fruits of both the varieties and whatever respiration was present was all cyanide-sensitive respiration. These findings are supported by the results reported earlier[9]. Furthermore, in Pusa *Ruby* the increase in cyanide-resistant respiration was fast whereas in Pusa *Gaurav* it was slow. This point explains the ripening behaviour in these two varieties. The addition of inhibitors of cyanide-sensitive and cyanide-resistant system completely stopped the respiration and no oxygen uptake was noted.

It was reported that during ripening, respiration in climacteric fruit increases and the main component of this respiration is cyanide-resistant respiration, which is involved in thermogenesis in many ripening fruits[4,5] and in flowers[10]. These observations indicate that cyanide-resistant respiration is the major part of total respiration and it increases during ripening of tomato fruit along with the activity of hydrolysing enzymes and both the processes seem to be related to each other.

1. Tucker, G. A., *Biochemistry of Fruit Ripening* (eds Seymour, G., Talor, J. and Tucker, G.), Chapman and Hall, London, 1993, pp. 1–51.
2. Awad, M. and Young, R. E., *Plant Physiol.*, 1979, **64**, 306–308.
3. Tucker, G. A. and Grierson, D., *The Biochemistry of Plants*, Academic Press Inc., 1971, vol. 12, pp. 265–317.
4. Kumar, S., Patil, B. C. and Sinha, S. K., *Biochem. Biophys. Res. Commun.*, 1990, **168**, 818–822.
5. Kumar, S. and Sinha, S. K., *J. Expt. Bot.*, 1992, **43**, 1639–1642.
6. Rouse, A. H. and Atkins, C. D., *Florida Agri. Expt. Station Bull.*, 1955, **570**, 1–9.
7. Hobson, G. E., *Nature*, 1962, **195**, 804–805.
8. Upmeyer, D. J. and Koller, H. R., *Plant Physiol.*, 1973, **51**, 871–874.
9. Pandey, M., Zeng, Y. R., Prasad, N. K. and Srivastava, G. C., *Indian J. Plant Physiol.*, 1995, **38**, 182–183.
10. Chen, J. and Meeuse, B. J. D., *Plant Cell Physiol.*, 1972, **13**, 831.

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Mapping of assembled epitopes with microgram quantities of antigen: Identification of an epitope at the receptor binding region of human follicle stimulating hormone

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Identification of epitopes by modification studies has been reported by us recently. The method requires milligram quantities of antigen and since several proteins are not available in large quantities they are not amenable for such an investigation. One such protein is human follicle stimulating hormone (hFSH) whose mapping of epitopes is of importance in reproductive biology. Here we report a method that uses microgram quantities of hFSH to map a β-specific epitope located at the receptor binding region. This identification has also been validated by the chemical modification method using heterologous antigen ovine follicle stimulating hormone (oFSH).

EPITOPE mapping is used to identify the region capable of binding to monoclonal antibodies (MAb) in an attempt to develop synthetic epitopes to be used in diagnostics, vaccines, etc.[1–4]. Linear peptide synthesis methodology, though popular, seldom provide, a sequence which has the same conformation as in the native molecule[5,6]. Recently, we reported a method for the identification of epitopic regions in their native conformation using chemical modification of antigen coupled to their immunoactivity as measured by solid phase radioimmunoassay (SPRIA)[7–9]. Even though this method is the first batch method reported for analysis of assembled epitopes, and compared to other existing methods is easier and adoptable in all laboratories, further improvement in the method can be envisaged in several directions. In the earlier method[7] need for antigen is rather high requiring milligram quantities and number of SPRIAs to be run are high. Any development which reduces both the quantity of antigen and number of SPRIAs required in epitope mapping would make the method extremely useful and economical. Several antigens are not available in mg quantities and one such antigen of importance in reproductive physiology is hFSH. Here we describe a method of epitope analysis which uses µg quantities of hSFH and eliminates the need for SPRIA in the analysis of assembled/sequential epitopes, and compare the results obtained with the conventional approach based on SPRIA of chemically modified antigen analogue, namely, oFSH.

MAb 68.K12.1D12 (referred to in this paper as 68.K12 – gift from Dr James A. Dias, Wadsworth Centre, WIH HD 18407) is a monoclonal antibody (MAb) against hFSH and binds to hFSH β-subunit. Displacement profile of [^{125}I]hFSH (obtained by iodination of hFSH by iodogen procedure) from immobilized MAb 68.K12 (Figure 1) shows that heterologous FSH, namely oFSH as well as its β-subunit binds to the antisera with 25% cross reactivity.

Chemical modification followed by gain or loss of activity with respect to a particular epitope has been used in our earlier studies[7,8] for mapping epitopes. Since hFSH or its subunits are not available in large amounts like human chorionic gonadotropin (hCG), we have modified iodinated hFSH and investigated the effect on

binding to the immobilized MAb. Modifications were done using the same procedures as already described[7]. About 30 lakhs cpm of [^{125}I]hFSH (40 ng) was taken in 100 µl of appropriate buffer containing 1 mg/ml of TNBS, 0.5 µl/ml TNM, 2–4 mg/ml PG (0.2 m $NaHCO_3$), 1 mg/ml of DEPC (0.2 M phosphate buffer pH 6.5) or freshly prepared 5 mg/ml EDAC (in 5% diamino ethane in 0.2 M phosphate buffer pH 5) for lysine (K), tyrosine (Y), arginine (R), histidine (H) and aspartic (D)/glutamic (E) acid modifications. Reactions were terminated after 30 min by addition of excess of ammonium acetate, tyrosine, arginine, imidazole and ammonium acetate (50 µl of 100 mg/ml) respectively. Control reactions were carried out without [^{125}I]hFSH and terminated followed by addition of [^{125}I]hFSH. All solutions were made up to 2 ml and binding of the control and reacted [^{125}I]hFSH with immobilized MAb 68.K12 was carried out at room temperature overnight. Table 1 indicates that binding of [^{125}I]hFSH is the same as in treated and untreated controls. Thus, the decrease/increase in binding of reacted [^{125}I]hFSH to the immobilized MAb can be ascribed to involvement of that amino acid residue in the epitopic region. Hence amino acids D/E, K and Y are in the core region of the epitope while H is in the proximity. Considering that we are using high concentration of modifying reagents, identification of R and H residues has been done based on the data of multiple modification (Table 2). In this approach lysine residues of the antigen are modified with TNBS followed by either H or R modification. This would render the two modifications very specific. These results (Table 2) indicate that D/E, K, Y and cys–cys (table under single modification) are at the core region and H and R are located at the proximal regions of the epitope (Table 2: multiple modification). Amino acid residues Y and K identified should also be enzyme-resistant as seen by retention of activity to tryptic and chymotryptic digestion (Table 2, under single modification). Regions fitting into this pattern are H68-K76 which have H, K and D in proximity but this region lacks Y. The nearest Y for this region is at Y33, too far away to be identified as proximal or at the core. Other region which has all the four amino acids near is βY31–Y33 attached to H83-D88 through the disulphide bond cycs32-cys84 (Figure 2). This has Y at 31,33: K at 86: and H at 83, both the K and Y being resistant to enzymatic digestion because of adjacent cys residues.

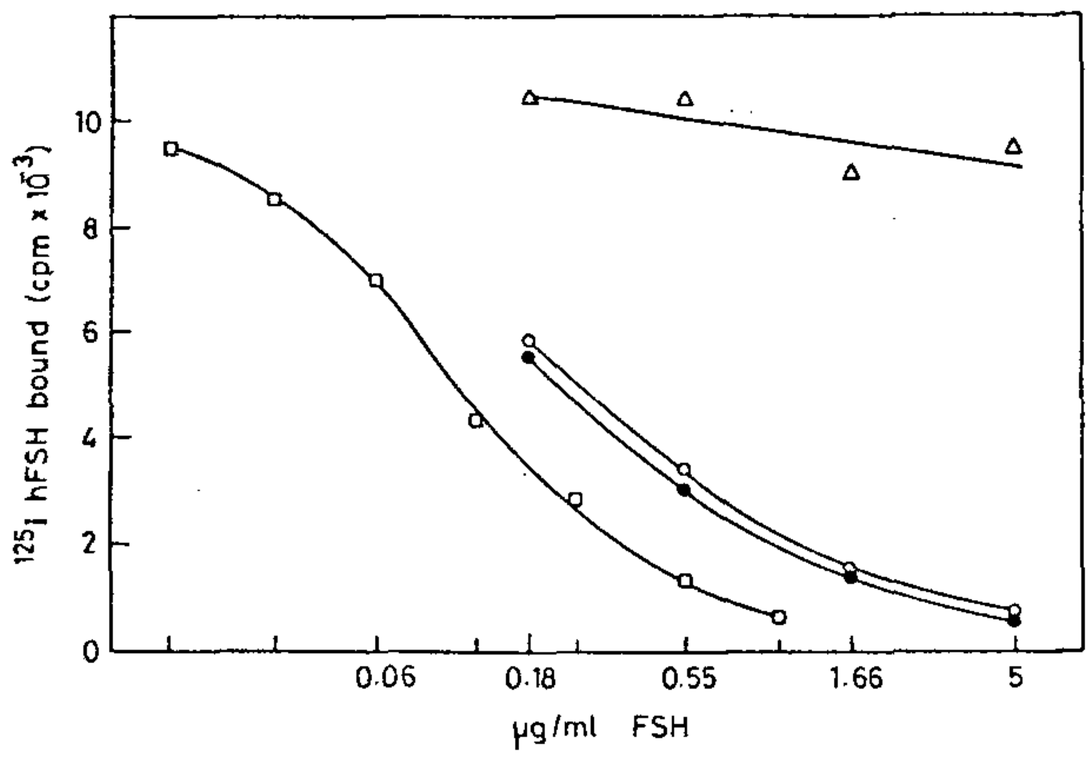

Figure 1. Displacement of [^{125}I]hFSH in SPRIA using MAb 68 coated wells by FSH and its subunits. (□)hFSH, (●)oFSH, (O)oFSH β and (Δ)hCG α. MAb 68 antisera was coated on the immunochemical bridge at a concentration of 1/1000 in microtiter wells and input count was 60,000 cpm/well.

Table 1. Binding of modified [^{125}I]hFSH to immobilized MAb 68.K12

Modification	Experimental (cpm)	Experimental (per cent)	Experimental control (cpm)	Experimental control (per cent)	Control (cpm)
None	–	–	–	–	43,000
TNBS (K)	70,900	165.0	42,600	99.1	
TNM (Y)	11,733	27.3	44,220	102.8	
PG (R)	32,785	76.2	40.567	94.3	
DEPC (H)	19,885	46.2	41,879	97.4	
CDI (D/E)	12,463	29.0	45,860	106.6	

MAb was immobilized on microtiter wells through immunochemical bridge[9]. All counts presented are average of three values. Input in each case was 128.600 cpm. Percentage bound are calculated against the control value of 100% (43,000 cpm). Chemical modifications were carried out by standard procedure.
TNBS – Trinitrobenzene sulphonic acid; TNM – tetranitromethane; PG – phenyl glyoxal; DEPC – diethyl pyrocarbonate; CDI – carbodiimide.

Table 2. Identification of amino acid residues at the epitopic region using [^{125}I]hFSH

Modification	Res. modified	[^{125}I]hFSH bound* (%)
Single modifications		
TNBS	K	165, 200, 197, 171
TNM	Y	27, 30
CDI	D/E	14, 16
Chymotrypsin	F, Y	80, 100, 86
Trypsin	K, R	100
RCM	S-S	< 1
*Multiple modifications***		
TNBS (control)	–	100
TNBS-PG	R	58, 56
TNBS-DEPC	H	55
Chym-TNBS (control)		100
Chym.TNBS-PG.	R	57, 56

*Values reported are of independent measurements.
**Multiple modifications were carried out in the order given. TNBS modification was carried out with 100 µg/ml TNBS for 30 min. To the same tube was added PG or DEPC to a final concentration of 2–4 mg/ml and reaction terminated after 2 h by addition of excess Arg or imidazole.

Amino acid residues identified at:
Core region : D/E, Y, K, S-S.
Proximal : R, H
Distant : F.

R at 35 and 97 are proximal to the epitopic region, placed four residues away from the core disulphides. Retention of activity by tryptic and chymotryptic digestion and partial loss of activity seen on R modification of the TNBS-modified chymotryptic digest indicate that R 97 is the residue involved in this epitope structure. In addition, elimination of R 35 and other K and R residues is based on the likelihood that the peptide loops 33–51 and 66–82 are easily accessible to proteolysis as in hCG β[10]. Based on these considerations region of the epitope in hFSH β is identified as shown in Figure 2. This region also explains partial crossreactivity (25%) with oFSH as the differences between oFSH and hFSH in this region are at G 85 and Q 81. One of the features of this epitope is that presence of K in the epitope has been based not on the loss of activity, but on increased activity. The increase seen in the cold modification is about 200–300% (Table 3) as against about 180% in the [^{125}I]hFSH modification approach. Considering that TNBS is a hydrophobic group this increase may indicate that peptide region β85–87 in FSH may be placed very close to a hydrophobic core of the paratope of MAb 68.K12 in the FSH–MAb complex. This is further substantiated by the observation that when the amino groups are modified with acetylation the change in activity is only marginal.

Data on inhibition of [^{125}I]hFSH binding to its receptor in presence of MAb 68.K12 indicate that as low as 0.03 µl of the MAb is able to inhibit binding to >50% (data not shown) and demonstrate that this epitopic region is situated near the receptor-binding region. The identification matches well with data reported for the region by several investigators using different methods and validates the identification of the epitope region[11–13].

Crossreactivity data shown in Figure 1 provide us with a possibility of using the heterologous ovine antigen for finding the epitopic region of this MAb in hFSH, using chemical modification method developed earlier[7,8]. Data on loss or retention of activity on chemical modification (Table 3) identifies the same region as the epitopic region. Thus these results prove that modification of [^{125}I] antigen will provide the same result as far as epitope identification is concerned, and when large quantities of purified antigen are not available, as in hFSH, above mentioned method is a viable alternative. In fact preliminary data obtained with several other MAbs using this method indicate that it has the potential to be a general method for epitope mapping.

Comparison between results from the two methods indicates that they are qualitatively comparable. The differences seen (TNM, 40% vs 29% : CDI, <5% vs 15% : TNBS, 200–300% vs 180% and DEPC 65% vs 55%) can be due to either experimental error or due to the difference in the two antigens (oFSH, hFSH). In spite of these small differences in the extent of inactivation/activation identification of amino acid residues at core and proximal regions is not different and indicates to the same region independently. Thus modification of iodinated probes provides an exceptionally easy way of analysis of conformation-specific epitope. In addition, this method has the same advantages for adoption to batch method.

One of the advantages of cold modification method[7] is that it can be used to delineate finer structural features in three-dimensional structures of the antigen, and pick fine differences in the specificity of MAbs apparently identifiable to the same region[8,14]. It would be interesting to find out whether the three-dimensional features of the molecule as obtained in hCG using the modification method can also be obtained in this methodology. One of the problems in the use of iodinated probes is that, unlike in cold modification procedure where SPRIA provides quantitative losses/gains of activity, methods need to be developed to convert simple binding data into rigorous quantitative tool. Further experiments are in progress to investigate the feasibility to extend this approach to pick the finer aspects of the three-dimensional structure using iodinated antigen.

This particular epitope assumes importance in relation to the use of oFSH as a vaccine for fertility control[15,16]. Considering that the epitope identified is at/or very close to the receptor binding region, this epitope, if synthesized, can become a potential substitute for oFSH as a vaccine.

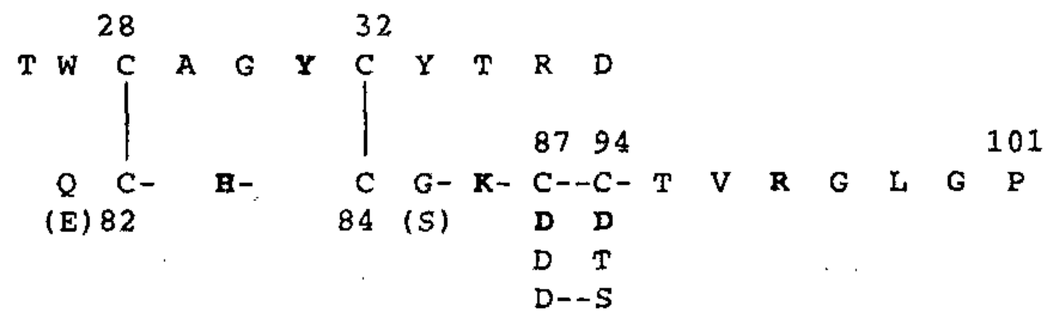

Figure 2. Epitopic region for MAb 68.K12. Amino acid residues in bold letter represents the residues identified by chemical modification approaches. Residues 81 and 85 are replaced by E and S respectively in oFSH. Rest of the amino acid residues are conserved in the hormone.

Table 3. Relative loss of activity of modified oFSH $\alpha\beta$, oFSH β to immobilised 68.K12 antisera

	Activity retained (%)	
Modifying agent	oFSH $\alpha\beta$	oFSH β
None	100	100
TNBS (K)	200	300
TNM (Y)	60	40
CDI (D/E)	<5	<5
DEPC (H)	65	65/55
Trypsin (K, R)	100	80
Chymotrypsin (F, Y)	80	80
RCM	<1	<1
Amino acids identified at:		
Core region	D/E, K	D/E, K
Proximity	H, Y	H, Y
Distant	F	F

Hence there is a need for synthesis of this disulphide-linked peptide and to investigate further the nature/specificity of the antibodies produced on immunization with such synthetic peptides in animal models. Data obtained in this and other papers[7,8] using cold modification method have clearly indicated importance of disulphide bonds in the integrity of epitopes and explains why most of the linear peptides show very low cross reactivity. Thus, it appears that a viable substitute for proteins in vaccine programmes or for diagnostic utility can only be a disulphide constrained peptide, which can be obtained at present by reoxidation of linear peptides synthesized by chemical methods or genetic engineering procedures. These data clearly demonstrate the importance of understanding the reoxidation of peptides/proteins with a view for field application.

1. Krichevsky, A., Birken, S., O'Conner, J. F., Bikel, K., Schlaterer, J. P. and Canfield, R. E., *Endocrine*, 1994, **2**, 511–520.
2. Dimhofer, S., Klieber, R., Leeuw, R. D., Bidart, J. M., Merz, W., Wick, G. and Berger, P., *FASEB J.*, 1993, **7**, 1381–1385.
3. Kaumaya, P. T. P., VanBuskirk, A. M., Goldberg, E. and Pierce, S. K., *J. Biol. Chem.*, 1992, **267**, 6338–6346.
4. Nataraj, U., Sabnis, T. N., Mahale, S. D. and Iyer, K. S. N., *Vaccine*, 1994, **12**, 431–434.
5. Weiner, R. S. and Dias, J. A., *Mol. Cell. Endocrinol.*, 1992, **85**, 41–52.
6. Dimhofer, S., Madersbacher, S., Bidart, J. M., Ten Kortenaar, P. B. W., Spottl, G., Mann, K., Wick, G. and Berger, P. J., *Endocrinology*, 1994, **141**, 153–162.
7. Venkatesh, N., Nagaraja, G. and Murthy, G. S., *Curr. Sci.*, 1995, **69**, 48–56.
8. Murthy, G. S. and Venkatesh, N., *Curr. Sci.*, 1995, **70**, 55–62.
9. Murthy, G. S., Lakshmi, B. S. and Moudgal, N. R., *J. Biosci.*, 1989, **14**, 9–20.
10. Birken, S., Kolks, M. A. G., Amr, S., Nisula, B. and Puett, D., *Endocrinology*, 1987, **121**, 657–666.
11. Weiner, R. S., Dias, J. A. and Anderson, T. T., *Endocrinology*, 1991, **128**, 1485–1495.
12. Lindau-Sheppard, B., Roth, K. E. and Dias, J. A., *Endocrinology*, 1994, **135**, 1235–1240.
13. Santa Coloma, T. A. and Reichert, L. E., *J. Biol. Chem.*, 1990, **265**, 5037–5042.
14. Venkatesh, N., Nagaraja, N. and Murthy, G. S., (manuscript in preparation).
15. Moudgal, N. R., Ravindranath, N., Murthy, G. S., Dighe, R. R. and Aravindan, G. R., in *Perspectives in Primate Reproductive Biology* (eds. Moudgal, N. R., Yoshinaga, K., Rao, A. J. and Adiga, P. R.), Wiley Eastern Ltd., New Delhi, 1991, pp. 297–306.
16. Moudgal, N. R., Ravindranath, N., Murthy, G. S., Dighe, R. R., Aravindan, G. R. and Martin, F., *J. Repro. Fertil.*, 1992, **96**, 91–102.

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Combined effect of cisplatin and α-tocopherol on mice bearing Dalton's lymphoma

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Inhibitory effect of cisplatin and α-tocopherol on the growth of murine Dalton's lymphoma was studied *in vivo*. Subtherapeutical dose (3 mg/kg) of cisplatin was combined with different concentrations of α-tocopherol in order to increase the therapeutical benefit of this antitumour compound. This combination therapy not only increased the survival time of tumour-bearing mice but also resulted in tumour-free survivors. Such a treatment of Dalton's lymphoma is safer compared to cisplatin alone at higher doses due to its dose-related nephrotoxicity.

CISPLATIN is effective in the treatment of a number of tumours if used at a therapeutical dose[1–4]. Despite its antitumour effects, nephrotoxicity and other side effects have been reported, limiting cisplatin's long term administration to experimental animals or humans[5–8]. Cisplatin-induced renal damage has been reported to be induced by oxygen-free radical reaction[9]. The cells can be protected against free radical mediated damage by antioxidants like ascorbic acid, vitamin E, glutathione and enzymes like superoxide dismutase, catalase and peroxidases. Sometimes ascorbic acid and α-tocopherol as well as ascorbic acid and glutathione function as partners in defence[10–14]. Besides antioxidant property, ascorbic acid and α-tocopherol have been reported to be protective against cancer[15–17]. It has been reported that incubations of tumour cells with sodium selenite or α-tocopherol succinate for 24 h prior to exposure to X-rays or chemical carcinogens resulted in an inhibition of transformation by each of the antioxidants[18]. Additive or synergistic effect or α-tocopherol with several antitumour agents has been reported on the growth inhibition of murine neuroblastoma cells[19,20]. In the present investigation we studied the combined effect of subtherapeutical dose of cisplatin with different concentrations of α-tocopherol on Dalton's lymphoma bearing mice to observe whether α-tocopherol can enhance the antitumour effect and tumour growth inhibition induced by cisplatin.

Transplantable Dalton's lymphoma in C3H/He mice was used in all experimental groups. Tumour (2×10^7 cells/mice) was transplanted i.p. on day zero. On day 6 post-transplantation, mice bearing palpable tumour received a single i.p. injection of 3 mg/kg cisplatin alone or in combination with two repeated injections either 20, 40 or 60 mg/kg α-tocopherol on alternate days. Control (without any treatment) was run for each group. High dose of α-tocopherol (60 mg/kg) was administered