How large a hormone can be measured by microencapsulated antibody?

F. A. Duffy and A. M. Wallace
Department of Clinical Biochemistry, Royal Infirmary, Castle Street, Glasgow G4 0SF
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ABSTRACT

The size of molecules able to be measured in immunoassays where antibody is encapsulated within semipermeable microcapsules is restricted by the pore size of the membrane. This study was performed to determine the approximate molecular weight cut-off of this membrane.

Permeability was assessed by measuring which labelled hormones were able to enter and bind their respective microencapsulated antibody. Hormones with molecular weights of less than 4000 (angiotensin II, thyroxine, 17-hydroxyprogesterone, progesterone, testosterone and androstenedione) passed freely through the pores but larger molecules, with molecular weights in excess of 10 000 (parathyroid hormone, human GH, TSH) could not. Insulin, with a molecular weight of 6000 (approximate minimum diameter 3-5 nm), had restricted entry while the next smallest hormone tested, the 1-34 amino acid portion of parathyroid hormone (molecular weight 4000; diameter 1-8 nm), was able to bind encapsulated antibody, suggesting that the pore diameter is between 1-8 and 3-5 nm. It can now be predicted that the method is able to measure compounds with a diameter within this range and with a molecular weight below 6000. Microcapsules may be useful for improving specificity of assays where a cross-reactant is too large to penetrate the membrane.

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INTRODUCTION

In a recent study, Wallace, Beastall, Cook et al. (1986) successfully used microencapsulated antibody for an extremely robust, large-scale screening assay for the measurement of 17-hydroxyprogesterone in babies. The technical simplicity of the procedure was of great advantage and allowed a large number (120 000) of samples to be screened. Antibodies are physically retained within the microcapsules because they are too large to escape through the pores in the semi-permeable membrane. Antibody-containing microcapsules, when used for radioimmunoassay (RIA), are light enough to remain suspended during the incubation stage yet are easily sedimented by centrifugation for separation of the antibody-bound fraction. The technique is useful for the measurement of compounds small enough to penetrate the membrane pores and interact with the entrapped antibody.

The potential of microencapsulated antibody in immunoassay was first recognized and explored by Halpern & Bordens (1979a), who reported a method using microcapsules for the measurement of digoxin. Assays soon followed for free thyroxine (Ashkar, Buehler, Chan & Hourani, 1979; Halpern & Bordens, 1979b), cortisol (Bordens & Halpern, 1980) and testosterone (Chan, Provost, Stella & Buehler, 1980). More recently, we have developed a procedure for antibody microencapsulation based on interfacial polymerization (Wallace & Wood, 1984) and reagents prepared in this way have been used for the measurement of both steroid and thyroid hormones (Duffy & Wallace, 1986; Wallace et al. 1986).

To date, radioimmunoassays using microencapsulated antibodies have been applied only to the measurement of antigens with a molecular weight below 800. The current study was undertaken to gain information on the size limits of molecules that can enter the capsule through the ultrathin nylon membrane and bind to the antibody. Establishment of the upper limit of molecular size for which this technique can be used will facilitate further development of similar RIA systems. A preliminary report of some of these results has been given previously (Duffy, Logue & Wallace, 1986).
MATERIALS AND METHODS

All the reagents required for the preparation of the microcapsules have been detailed previously (Wallace & Wood, 1984). With the exceptions noted below, antisera were raised within this Department or obtained from the Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarkshire. Angiotensin II antiserum was a gift from Dr B. Williams, Department of Medicine, Western General Infirmary, Edinburgh, Lothian and testosterone antiserum was a gift from Dr R. Webb, AFRC Animal Breeding Research Organisation, Roslin, Midlothian. All other chemicals were general laboratory chemicals of analytical grade obtained from either Sigma Chemical Co., Poole, Dorset, or BDH, Poole, Dorset.

Preparation of microencapsulated antibodies

The procedure for microencapsulating antibodies within semipermeable nylon membranes by interfacial polymerization has been detailed elsewhere (Wallace & Wood, 1984). All antibodies to the hormones listed in Table 1 were encapsulated in this way. In brief, microencapsulation first required emulsification of an aqueous solution of antibody and diamine in an organic solvent. Dic acid was then added dropwise, to produce an ultrathin nylon membrane by polymerization of diamine and dic acid at the interface of the aqueous droplets and organic solvent. The antibody-containing semipermeable microcapsules (mean diameter 25 µm) thus produced were transferred from the organic phase to an aqueous phase, thoroughly washed, and finally suspended and stored in 20 ml phosphate-buffered saline (0-1 mol/l, 0-9% (w/v) NaCl, pH 7-4) containing 0-02% (w/v) sodium azide as preservative, at 4°C. In addition, microcapsules were prepared for the determination of non-specific binding and these contained only normal rabbit serum with no specific antibody.

Radiolabelled hormone tracers

All hormone tracers used in this study were labelled with 125I by conventional procedures for the iodination of steroid (Cook & Beastall, 1987) and protein (Edwards, Laloz & Pull, 1983) hormones. The radiolabelled hormones were diluted with phosphate-buffered saline to give approximately 10 000 c.p.m. per 200 µl and used for the determination of antibody binding.

Determination of antibody binding

Each standard antibody-containing microcapsule preparation was serially diluted 1:5, 1:10, 1:20, 1:40, 1:80, 1:160 and 1:320 with buffer (0-1 mol phosphate buffer/l, pH 7-4), containing 0-9% (w/v) NaCl and 0-25% (w/v) bovine serum albumin or for parathyroid hormone (PTH) only, 0-03 mol barbitone buffer/l, pH 8-6) and duplicate 200 µl aliquots of each dilution were incubated for 45 min at 37°C with 200 µl (10 000 c.p.m.) of the respective 125I-labelled hormone. Unbound radiolabelled antigen was removed by washing which involved addition of 0-9% (w/v) saline containing 0-2% (v/v) polyoxyethylene sorbitan monolaurate (Tween 20), centrifugation (1000 g, 5 min, 4°C) and aspiration of the supernatant. Non-specific binding was determined by also including in the assays appropriate dilutions of microcapsules containing only normal rabbit serum. If no binding was detected, the incubation time was extended to 18 h at 4°C. This precaution was taken to demonstrate that lack of labelled-hormone binding was due to inability to penetrate the capsule membrane rather than a slow rate of entry. If binding remained undetectable after the prolonged incubation time, as occurred with labelled insulin, PTH, thyrotrophin and human growth hormone (hGH), the capsules were sonicated (MSE Ultrasonicator) causing complete disintegration of the ultrathin nylon membrane and release of the 'captured' antibody. All sonicated preparations were examined visually under a light microscope to confirm complete membrane disruption. Antibody binding was subsequently tested, but it was now necessary to employ a second antibody to separate the antibody bound from the free fraction.

With the exception of the insulin antibody, which was raised in a guinea-pig, the antisera were raised in sheep. Consequently, anti-species antisera, either donkey anti-sheep globulin or sheep anti-guinea-pig globulin, linked to Sepharose (Cook & Beastall, 1987) were used. All second antibody separations were carried out in a similar manner. Following incubation (45 min at 37°C or 18 h at 4°C) of radiolabelled hormone with the released antibody, 100 µl of appropriately diluted anti-species antibody linked to Sepharose was added to the tubes and further incubated at room temperature with continuous shaking for 60 min. Addition of 4 ml 0-9% (w/v) NaCl to each tube preceded centrifugation (1500 g, 15 min, 4°C) and aspiration of the supernatant. The washing process was repeated before determination of the bound fraction.

RESULTS

The ability of radiolabelled hormones to enter microcapsules and bind their respective antisera is documented in Table 1. A range of antibodies was selected for encapsulation on the basis of the molecular weight of the hormone against which the antibody was directed (molecular weight range from 286
to 28,000). Separate preparations of microcapsules were made for each antibody. Binding occurred if the molecular weight was less than 6000 and was inhibited if it was more than 10,000. For insulin, molecular weight 6000, the binding achieved at all microcapsule dilutions was greatly reduced when compared with what was obtained if the microcapsules were disrupted and the antibody binding measured using a second antibody. At a dilution of 1:5, binding fell from 8-5% bound/total (B/T) to undetectable beyond a dilution of 1:80 for the intact insulin antibody-containing microcapsules. The same antibody, when released from the microcapsules by disrupting the nylon membrane, produced binding which decreased, in a uniform manner from 57-3% B/T at a 1:5 dilution to 2-7% B/T at a 1:320 dilution.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Molecular weight</th>
<th>Measured binding* with intact capsules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Androstenedione†</td>
<td>286</td>
<td>+</td>
</tr>
<tr>
<td>Testosterone†</td>
<td>288</td>
<td>+</td>
</tr>
<tr>
<td>Progesterone†</td>
<td>314</td>
<td>+</td>
</tr>
<tr>
<td>17-Hydroxyprogesterone†</td>
<td>330</td>
<td>+</td>
</tr>
<tr>
<td>Thyroxine</td>
<td>777</td>
<td>+</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>1000</td>
<td>+</td>
</tr>
<tr>
<td>Parathyroid hormone fragment</td>
<td>4000</td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>6000</td>
<td>+/-</td>
</tr>
<tr>
<td>Parathyroid hormone</td>
<td>10,000</td>
<td>-</td>
</tr>
<tr>
<td>Human growth hormone</td>
<td>21,000</td>
<td>-</td>
</tr>
<tr>
<td>Thyrotrophin</td>
<td>28,000</td>
<td>-</td>
</tr>
</tbody>
</table>

*Radiolabelled hormone was incubated (37°C, 45 min) with intact capsules containing the appropriate antiserum. Unbound hormone was washed from the system by adding physiological saline, centrifuging (1050 g, 5 min, 4°C) and aspirating the supernatant. Two washes were carried out for each preparation.

†When radiolabelled with 125I by a 3-CMO-iodohistamine linkage, the molecular weight is increased by 290.

Binding of label for hormones with molecular weights greater than insulin was greatly depressed (<5% B/T) when compared with the binding achieved following disruption of the microcapsular membrane, as exemplified by hGH in Fig. 1.

**DISCUSSION**

Determination of the pore diameter of semipermeable microcapsules poses a number of technical problems. In elucidating the permeability characteristics of dialysis membrane tubing, access to both sides of the membrane is possible. Therefore, a range of standard molecules, such as dextrans of increasing molecular weight, can be tested for their ability to pass through the membrane, thus enabling the size of the pores to be determined. Unfortunately, if the membrane under study is spherical, free access to the interior is restricted. Our interest in the use of microencapsulated antibody in immunoassay and the local availability of a large number of antisera, led us to investigate the problem by encapsulating antibodies and determining whether the hormone against which they were raised was able to cross the membrane and be retained within the capsules by binding to the antibody. As far as we are aware, this is the first report describing estimates of microcapsule pore size by this means.

The results presented above suggest that molecules capable of freely penetrating the semipermeable nylon microcapsules have a molecular weight below 4000. This determination, however, does not take account of peculiarities in three-dimensional shape or electrical charge of the pores. Furthermore, the assumption is made that just as there is a range in the sizes of microcapsules produced within each batch (Wallace & Wood, 1984), so there is likely to be a range of pore sizes within batch. The range in size of microcapsules is the same within and between batches and it is probable that the pore size behaves similarly. We obtained no evidence for the binding of hormones varying between different preparations of microcapsules. Of all the hormones tested, insulin (molecular weight 6000) appeared to be closest to the pore size. As a result of X-ray crystallography, much is known about the molecular structure of insulin. It is probable that this hormone, a globular protein, is present in solution in its larger form as a hexamer, which produces an ellipsoid 5-0 nm in diameter and 3-5 nm in length, but smaller dimers and monomers might also be present (Blundell, Dobson, Dobson et al. 1971). This information suggests that the maximum pore diameter in the microcapsule membrane is below 3-5 nm since some insulin was able to bind with its microencapsulated antibody, but not to the extent achieved when the antibody was released from the microcapsules by sonication.

Parathyroid hormone, the next largest hormone examined, molecular weight 10,000, was unable to enter the microcapsules, although the 1-34 amino acid fragment of PTH entered freely. The exact three-dimensional shape of these molecules has not been determined by crystallographic analysis, but structural information has been inferred from hydrodynamic and dark-field electron microscopic studies (Cohn & Elting, 1983). It appears that PTH has two domains connected by a fairly rigid stalk, it migrates electrophoretically as an asymmetric rather than globular protein and has a Stokes radius of 2-0 nm although, from dark-field electron microscopy, the
maximum dimension is 3.6 nm (Fiskin, Cohn & Peterson, 1977). It would have been expected that a proportion of the PTH molecules would be in a favourable orientation to penetrate the microcapsular pores since PTH is very close in its longest dimension to insulin and is asymmetric rather than globular. The fact that radiolabelled PTH molecules were not detectable within capsules suggests that the pore diameters are much less than 3.5 nm. On the other hand, the 1-34 amino acid fragment freely entered the capsules, and studies with this synthetic peptide indicate that it contains the biologically active domain of PTH plus a piece of the stalk and is at least 1.5 nm long. It migrates electrophoretically as a globular peptide and has a Stokes radius of 0.9 nm (Fiskin et al. 1977). Therefore, it is likely that the upper limit of the pore size is greater than 1.8 nm.

Our findings are in general agreement with those of Chang (1972). He calculated the pore diameter of semipermeable nylon microcapsules to be approximately 1.8 nm by examining the permeability characteristics of various test solutions in the microcapsules. In hypertonic solutions, microcapsules collapse (crenate), but as equilibrium is reached between the internal hydrostatic pressure and the external osmotic pressure, the capsules regain their shape (Chang, 1964). Using a number of solutions, i.e. sucrose, dextrose, ethylene glycol and propylene glycol, at different concentrations, Chang (1964) observed the percentage of capsules crenated after a set time-period and calculated the equivalent pore diameter from these studies.

Knowledge of the size range of molecules able to penetrate the semipermeable membrane allows us to consider novel uses for microencapsulated antibody, a few of which are outlined below. Microencapsulated antibody has the potential for measuring hormone fragments in the presence of an interfering parent hormone. For example, β-endorphin (molecular weight 3500) is able to penetrate the microcapsule membrane whereas the larger parent hormone, β-lipotrophic hormone (molecular weight 10,000), should not. Microencapsulated antibody can also be used as a purification agent in, for example, removing hormone fragments which impede measurement of the parent molecule. This is illustrated by the binding

Figure 1. Antiserum dilution curves for human GH (hGH). Anti-human GH antiserum was microencapsulated and the resultant microcapsules serially diluted with assay buffer (0.1 mol phosphate buffer/l, pH 7.4, containing 0.25% (w/v) bovine serum albumin). Radiolabelled hGH was incubated at 37 °C for 45 min or at 21 °C for 18 h with the dilutions of microcapsules. Unbound hormone was washed out of the system (by adding physiological saline (4 ml), centrifuging at 1000 g for 5 min at 4 °C, aspirating and then repeating the process) ( ● — ● ). Portions of the original microcapsules were then sonicated (membrane disruption checked by light microscope), diluted and binding to antibody was measured using a second (anti-species) antibody-separation procedure ( ● — ● ). This involved a further incubation at room temperature for 60 min with continuous shaking after addition of the anti-species second antibody linked to Sepharose. Separation of the bound from the unbound fraction was carried out as above but with centrifugation for 15 min at 1500 g. Ab, antibody; Ag, antigen.
of the 1–34 amino acid fragment of PTH when the parent molecule was unable to bind (Table 1). These potential uses have yet to be fully explored. The immediate advantage of microencapsulation in these circumstances is that it would eliminate any need for partial purification of samples before analysis and effectively increase the specificity of the antibody in the RIA system.

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REFERENCES


