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USE OF CELLULOSE CARBONATE FOR THE PREPARATION OF IMMUNOSORBENTS: THE RADIOIMMUNOASSAY OF FOLLICLE-STIMULATING HORMONE

J. F. KENNEDY AND H. CHO TUN

Department of Chemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT (Great Britain)

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ABSTRACT

The use of cellulose 2,3-carbonate as a matrix for the insolubilisation of biologically active molecules has been extended to the preparation of insoluble antigen and antibody (immunosorbents). Both human pituitary follicle-stimulating hormone (FSH) and its homologous antibody have been covalently attached to cellulose carbonate by the nucleophilic attack of their primary amino groups on the cyclic carbonate groups. Antibody to FSH retains its immunological reactivity on insolubilisation, and is therefore suitable for use in the solid phase radioimmunoassay of unknown amounts of FSH by competitive binding of radioactively labelled and unlabelled FSH. Acceptable inhibition curves can be obtained, and the low, non-specific adsorption characteristics have advantages over other systems. FSH also retains immunological reactivity on insolubilisation, and the derivative holds potential for the radioimmunoassay of FSH as it can be layered immunologically with anti-FSH and then FSH itself.

INTRODUCTION

Many macromolecules can be specifically determined by utilising their reactions as antigens with homologous antibodies, coupled with radioactive labelling. The basic principle of the radioimmunoassay[1] therefore involves binding of a known amount of the labelled antigen to antibody, and the competitive inhibition of this reaction by the unknown amount of unlabelled antigen. The radioimmunoassay procedure must therefore be designed so as to separate, at some stage, the antigen bound to antibody from free antigen so that either can be determined.

Since the determination of the macromolecule usually has to be performed on a small scale, it is impractical to attempt a physical separation of the antigen–antibody complex. It has therefore been found useful to insolubilise the antibody, thereby permitting a simple phase separation of the free and bound antigen. Polymers that have been employed in this respect may be classified according to whether they bind the antibody physically or covalently. Examples of the former type are plastics[2] and bentonite[3], which have been used for the radioimmunoassay of follicle-stimulating

hormone and growth hormone, respectively. However, to avoid possible loss of bound material from the immunosorbent in response, for example, to change of the ionic environment, covalent insolubilisation is often to be preferred. Polysaccharides have been used extensively for covalent insolubilisation of antibody to provide the immunosorbent[4], and Wide and Porath[5] have investigated the coupling of antibodies to Sephadex cyclic imidocarbonate for the radioimmunoassay of several glycoprotein hormones.

The strained carbonate rings of cellulose 2,3-carbonate[6] undergo nucleophilic attack by compounds containing mercapto and amino groups, with concomitant attachment of the nucleophilic species[7]. These reactions of cellulose carbonate have been successfully extended to the insolubilisation of an enzyme with retention of activity[8]. The utility of cellulose carbonate as a matrix for immunosorbent preparation and its application in the radioimmunoassay of human pituitary, follicle-stimulating hormone (FSH), the fertility glycoprotein, is now reported.

EXPERIMENTAL AND RESULTS

Materials. — Human pituitary FSH type CPI (700 i.u./mg) was prepared as described previously[9], and FSH type CPD150 (7,000 i.u./mg) was prepared from this material[10]. FSH-CPD150 was labelled with ^{125}I-iodine in a way analogous to that described for human serum albumin[7], giving ^{125}I-FSH-CPD150.

Rabbit anti-human FSH (500 μl) was partially purified by precipitation[11] of the antiserum with 18% aqueous sodium sulphate for 1 h at 20°. The precipitated material was re-dissolved in 0.1M sodium hydrogen carbonate (500 μl) to give a concentration equivalent to that in the original serum. This antibody preparation was shown by the bentonite solidphase radioimmunoassay[3] to cross-react with human pituitary luteinizing hormone (LH), human chorionic gonadotrophin (HCG), and human pituitary thyroid-stimulating hormone (TSH) to minor extents, values of 5.0, 0.29, and 0.58% being obtained when the 2nd IRP-HMG was used as the standard for FSH. No cross-reaction was observed when high TSH serum was employed.

Cellulose 2,3-carbonate was prepared by the method of Barker *et al.*[6], using the standard reaction conditions and a reaction time of 10 min.

Insolubilisation of anti-FSH by reaction with cellulose carbonate. — Samples of cellulose carbonate (9 × 50 mg) were suspended in 50mM sodium phosphate buffer (pH 7.4; 400 μl), and anti-FSH (diluted with the same buffer; 100 μl) was added. The mixtures were stirred at 20° for various times, after which they were centrifuged, and the solid samples were washed by stirring and centrifuging with 0.25M sodium phosphate buffer (pH 7.4; 5 × 5 ml), 0.1M sodium acetate buffer (pH 4.0; 5 × 5 ml), and 50mM sodium phosphate buffer (pH 7.4; 3 × 5 ml). Before the last wash was removed, the samples were reduced to finer particles by using a tissue homogeniser, and centrifuged, and the supernatant liquors were rejected. To each of the damp samples and cellulose carbonate (50 mg), a 1% solution of human serum albumin in 50mM sodium phosphate buffer (pH 7.4; 1 ml) was added, followed by stirring for 24 h at

20°. The solids were then washed as above, and phosphate buffer was added to give a 1% suspension.

Investigation of antibody reactivity of insolubilised anti-FSH. — Suspensions of the insolubilised anti-FSH and cellulose carbonate–human serum albumin control (4–60 μl) were mixed in disposable plastic tubes with 0.2% human serum albumin in 50mM sodium phosphate buffer (pH 7.4) (HSA, 200 μl) and ^{125}I-FSH-CPD150 solution which had been diluted with HSA to give a count rate of $\sim 1.0 \times 10^4$ counts/min (200 μl). A blank, in which suspension was replaced with buffer, was also prepared. The tubes were counted for radioactive content by using a Panax automatic gamma counter, then incubated for 6 h at 20° and centrifuged. After removal of the supernatant liquors, the tubes were re-counted, and the percentages of ^{125}I-FSH taken up by the immunosorbent were calculated (Table I).

TABLE I

ANTIBODY REACTIVITY TO FSH OF ANTI-FSH INSOLUBILISED ON CELLULOSE CARBONATE

Conditions of insolubilisation[a]		*Uptake (%) of ^{125}I-FSH by various amounts of insolubilised anti-FSH suspension (μl)*					
Dilution of cellulose carbonate–anti-FSH	*Coupling time (h)*	*60*	*30*	*15*	*4*	*2*	*1*
1/10	3	21.4	14.1	10.6	6.8	5.1	5.5
1/10	6 A	29.2	18.2	11.9	8.0	7.0	7.7
1/10	24 B	27.7	17.3	10.2	7.6	6.2	3.5
1/100	3	7.1	4.8	5.8	4.8	4.3	5.8
1/100	6	8.2	6.0	4.9	4.5	4.2	4.4
1/100	24	8.3	6.1	5.0	4.8	4.6	4.7
1/500	3	5.9	5.5	5.3	4.9	4.4	4.0
1/500	6	5.5	4.9	4.5	4.5	4.4	4.6
1/500	24	6.0	5.5	4.8	4.5	5.3	4.5
Control (cellulose carbonate–albumin)	24	5.0	—	4.8	—	4.5	—
Blank	24	4.8[b]	—	—	—	—	—

[a]Capital letters denote particular types of anti-FSH referred to subsequently. [b]Buffer used in place of suspension.

Investigation of the effect of calf serum on the reaction of insolubilised anti-FSH with FSH. — Suspensions of insolubilised anti-FSH (Type B, 10×50 μl) were added to HSA (200 μl). In the mixtures, solutions of FSH-CPI in HSA (containing $0–100 \times 10^{-3}$ i.u. of FSH/ml, 200 μl) and ^{125}I-FSH-CPD150 (200 μl) were added, followed by counting, and incubation for 6 h at 20°. A second set of tubes, in which the first HSA was replaced by calf serum (200 μl), was similarly prepared. The percentage uptake of ^{125}I-FSH by the immunosorbents (Table II) was estimated as described in the previous section.

Investigation of the dependence upon time of the reaction of insolubilised anti-FSH with FSH. — Suspensions of insolubilised anti-FSH (Type A, 18×30 μl) were mixed

TABLE II

EFFECT OF CALF SERUM ON THE ANTIBODY REACTIVITY TO FSH OF ANTI-FSH INSOLUBILISED ON CELLULOSE CARBONATE

Samples	*Uptake (%) of ^{125}I-FSH by insolubilised anti-FSH in the presence of various amounts of FSH (i.u. × 10^{-3})*					
	20.2	*5.05*	*1.26*	*0.32*	*0.00*	*λ[a]*
Calf serum absent						
Cellulose carbonate–anti FSH	6.5	8.0	11.9	13.0	16.8	0.116
	6.0	9.1	11.5	13.6	18.5	
Control (cellulose carbonate–albumin)	6.8	—	—	—	4.9	
	4.8	—	—	—	4.9	
Blank	3.6	—	—	—	—	
	3.8	—	—	—	—	
Calf serum present						
Cellulose carbonate–anti FSH	5.7	9.0	13.1	13.6	16.2	0.051
	5.9	9.0	12.4	12.5	17.4	
Control (cellulose carbonate–albumin)	4.3	—	—	—	—	
	6.0	—	—	—	—	
Blank	3.2	—	—	—	—	
	3.3	—	—	—	—	

[a]Index of precision

with calf serum (200 μl), HSA (200 μl), and ^{125}I-FSH-CPD150 (200 μl), counted, and incubated for various times at 20°, after which the percentage of ^{125}I-FSH taken up by the immunosorbent (Table III) was estimated as described above.

TABLE III

DEPENDENCE UPON TIME OF THE REACTION WITH FSH OF ANTI-FSH INSOLUBILISED ON CELLULOSE CARBONATE

Samples	*Incubation time (h)*	*Uptake of ^{125}I-FSH (%)*
Cellulose carbonate–anti FSH	3	13.7, 15.3, 12.2
Cellulose carbonate–anti FSH	6	17.5, 17.7, 16.7
Cellulose carbonate–anti FSH	9	23.5, 21.4, 19.9
Cellulose carbonate–anti FSH	24	25.9, 26.1, 26.9
Cellulose carbonate–anti FSH	48	34.8, 32.3, 30.6
Cellulose carbonate–anti FSH	72	34.8, 35.5, 34.9
Control (cellulose carbonate–albumin)	24	4.9, 4.9
Blank	72	4.2, 3.9

Investigation of the reaction of insolubilised anti-FSH with FSH using different routines. — (*a*) Suspensions of insolubilised anti-FSH (Type A, 10 × 50 μl) were mixed with calf serum (200 μl), solutions of FSH-CPI in HSA (containing 0–100 × 10^{-3} i.u. of FSH/ml, 200 μl), and ^{125}I-FSH-CPD150 (200 μl). The percentages of ^{125}I-FSH

taken up by the immunosorbents were estimated as described above, using an incubation time of 24 h; Fig. 1*i*: index of precision $\lambda = 0.036$.

(*b*) Suspensions of insolubilised anti-FSH were treated as in (*a*), but without addition of ^{125}I-FSH, and incubated for 24 h at 20°. ^{125}I-FSH-CPD150 (200 µl) was then added, and the percentages of ^{125}I-FSH taken up were estimated as described above, using an incubation time of 6 h; Fig. 1*ii*: index of precision $\lambda = 0.038$.

(*c*) Suspensions of insolubilised anti-FSH were treated as in (*a*), but without addition of ^{125}I-FSH, and incubated for 24 h at 20°. The suspensions were centrifuged, the supernatants were removed, ^{125}I-FSH-CPD150 (200 µl) was added, and ^{125}I-FSH uptake was determined as in (*b*); Fig. 1*iii*: index of precision $\lambda = 0.256$.

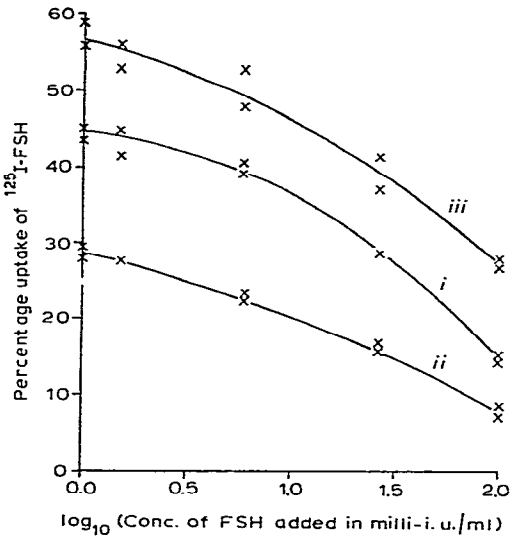

Fig. 1. Inhibition curves for the radioimmunoassay of FSH, using anti-FSH insolubilised on cellulose carbonate and three incubation routines (See Experimental). Points representing $-\infty$ on the log scale (zero addition of FSH) are plotted at 0.0 on the log scale for the sake of convenience.

Application of insolubilised anti-FSH to the determination of FSH in clinical sera. — Suspensions of insolubilised anti-FSH (Type A, 10×50 µl) were added to serum specimens, diluted with 50mM sodium phosphate buffer (pH 7.4; 200 µl), HSA (200 µl), and ^{125}I-FSH-CPD150 (200 µl). Estimation of the percentages of ^{125}I-FSH taken up was effected as described above, using an incubation time of 24 h; this gave an inhibition curve similar to Fig. 1*i*. Using a calibration curve constructed as in (*a*) in the preceding experiment, two sera were estimated to contain 8 and 50×10^{-3} i.u. of FSH/ml, respectively. It was shown that a recovery value of 85% could be obtained when small amounts of known quantities of FSH-CPI had been added to the sera. Comparable results were obtained by using a double antibody technique.

Insolubilisation of FSH by reaction with cellulose carbonate, and investigation

of its retention of antigenicity. — Cellulose carbonate (50 mg) was stirred with a solution of FSH-CPI (20 mg) in 50mM sodium phosphate buffer (pH 7.4; 500 µl), or FSH-CPD150 in a mixture of 0.1M ammonium acetate (600 µl) and 0.25M sodium phosphate buffer (pH 7.4; 150 µl) for 24 h at 20°. After centrifugation, the solids were washed with a mixture of equal volumes of 0.2M sodium phosphate buffer (pH 7.4) and 0.9% aqueous sodium chloride (15 × 5 ml), and then with 50mM sodium phosphate buffer (7.4; 3 × 5 ml). To the centrifuged solids and a control sample of cellulose carbonate, a solution of human serum albumin (20 mg) in 50mM phosphate buffer (pH 7.4; 500 µl) was added and the suspensions were stirred for 24 h at 20°. The solids were centrifuged off and washed as described above.

The damp solids were then suspended in a solution of anti-FSH (1/100 dilution) in 50mM phosphate buffer (pH 7.4; 500 µl), stirred again for 24 h at 20°, washed as described above, and re-suspended in buffer (5 ml).

The ability of these cellulose carbonate–FSH–anti-FSH samples to react with FSH was assessed (Table IV) as described for cellulose carbonate–anti-FSH. Inhibition curves were obtained as described previously and were analogous to those shown in Fig. 1.

TABLE IV

ANTIBODY REACTIVITY TO FSH OF ANTI-FSH INSOLUBILISED ON CELLULOSE CARBONATE–FSH

Sample	*Uptake (%) of ^{125}I-FSH by various amounts of insolubilised anti-FSH suspension (µl)*						
	60	*30*	*15*	*8*	*4*	*2*	λ^a
Cellulose-antigen (FSH-CPI)-antibody	34.0	29.0	20.3	11.4	11.5	7.2	0.119
	34.5	25.6	19.5	13.4	11.9	7.6	
Cellulose-antigen (FSH-CPD150)-antibody	33.6	31.6	25.2	19.1	15.4	11.9	0.252
	33.4	26.8	21.8	17.4	16.5	12.2	
Cellulose carbonate–albumin	13.5	—	—	—	—	—	
	13.8	—	—	—	—	—	
Blank	4.8	—	—	—	—	—	
	5.1	—	—	—	—	—	

[a]Index of precision.

DISCUSSION

In the field of insolubilisation of biologically active molecules, retention of activity on insolubilisation will never be a certainty until the primary structure and active site of the molecule are known, thereby enabling tailoring of the coupling to the reactive matrix such that the active site is not disturbed. However, the reaction of anti-FSH with cellulose carbonate occurs in such a way that immunological activity is retained to a certain extent, as judged by the ability of the insolubilised antibody to react with homologous antigen (Table I). All insolubilised anti-FSH samples were treated with human serum albumin before use, in order to saturate any unfilled

combining sites on the matrix and to minimise any subsequent non-specific adsorption, for example, by physical association with the matrix. The presence of albumin was also necessary to avoid significant, non-specific uptake of the labelled FSH by the tube walls. As can be seen from these results, whilst insolubilised anti-FSH prepared from highly diluted antibody was not significantly active, the use of higher concentrations of antibody protein for the coupling yielded products having appreciable immunological activity. This dependence of the success of the coupling reaction upon protein concentration has also been shown for the coupling of enzymes to cellulose carbonate[12]. Variation of the time of the coupling reaction showed that the maximal manifestation of antibody reactivity had been achieved after 6 hours. Whether or not further binding of protein occurred after 6 hours is uncertain, but it is clear from the results that the immunological activity was not enhanced. This may be due to overcrowding of the immunological sites, and the apparent slight drop in activity after coupling for more than 6 hours may be interpreted in this way.

Results for the control samples of albumin alone coupled to cellulose carbonate showed that the net, non-specific uptake of labelled FSH added was very low (0.2–2%; Tables I–III). This confirmed that the reaction between insolubilised anti-FSH and FSH was indeed an immunological one. Furthermore, the low, non-specific uptake is an advantage from the point of view of use of cellulose carbonate as the matrix for solid-phase radio-immunoassay over other matrices, *e.g.*, bentonite, where higher, non-specific uptakes (8% of labelled FSH added) are experienced[3,13].

Also, with a view to using anti-FSH insolubilised on cellulose carbonate as an immunosorbent, it was important to ascertain if the presence of any of the constituent molecules of serum had any effect upon the insoluble antibody–antigen reaction. Investigation of the effect of calf serum, which is not expected to contain any molecule resembling human FSH, demonstrated (Table II) that serum molecules in general do not interfere in the antibody–antigen reaction. Variation of the time allowed for the insoluble antibody to bind FSH (Table III) showed that the binding did not reach a maximum until after 48 hours. However, in spite of the lower amount of FSH bound, a reaction time of 24 hours was selected because of greater convenience and reduced risk of damage to the reactants and product. Furthermore, it is noteworthy that the sensitivity of the system is not necessarily decreased by a lower ability to bind FSH.

Using three different incubation routines, it was shown that inhibition curves for the competition of labelled and unlabelled FSH for insolubilised antibody could be constructed (Fig. 1), and therefore that insolubilisation of anti-FSH by reaction with cellulose carbonate can provide an immunosorbent for the solid-phase radio-immunoassay of FSH. Although different levels of uptake were experienced for the different routines (Figs. 1*i*, *ii*, and *iii*), it can be seen that their sensitivities were not vastly different; the differences in the percentage uptake of ^{125}I-FSH for minimal and maximal amounts of FSH present were 28, 21, and 29 for routines (*a*), (*b*), and (*c*), respectively. Thus, for use in a radioimmunoassay procedure, routine (*a*) was chosen because of its greater simplicity and possible greater accuracy than (*c*) (see later). By combining (*a*) with the optimal method of producing insolubilised anti-FSH (1/10

dilution of purified antiserum, 6-h coupling), a basis for the procedure was established. As a test of its validity, the system was applied to the measurement of unknown levels of FSH in human serum, and the results obtained are comparable to those obtained by the double antibody technique. Although the upper parts of the inhibition curves exhibit low sensitivity to changes in FSH concentration, this is not a very serious drawback as it was shown that low levels of FSH could be satisfactorily augmented to more sensitive parts of the scale. Thus, the insolubilised anti-FSH was suitable for the assay of FSH concentrations of 6×10^{-3} i.u./ml or more (equivalent to 1.2×10^{-3} i.u. of FSH; this corresponds to 0.78 on the log scale in Fig. 1), which represents approximately 100 pg of purified FSH.

An estimate of the degree of precision of the assay may be obtained by calculating the index of precision (λ) due to Gaddum[14]. For the results in Table II (presence and absence of calf serum), λ was calculated for the range where response to log(dose of FSH) was approximately linear and was assigned values of 0.116 and 0.051, respectively. For the standard curves shown in Fig. 1, the logistic transformation was applied to points on the more sensitive parts of the curve, in order to obtain linearity for calculation of λ, which was assigned values of 0.035 (*i*), 0.038 (*ii*), and 0.256 (*iii*). The indices of precision are, of course, subject to experimental error, especially due to the small number of observations used. Whilst this may explain the large value for curve *iii* (Fig. 1), compared with curves *i* and *ii*, the discrepancy may also be attributable to the technique used to obtain curve *iii* being less satisfactory. Values obtained for λ for the solid-phase radioimmunoassay of FSH with betonite[3] lay within the range 0.0777–0.242 (average $\lambda = 0.156$).

The attempts to attach FSH to cellulose carbonate, with retention of immunological reactivity, were also successful. Clearly, the demonstration of this retention (Table IV) involved a more complex procedure—it is not possible to make a test using labelled antiserum since pure anti-FSH, rather than anti-FSH serum, is not available. However, the results further indicate that an insolubilised anti-FSH can also be obtained by layering of the antibody on to the insolubilised antigen. Typical inhibition curves could be obtained for this insolubilised anti-FSH, but with low sensitivity (6 and 4% for FSH-CPI and FSH-CPD150, respectively), which may be due to an initially low binding of FSH or to a low availability of antibody sites. Values for the index of precision were calculated for the range where response to log(dose of insolubilised anti-FSH) was approximately linear, and λ was assigned values of 0.119 and 0.252, such values being subject to the factors already discussed.

In conclusion, it may be stated that cellulose carbonate is suitable for the insolubilisation of both an antigen and its antibody, with retention of immunological activity. Since amino groups are considered to be those groups on the polypeptide chains of the two glycoproteins that react with the cyclic carbonate groups, it appears that at least some amino groups are unimportant for manifestation of immunological activity. For FSH, this is in agreement with the finding that, although *in vivo* biological activity of FSH is destroyed by specific derivatisation of free amino groups, such

derivatisation only partially abolished immunological activity[15]. From this work, it may also be concluded that cellulose carbonate holds potential for use as the matrix in insolubilisation of antigens and antibodies for immunoadsorbent techniques, including radioimmunoassay and purification.

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