growth hormone and offered convincing evidence for their existence as separate molecular entities[18]. This had been a vigorously contested and debated issue until 1971.

It should be possible to adapt affinity chromatography techniques in hormone research at any level —either on a micro or macro scale and for application to either partially purified fractions or crude extracts. At the present state of the art, the technique is still not amenable to wide application for routine isolation of hormones* on a large scale, mainly because of the difficulties in producing large quantities of high titer specific antibodies. Hitherto, in most applications, antibodies (heterogeneous) produced in small animals such as rabbits or guinea pigs have been utilized. But the rapidly emerging powerful technology of the hybridoma technique offers the potential of producing large amounts of monoclonal antibodies, and hence we may soon expect the trend to change. It may be possible in the not too distant future to have large columns of immobilized specific monoclonal antibody against each hormone, for instance from the pituitary, connected in series. A single step purification may be envisaged by serial passage of the tissue extract containing the mixture of hormones through these columns and their subsequent elution for recovery of the product (Fig. 1). This has the promise of being rapid and specific. The versatility of the approach lies in the fact that the columns will obviously be reusable and very economical in the long run. Such a technique will be particularly useful in the fractionation of hormones from valuable tissues such as the human pituitary which are always scarce and not easy to procure. The technique will also find application in hormone production by

* Glycoprotein hormones are an exception as they may be concentrated fairly easily by using con-A–Sepharose.

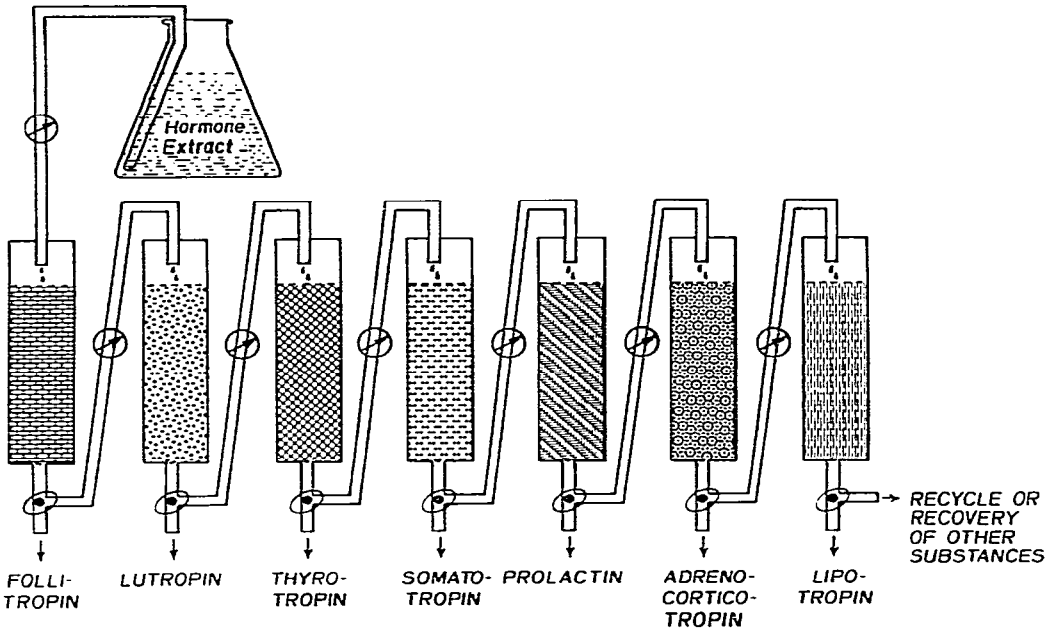


Fig. 1. Hypothetical single step isolation of pituitary hormones. Each column contains gels on which highly specific antibody against the respective hormone is immobilized. An extract of hormones is continuously pumped through these columns connected in series. In the first column, follitropin may be expected to be removed, followed by lutropin on the second and so on. The process may require recycling for complete removal of each hormone. The elution from each column might require different methods, with the glycoprotein hormones demanding the mildest possible conditions whereas hormones such as adrenocorticotropin or lipotropin are easily capable of withstanding low pH or denaturants. To increase specificity of adsorption for the glycoproteins, the use of IgGs against the respective hormone specific β-subunits may be desirable. As this class of hormones is effectively adsorbed on con-A–Sepharose, the group specific adsorbent can also be profitably incorporated into the above scheme to enhance both specificity and efficiency of the large scale operations.

synthetic methods, especially after assembly of the peptide or protein by the solid phase technique or by novel means such as DNA recombination technology. It is obvious that efficient purification systems play an important rôle in the success of the synthetic methods.

The small scale affinity chromatography method using immobilized hormones will continue to be useful to the individual researcher. In instances where cells in culture are to be studied over long periods in presence of sera, affinity chromatography might be one of the possible ways of rendering them hormone free by passage on immobilized antibody columns.

In regular affinity chromatography of the glycoprotein hormones, it is advisable to avoid rather harsh conditions of elution such as low pH and or the use of denaturing agents such as guanidine hydrochloride. Despite claims of recovery of activity[6,19], the success rates may be widely variable. Another caveat related to glycoprotein hormones and their behavior on con-A–Sepharose is worth stressing. While effective adsorption of the hormone on con-A–Sepharose is suggestive of the presence of significant amounts of carbohydrate in the product to be purified, a negative result

showing non-retention on the column need not always be due to lack of carbohydrate. The latter may simply reflect the fact that sugar residues, in this instance, mannoses, were not present or, if so, were not oriented in the right configuration for effective binding to con-A–Sepharose. This generalization can be extended to other lectins with different specificity(ies).

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REFERENCES

1 R. Axén, J. Porath and S. Ernback, *Nature (London)*, 214 (1967) 1302.
2 W. B. Jakoby and M. Wilchek (Editors), *Methods Enzymol.*, 37 (1974).
3 J. Porath, *Methods Enzymol.*, 37 (1974) 13.
4 M. R. Sairam, W. C. Clarke, D. Chung, J. Porath and C. H. Li, *Biochem. Biophys. Res. Commun.*, 61 (1974) 355.
5 M. R. Sairam and J. Porath, *Biochem. Biophys. Res. Commun.*, 69 (1976) 190.
6 D. Gospodarowicz, *J. Biol. Chem.*, 247 (1972) 6491.
7 H. Lis and N. Sharon, *Annu. Rev. Biochem.*, 42 (1973) 541.
8 M. L. Dufau, J. Tsuruhara and K. J. Catt, *Biochim. Biophys. Acta*, 278 (1972) 281.
9 G. A. Bloomfield, M. R. Faith and J. G. Pierce, *Biochim. Biophys. Acta*, 533 (1978) 371.
10 N. Ui, H. T. Takahashi, T. Yora and P. G. Condliffe, *Biochim. Biophys. Acta*, 497 (1977) 812.
11 D. R. Idler, L. S. Buzar and S. J. Hwang, *Endocrin. Res. Commun.*, 2 (1975) 215.
12 J. G. Pierce, M. R. Faith and E. M. Donaldson, *Gen. Comp. Endocrinol.*, 30 (1976) 47–60.
13 M. R. Sairam, *Arch. Biochem. Biophys.*, 194 (1979) 63.
14 M. R. Sairam, unpublished results.
15 M. R. Sairam and P. W. Schiller, *Arch. Biochem. Biophys.*, 197 (1979) 294.
16 G. Kronvall, U. S. Seal, S. Finstad and R. C. Williams, Jr., *J. Immunol.*, 104 (1970) 140.
17 J. J. Langone, *J. Immunol. Methods*, 24 (1978) 269.
18 H. J. Guyda and H. G. Friesen, *Biochem. Biophys. Res. Commun.*, 42 (1971) 1068.
19 F. Pekonen, D. M. Williams and B. D. Weintraub, *Endocrinology*, 106 (1980) 1327.
20 G. I. Tesser, H. U. Fish and R. Schwyzer, *Helv. Chim. Acta*, 57 (1974) 1718.
21 P. Hwang, J. B. Murray, J. W. Jacobs, H. D. Niall and H. G. Friesen, *Biochemistry*, 13 (1974) 2354.
22 B. D. Weintraub, *Biochem. Biophys. Res. Commun.*, 39 (1970) 83.
23 Y. M. Choy, K. M. Lau and C. Y. Lee, *J. Biol. Chem.*, 254 (1979) 1159.
24 C. Y. Lee, S. Wong, A. S. K. Lee and L. Ma, *Hoppe-Seyler's Z. Physiol. Chem.*, 358 (1977) 910.
25 T. Maruo, H. Cohen, S. J. Segal and S. S. Koide, *Proc. Nat. Acad. Sci. U.S.*, 76 (1979) 6622.
26 M. R. Sairam, M. R. Ranganathan and P. Lamothe, *J. Endocrinol.*, 84 (1980) 17.
27 Y. Akanuma and M. Hayashi, *Methods Enzymol.*, 37 (1974) 746.
28 R. F. Murphy, K. D. Buchanan and D. T. Elmore, *Biochim. Biophys. Acta*, 303 (1973) 118.