Means for the assessment of radioligand quality and its importance in receptor-binding studies

Observations with radiolabelled formylmethionyl-leucyl-phenylalanine

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Various methods for testing the quality of radioligands were applied to two different radiolabelled forms of formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe). The purpose of the study was both to examine the value of these methods for assessing radioligand quality and to determine the suitability of these particular radioligands for studying the chemotactic formylpeptide receptors on the rabbit neutrophil. It is useful in this context to distinguish two different aspects of radioligand quality: these are purity and equivalence to the native ligand. The two methods described for measuring receptor-reactivity (or 'bindability'), by measuring binding to an increasing excess of receptors and by a re-incubation procedure, provide a reliable measure of purity that should readily be applicable to other radioligands. Equivalence to the native ligand is more difficult to establish, and any uncertainty about the specific radioactivity of the radioligand can pose serious problems with this assessment. Commercial preparations of both tritiated and ^{35}S -labelled fMet-Leu-Phe were found to be inadequately pure for detailed receptor studies. Repurification by t.l.c., however, consistently yielded radioligand preparations of high purity and close equivalence to the native ligand. Other radioligands may often also require a suitable repurification step before use for detailed receptor studies; this is especially important whenever a complex receptor-binding pattern is envisaged.

INTRODUCTION

Most studies of the interaction between a biologically active ligand and its receptors employ a radiolabelled form of the ligand to trace the behaviour of the native ligand. It is usually an implicit assumption of such studies that the radiolabelled ligand will behave in the same manner as the native ligand [1–4]. Although the importance of radioligand quality is tacitly accepted by most investigators, only rarely is this fundamental assumption of equivalence in behaviour between radioligand and native ligand tested in a more than cursory manner. (In a survey of recent articles on receptor binding, only 29 out of 163 papers provided any indication of radioligand quality and in most cases this information was very incomplete.) Furthermore, when radioligand quality has been assessed in a reasonably adequate manner imperfections have often been revealed in many radioligands [3,5–17].

Studies by different investigators of the receptors for many substances [18,19], including chemotactic formylpeptides [20–24], have often yielded widely varying estimates of affinity and receptor number; it is not unusual, for example, for estimates of binding affinity for a particular ligand–receptor interaction to vary at least 10-fold. Many factors contribute to this variability; a major one is the use of radioligands of differing quality. Several other factors have been discussed in detail in a previous paper [24].

Several problems are encountered in assessing the suitability of a radioligand for receptor-binding study.

The first is deciding on exactly what is required of a quality radioligand. It is useful, in this context, to distinguish two quite separate aspects of quality: these are the purity of the radioligand, and its equivalence in receptor-binding activity to the native ligand. A pure radioligand, for example, may not be equivalent to the native ligand if the radionuclide introduced is a foreign atom whose presence changes the properties of the ligand, or if the labelling process itself causes some alteration of the ligand. Similarly, equivalence of the radioligand to the native ligand is not adequate proof of radioligand purity if the available native ligand is itself impure, as is often the case, for example, with complex polypeptide and glycoprotein hormones. Although both these aspects of radioligand quality are important if the binding properties of the radioligand are to provide a true indication of the behaviour of the native ligand, it is rare for investigators to make separate assessments of both aspects. (This was done in only five of the 163 papers in the above-mentioned survey.)

The present paper examines various experimental procedures for the assessment of both aspects of radioligand quality. The strengths and limitations of these procedures are illustrated by data from a thorough evaluation of the quality of two different forms of radiolabelled *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMet-Leu-Phe), one labelled with ^3H and the other with ^{35}S . The radioligands were evaluated in terms of their suitability for studying the interaction between fMet-Leu-Phe and the chemotactic formylpeptide receptors on the rabbit neutrophil.

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EXPERIMENTAL

Chemicals

Shellfish glycogen, crystalline bovine serum albumin, *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide and the chemotactic formylpeptide fMet-Leu-Phe were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.); the radiolabelled formylpeptides fMet-Leu-[^3H]Phe (1.8–2.2 Bq/fmol) and f[^{35}S]Met-Leu-Phe (7–31 Bq/fmol) were purchased from New England Nuclear (Boston, MA, U.S.A.); cytochalasin B was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.).

Rabbit neutrophils

Rabbit peritoneal neutrophils were collected 5–7 h after the injection of 300 ml of a sterile solution (1 g/l) of shellfish glycogen in 150 mM-NaCl, as previously described [24,25]. The neutrophils were washed twice and resuspended in Mg^{2+} -free Hanks balanced salt solution (137 mM-NaCl/5 mM-KCl/0.6 mM- Na_2HPO_4 /0.7 mM- KH_2PO_4 /15 mM- NaHCO_3) containing 1.7 mM- CaCl_2 , 1 g of D-glucose/l, 0.1 g of bovine serum albumin/l and 10 mM-Hepes, pH 7.2, for all studies.

Repurification of radiolabelled fMet-Leu-Phe

Except in the case of preliminary experiments to characterize the initial purity of the radioligands, each batch of radiolabelled fMet-Leu-Phe was repurified by t.l.c. on 10 cm $\times$ 20 cm high-performance silica-gel plates (Whatman, Clifton, NJ, U.S.A.). The developing solvent was butan-1-ol/acetic acid/water (4:1:1, by vol.) containing 0.2% (v/v) 2-mercaptoethanol. After development, the solvent was evaporated from the plate *in vacuo* and the location of authentic radiolabelled fMet-Leu-Phe (R_f 0.75) established by means of a t.l.c. scanner (Bioscan, Washington, DC, U.S.A.). The silica gel was scraped from this region of the plate, and the radiolabelled formylpeptide was extracted (for 30 min at -20°C) into 1 ml of methanol containing 0.01% 2-mercaptoethanol. The silica gel was removed by filtration and rinsed with a further 1 ml of methanol. The resulting repurified stock of radiolabelled fMet-Leu-Phe was concentrated by evaporation under N_2 and stored at -20°C in methanol containing 0.1% 2-mercaptoethanol. Repurification was repeated after storage for a period of 3 months.

In some studies the major impurity (R_f 0.48) found in radiolabelled fMet-Leu-Phe was separately extracted from the t.l.c. plate and similarly concentrated for further analysis.

Receptor-binding assay

The binding of radiolabelled fMet-Leu-Phe to the chemotactic formylpeptide receptors on intact rabbit neutrophils was assessed by the silicone oil assay previously described [24]. In brief, 50 μl of radiolabelled fMet-Leu-Phe solution was layered at 4°C over 500 μl of Versilube F50 silicone oil (General Electric, Silicone Products Division, Waterford, NY, U.S.A.) in a micro-centrifuge tube. The binding reaction was started by the addition of 50 μl of neutrophil suspension, and was stopped after 15 min at 4°C by centrifugation (13000 *g* for 2 min). The radionuclide content of the cell pellet was determined by scintillation counting [24]. Binding measurements were performed at least in duplicate. Non-specific binding was measured by parallel incubations in

the presence of 10 μM non-radioactive fMet-Leu-Phe. The specific binding percentage was calculated as $100 \times (B - N)/(T - N)$, where *B* represents the radioactivity (d.p.m.) bound to the experimental sample, *N* that non-specifically bound and *T* the total amount of radioactivity incubated [7,24,26].

In studies to characterize the competitive nature of the binding reaction the concentration of fMet-Leu-Phe (radioligand alone or radioligand together with non-radioactive ligand) was varied (from 0.1 nM to 0.1 μM) with a constant neutrophil concentration (2×10^7 cells/ml). The receptor-binding data from these studies were analysed by using a version of the LIGAND computer program [27,28] that had been adapted for use on the Apple II computer by Dr. M. H. Teicher (Harvard Medical School, Cambridge, MA, U.S.A.). This program was kindly supplied by the Biomedical Computing Technology Information Center (Vanderbilt Medical Center, Nashville, TN, U.S.A.).

Assay of granule-enzyme release from neutrophils

The stimulation by fMet-Leu-Phe of the release of *N*-acetyl- β -glucosaminidase (EC 3.2.1.30) from the granules of rabbit neutrophils was assayed by a modification of the procedures previously described [29,30]. Rabbit neutrophils (5×10^6 cells/ml final concentration; pre-warmed to 37°C) were incubated for 5 min at 37°C with fMet-Leu-Phe (3 pM to 0.1 μM ; measured in duplicate) in the presence of cytochalasin B (5 mg/l). Granule-enzyme release induced by radiolabelled fMet-Leu-Phe was compared directly with that induced by non-radioactive fMet-Leu-Phe, the use of closely spaced doses ensuring a precise measure of the relative potencies. The reaction volume was normally 1 ml, but was decreased to 0.5 ml for more economical use of the radioligand in the studies with f[^{35}S]Met-Leu-Phe. After incubation, the cells were removed by centrifugation (1000 *g* for 5 min at 4°C). The β -glucosaminidase content of each supernatant (100 μl portion) was then determined by incubation for 15 min at 37°C with the chromogenic substrate *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide (100 μl of 4 mM stock solution in 50 mM-sodium citrate buffer, pH 4.5), the reaction being stopped and the colour developed by addition of 200 μl of cold (4°C) 0.4 M-glycine buffer, pH 10.5. The absorbance at 410 nm of the product (250 μl portion) was determined by means of a micro-titre plate reader (model MR580; Dynatech, Cambridge, MA, U.S.A.). All results are expressed as a percentage of the total β -glucosaminidase content of the neutrophils, the latter being measured with cells lysed with 0.12% (v/v) Triton X-100.

The enzyme-release data were analysed by using the ENZREL computer program (copies of this program for the Apple II computer being available upon request). This program, which calculates percentage release values from the experimental data and fits sigmoidal dose-response curves, was adapted from the ALLFIT program of de Lean *et al.* [31].

RESULTS

Assessment of radioligand purity

Radiochemical analysis. The radiochemical purity of each batch of radiolabelled fMet-Leu-Phe was assessed by using the same t.l.c. system as for repurification (see the Experimental section). No detectable degradation of

Fig. 1. Assessment of receptor-reactivity

(a) Binding of radioligand (0.2 nM) was measured with increasing concentrations of receptors (up to 5×10^8 neutrophils/ml). The double-reciprocal plot allows extrapolation to infinite receptor concentration (zero value for reciprocal), yielding a receptor-reactivity estimate of 85%. (b) Binding to a high concentration of receptors (5×10^8 neutrophils/ml) was measured both for an initial incubation of the radioligand (B_1 ; left-hand column), and for a re-incubation of the radioligand remaining free after the initial incubation (B_2 ; right-hand column). This allows the initial binding data ($\square$) to be corrected to an infinite receptor concentration ($\square$), yielding a receptor-reactivity estimate of 87%. Both sets of results are from a typical study with $f[^{35}\text{S}]\text{Met-Leu-Phe}$. Data are means $\pm$ S.E.M for triplicate measurements.

$f\text{Met-Leu-Phe}$ occurred during this t.l.c. analysis: this was shown by two-dimensional chromatography, in which the authentic radiolabelled $f\text{Met-Leu-Phe}$ separated by development in the first dimension was seen to migrate as a single spot (containing $> 99\%$ of radioactivity, by t.l.c. scanning) when the plate was developed in the second dimension with the same solvent. When tested by t.l.c., both radioligands were invariably found to be impure on receipt from the manufacturer; tests were performed within 3 days of receipt, with the radioligand batch stored unopened at -20°C . Five different batches of $f\text{Met-Leu-}[^3\text{H}]\text{Phe}$ showed initial purities ranging from 25% to 87% (mean 61%), whereas seven batches of $f[^{35}\text{S}]\text{Met-Leu-Phe}$ gave values from 1% to 95% (mean 63%). Two separate lots of the latter radioligand were found to be comprised almost entirely ($> 95\%$) of a radiochemical that was not $f\text{Met-Leu-Phe}$; these were not used for further studies. Gradual deterioration of each radioligand occurred during storage, the half-time for decline of radiochemical purity being estimated to be about 1 year (with storage at -20°C in the original solvent).

Repurification of the radiolabelled $f\text{Met-Leu-Phe}$ by t.l.c. was effective at removing the major impurity. So long as the repurification process was repeated at 3-month intervals, a radioligand stock of consistently high purity could be maintained. Seventeen separate assessments of the repurified $f\text{Met-Leu-}[^3\text{H}]\text{Phe}$ (made at various times during the repurification interval) yielded a purity estimate of $92 \pm 5\%$ (mean $\pm$ S.D.); a comparable estimate of $91 \pm 4\%$ (seven assessments) was obtained with repurified $f[^{35}\text{S}]\text{Met-Leu-Phe}$. These t.l.c. studies also provided limited evidence of equivalence to authentic $f\text{Met-Leu-Phe}$ insofar as each radioligand co-migrated with the native compound.

The purity of the non-radioactive $f\text{Met-Leu-Phe}$ used for the radioreceptor assays and bioassays in the present study was also assessed by this t.l.c. system. Single spots were seen on chromatograms of 10–100 nmol of $f\text{Met-Leu-Phe}$ when sprayed with either a non-specific starch/iodide reagent [32] or a palladium chloride reagent specific for sulphur-containing compounds [33], implying that the purity was greater than 90%.

Analysis of receptor-reactivity. The receptor-reactivity ('bindability') of each repurified stock of radiolabelled $f\text{Met-Leu-Phe}$ was assessed by measuring its binding (at a radioligand concentration of 0.2 nM) to increasing concentrations (near excess) of rabbit neutrophils (from 6×10^7 to 5×10^8 cells/ml), with the use of the procedure previously described [24]. The principle of these measurements was to extrapolate the binding to a theoretical infinite excess of receptors, defining the extrapolated binding percentage as the receptor-reactivity [34]. Extrapolation was performed on a plot of the reciprocal of the binding percentage against the reciprocal of the neutrophil concentration, since the relationship between these two reciprocal quantities becomes virtually linear at high cell concentrations (Fig. 1a). Ten measurements made with repurified $f\text{Met-Leu-}[^3\text{H}]\text{Phe}$ gave a receptor-reactivity estimate of $84 \pm 5\%$ (mean $\pm$ S.D.), whereas six measurements made with $f[^{35}\text{S}]\text{Met-Leu-Phe}$ provided a value of $85 \pm 4\%$. These estimates were slightly, but significantly, lower than the estimates of radiochemical purity ($P < 0.001$ and $P < 0.05$ respectively, t test).

Receptor-reactivity was also measured for some preparations by an alternative approach (Fig. 1b). A tracer concentration of radiolabelled $f\text{Met-Leu-Phe}$ (0.2 nM final concentration) was incubated (in the normal manner) with a high concentration of rabbit neutrophils

Fig. 2. Equivalence in receptor-binding activity between radioligand and native ligand

A dose-response curve for competition of $f[^{35}\text{S}]$ Met-Leu-Phe binding to rabbit neutrophils (2×10^7 cells/ml) by non-radioactive fMet-Leu-Phe (○) was compared with a self-competition curve for fMet-Leu- $[^3\text{H}]$ Phe binding (●) to the same neutrophil preparation. The potency of fMet-Leu- $[^3\text{H}]$ Phe in this typical study is estimated to be 96% (relative to fMet-Leu-Phe). The line, however, shows the optimal fitted curve assuming equal potencies. The error bar on the lowest dose point represents the pooled S.E.M. for the complete set of duplicate measurements (by analysis of variance).

(5×10^8 cells/ml) and the specific binding percentage (B_1) was determined. The free radioligand was carefully recovered from the supernatants of these incubations and any traces of silicone oil were removed by re-centrifugation. This free radioligand stock was then further incubated with an equal concentration of neutrophils and its specific binding percentage (B_2) was determined. If it is assumed that the radiolabelled fMet-Leu-Phe does not suffer significant degradation during incubation with neutrophils at 4 °C, as evidenced by our previous studies [24], then any decrease in binding observed for the re-incubation period can be attributed to the presence of non-reactive radioligand that has effectively been concentrated in the free fraction. This allows the receptor-reactivity to be calculated (by solution of the appropriate pair of simultaneous equations) as $100 B_1^2 / (100 B_1 - 100 B_2 + B_1 B_2)$. Three measurements of the receptor-reactivity of repurified $f[^{35}\text{S}]$ Met-Leu-Phe made by this alternative approach yielded a value of $88 \pm 3\%$. This estimate did not differ significantly from the principal estimate of receptor-reactivity ($P > 0.2$, t test), the two approaches thus each tending to validate the other. Nor did the estimate differ significantly from the estimate of radiochemical purity ($P > 0.2$).

Assessment of equivalence of the radioligand to the native ligand

Radioreceptor assay. A common method used to test the equivalence of a radioligand to its non-radioactive counterpart, particularly in radioimmunoassays, is to compare the ability of the radioligand to compete with itself for binding (saturation binding curve) with the ability of the native ligand to competitively inhibit

binding of the radioligand [35]. This approach, however, was not directly practicable with either fMet-Leu- $[^3\text{H}]$ Phe (specific radioactivity too low to allow it to be used effectively at a tracer concentration) or $f[^{35}\text{S}]$ Met-Leu-Phe (inadequate quantities available). An indirect approach was thus adopted. First, equivalence between repurified $f[^{35}\text{S}]$ Met-Leu-Phe and repurified fMet-Leu- $[^3\text{H}]$ Phe was established by direct comparison of their binding at a near-tracer concentration (0.2 nM): in six studies binding of the $f[^{35}\text{S}]$ Met-Leu-Phe averaged $103 \pm 11\%$ (mean $\pm$ S.D.) of that of the tritiated ligand. Secondly, dose-response curves for competition of $f[^{35}\text{S}]$ Met-Leu-Phe binding by non-radioactive fMet-Leu-Phe were compared with self-competition (saturation binding) curves for fMet-Leu- $[^3\text{H}]$ Phe. The results of three such comparisons revealed no significant differences in the binding-competition curves (Fig. 2), the potency of repurified fMet-Leu- $[^3\text{H}]$ Phe averaging $100 \pm 9\%$ of that of the native ligand. The close similarity ($P > 0.5$ for each site, t test) of affinity estimates for fMet-Leu-Phe obtained in six other self-competition studies with fMet-Leu- $[^3\text{H}]$ Phe (geometric mean K_a estimates, assuming an independent 'two-binding-site' model [24], of 2.0×10^9 and $2.7 \times 10^8 \text{ M}^{-1}$ for the high-affinity and low-affinity sites) and 16 other studies of the competitive inhibition of $f[^{35}\text{S}]$ Met-Leu-Phe binding by non-radioactive fMet-Leu-Phe (1.9×10^9 and $2.4 \times 10^8 \text{ M}^{-1}$ respectively) further confirmed the close equivalence in receptor-binding properties between the two radioligands and the native ligand.

Bioassay. The biological potency of each form of radiolabelled fMet-Leu-Phe was determined by comparing its ability to induce the release of a granule enzyme, *N*-acetyl- β -glucosaminidase, from rabbit neutrophils with the ability of non-radioactive fMet-Leu-Phe to induce release. Each of four studies with fMet-Leu- $[^3\text{H}]$ Phe (with four different batches of this radioligand) showed a close equivalence between the radioligand and the native ligand (Fig. 3). Analysis by the ENZREL computer program indicated good parallelism between the dose-response curves for fMet-Leu-Phe and fMet-Leu- $[^3\text{H}]$ Phe ($P > 0.2$, partial F test). On average, the potency of fMet-Leu- $[^3\text{H}]$ Phe was $107 \pm 9\%$ (mean $\pm$ S.D.) of that of fMet-Leu-Phe.

Four batches of repurified $f[^{35}\text{S}]$ Met-Leu-Phe were also tested. Two proved to be of similar potency (100% and 148%) to non-radioactive fMet-Leu-Phe; each of the other two batches, however, consistently appeared to be much more potent than the native compound, yielding relative potency values of 278% and 399%. These observations could not be attributed to an impure preparation of non-radioactive fMet-Leu-Phe, this being at least 90% pure by t.l.c. (see first section of the Results section). Radioligand concentrations throughout these studies, and also in the radioreceptor-assay studies reported above, were based on the manufacturer's estimates for the specific radioactivity of each radioligand. The limited quantities of $f[^{35}\text{S}]$ Met-Leu-Phe available unfortunately precluded an independent direct assessment of specific radioactivity for the two anomalous batches. Nevertheless, the results of some additional receptor-binding studies provided strong, albeit indirect, evidence that it was the specific-radioactivity estimates for these batches that were erroneous rather than their biological potencies being anomalous. Their specific

binding percentages were closely comparable with those for fMet-Leu-[^3H]Phe when measured at a near-tracer concentration (0.2 nM, where receptor occupancy is minimal and the binding percentage is little affected by the precise ligand concentration), but were substantially lower than those for fMet-Leu-[^3H]Phe when measured

at a higher concentration (nominally 10 nM, where receptor occupancy is considerable and the binding percentage is inversely related to the ligand concentration). These observations imply that the true concentrations of f[^{35}S]Met-Leu-Phe were greater than the calculated values, and thus that the quoted specific-radioactivity values were substantial overestimates. The observations cannot be explained by a higher potency of the ^{35}S -labelled ligand for receptor binding, which would have resulted in higher binding percentages at both ligand concentrations; nor could they arise from a higher biological efficacy for this ligand, in which case there would have been no differences in binding.

Properties of the impurities in radiolabelled fMet-Leu-Phe preparations

All batches of fMet-Leu-[^3H]Phe contained a similar major impurity (R_F 0.48); some also contained a minor impurity that migrated at an intermediate position between the major impurity and the authentic radioligand. Five batches of f[^{35}S]Met-Leu-Phe showed a similar pattern, but two were found to be composed almost entirely of a compound whose migration (R_F 0.59) was similar to that of the normal minor impurity.

The normal major impurity appeared to be the sulphoxide. It co-migrated with non-radioactive sulphoxide prepared by the method of Harvath & Aksamit [36]; also it was found in an increased proportion when chromatography was performed in the absence of reducing agent (2-mercaptoethanol). A limited assessment was made of the receptor-binding properties of this impurity, which was obtained in a homogeneous form (99% pure) by extraction from the appropriate band of a t.l.c. plate on which had been run a particularly poor stock of fMet-Leu-[^3H]Phe. Three studies of the

Fig. 3. Equivalence in biological activity between radioligand and native ligand

Dose-response curves were compared for the stimulation of *N*-acetyl- β -glucosaminidase release from rabbit neutrophils (5×10^6 cells/ml) by non-radioactive fMet-Leu-Phe (○) and fMet-Leu-[^3H]Phe (●). The relative potency of fMet-Leu-[^3H]Phe in this typical study is estimated to be 114%. The line shows the optimal fitted curve assuming equal potencies. The error bar on the zero dose point represents the pooled S.E.M. for the complete data (duplicate measurements).

Fig. 4. Effect of radiolabelled impurity on the observed binding pattern for a radioligand-receptor interaction

A simple 'one-binding-site' model is assumed for the interaction. All data are for equilibrium binding in a 'labelled-ligand-only' study with differing concentrations of a radioligand of affinity 10^9 M^{-1} . The receptor concentration is taken as 2 nM and the partially reactive impurity assumed to have an affinity of 10^8 M^{-1} . (a) Theoretical Scatchard plots for observed binding with a pure radioligand (—), and with an impure radioligand containing 50% of either an unreactive impurity (---) or a partially reactive impurity (.....). (b) Data for the last case corrected for an assumed receptor-reactivity of 50%. (Note the different scale on the ordinate axis).

binding to rabbit neutrophils (2×10^7 cells/ml) of a near-tracer concentration (0.2 nM) of this impurity showed a specific binding percentage ($2.8 \pm 0.6\%$) that was 16-fold lower, on average, than that of authentic fMet-Leu-[^3H]Phe ($44.7 \pm 12.0\%$). The results of a detailed self-competition study suggested that this major impurity bound to the same receptors as fMet-Leu-Phe, but with a much lower affinity.

The nature of the minor impurity was not established, but, like the major impurity, it was found to have a much lower receptor-binding activity.

Theoretical analysis of the effect of a radiolabelled impurity on the observed radioligand-receptor binding pattern

Equilibrium-binding patterns were calculated, on the basis of the Law of Mass Action, for interaction between either a pure or an impure radioligand and a single set of independent receptors (Fig. 4): values for binding affinity and capacity were chosen so that this 'one-binding-site' model would approximate the observed binding pattern between fMet-Leu-Phe and rabbit neutrophils. The presence of a non-reactive impurity (comprising 50% of the radiolabel) is seen to result in a slight downward concavity in the Scatchard plot (Fig. 4a), severe underestimation of binding affinity and slight overestimation of binding capacity (assuming that a straight-line fit is applied). These errors would be completely removed on correcting the data for 50% receptor-reactivity. The presence of a partially reactive impurity (assumed to have a 10-fold lower affinity than the true ligand) results in a similar, albeit slightly less distorted, binding pattern when the data are uncorrected. Correction for an estimated 50% receptor-reactivity, however, would in this case produce the opposite pattern of an upwardly concave Scatchard plot (Fig. 4b), apparently suggesting the presence of two classes of receptors (or of another form of heterogeneous binding). An appropriate correction cannot realistically be made under these circumstances.

DISCUSSION

A relatively impure form of labelled ligand, or one that is not equivalent to the native ligand, may be adequate for initial studies to demonstrate the existence of a particular receptor on its target tissue. When the study progresses to a more detailed examination of the nature of the ligand-receptor interaction, however, the requirements on the labelled ligand should be regarded as much more stringent. In particular, whenever a complex model is proposed for the ligand-receptor interaction, it is mandatory that the labelled ligand used to obtain the experimental receptor-binding data be both essentially pure and equivalent to the native ligand in its receptor-binding properties. Only when these latter two conditions are met can any real credence be placed in the complex binding model.

The presence of a labelled impurity in a ligand preparation inevitably causes some distortion of the observed ligand-receptor interaction even if the impurity itself possesses no receptor-binding activity [18]. Not only is the distortion quantitative, leading to an underestimate of binding affinity or an overestimate of binding capacity, but it may also be qualitative, for example altering the shape of the Scatchard plot and thus apparently changing

the nature of the interaction process (Fig. 4). So long as the impurity is inactive and its extent is known, the observed data can be corrected for the presence of the impurity to yield truly representative receptor-binding data: an example of this is the 'bindability' correction available in the LIGAND computer program. More frequently, however, the radiolabelled impurity may itself also be capable of binding to the receptor, albeit with a different affinity from the true ligand. Under such circumstances, a correction for the apparent 'bindability' may be very misleading (Fig. 4); the only real recourse, therefore, is to remove the impurity.

Equivalence in receptor-binding properties between the labelled ligand and its native counterpart is also essential if the observed receptor-binding data are to yield realistic information on the nature of the ligand-receptor interaction. This is particularly true of studies in which dose-response curves for receptor binding are obtained by competition between labelled and native ligands. For example, studies of this type will yield a curved Scatchard plot for binding to a single set of independent receptors whenever the labelled and native ligands have significantly different receptor-binding affinities [1,2].

Despite the acceptance of most investigators, in principle, of these potential problems in the interpretation of receptor-binding data obtained with a labelled ligand, extremely few of the radioligands used for receptor studies have, in practice, been subjected to rigorous tests of their purity and equivalence to the native ligand. Moreover, those radioligands that have been tested, albeit often in an incomplete manner, have frequently been found to be impure [8,11-14] or non-equivalent to the native ligand [5-7,9,10,15-17,37]; some, for example, have been no more than 30% pure [8,13,14]. One reason for the inadequate characterization of many radioligands is the paucity of well-established, but widely applicable, techniques for such characterization. Another difficulty is that there appears to be a poor understanding of the distinction between the two separate aspects of radioligand quality and of the need for different experimental procedures to evaluate these two aspects. A further problem is that some of the available techniques, particularly those for demonstrating equivalence to the native ligand, may not always yield reliable information.

The present study of two different radiolabelled forms of fMet-Leu-Phe illustrates the strengths and limitations of some techniques for analysing in a more rigorous manner the suitability of a radioligand for receptor studies. Radiochemical assessments of purity are usually the most convenient to perform, but their suitability depends on whether the chosen technique can differentiate the true radioligand from potential impurities of different receptor-binding potency. In contrast, quantification of receptor-reactivity (or 'bindability'), by either of the techniques described, provides a measure of radioligand purity that is clearly meaningful to receptor studies. The present techniques differ from those used by other investigators [8,38] in that each includes an effective extrapolation to infinite receptor concentration (Fig. 1); such extrapolation is essential whenever the radioligand possesses a relatively low binding affinity. In the case of the simple formyltripeptide fMet-Leu-Phe both techniques for assaying receptor-reactivity yielded similar results to the t.l.c. estimates of radiochemical purity, the small difference between these purity estimates probably

reflecting solubilization of a few receptors during the binding assay. It was noteworthy, however, that the manufacturer's estimates of radiochemical purity (95–99 % without the repurification step) were unduly optimistic, their h.p.l.c. procedure apparently not resolving the impurities sufficiently from the authentic fMet-Leu-Phe. Thus, if a radiochemical technique is to be used to determine radioligand purity, it becomes very important that an appropriate technique be chosen. Furthermore, it is often impractical to measure radiochemical purity directly with radioligands of greater structural complexity, such as radiolabelled polypeptide hormones, because the potential forms of impurity are too numerous. The techniques described for measuring receptor-reactivity, in contrast, can conveniently be applied to characterize any radioligand; their applicability to ^{125}I -labelled thyroid-stimulating hormone (thyrotropin), for example, has already been shown [19,34].

It is usually more difficult to establish the equivalence of a radioligand to its native counterpart than to ascertain its purity. Moreover, not only do the techniques employed, primarily bioassays and self-displacement radioreceptor assays, tend to lack the precision of the assays of purity, they also depend both on the availability of a pure preparation of the native ligand and on an accurate knowledge of the specific radioactivity of the radioligand. Although pure preparations of simple native ligands are often available, this is usually not the case with the complex polypeptide or glycoprotein hormones. Furthermore, the limited quantity of radioligand available usually precludes a direct chemical assessment of specific radioactivity, except in the case of large-scale commercial preparations of simple ligands. The specific radioactivity is thus either estimated from the yield of the labelling procedure or assessed by a comparison with the native ligand in a bioassay or self-displacement radioreceptor assay [9,39]. Implicit in the latter procedure is an assumption of equivalence in behaviour between radioligand and native ligand, so that this method of determining specific radioactivity effectively prevents any realistic assessment of this equivalence. Although the accuracy of the specific-radioactivity estimate cannot usually be assessed, the availability of two different radiolabelled forms of the same ligand made this practicable in the present study. All preparations of f[^{35}S]Met-Leu-Phe tested were equivalent to those of fMet-Leu-[^3H]Phe in receptor-binding activity at a near-tracer concentration, yet two batches of f[^{35}S]Met-Leu-Phe showed greater apparent potency than fMet-Leu-[^3H]Phe and non-radioactive fMet-Leu-Phe both as self-competitors of binding and as stimulators of granule-enzyme release. These observations implied that the specific-radioactivity values for some batches of f[^{35}S]Met-Leu-Phe were seriously in error (see the Results section). That errors of such magnitude (up to 4-fold) could arise with a commercial radioligand preparation emphasizes the likelihood of unreliable specific-radioactivity estimates for radioligands prepared by an individual investigator, for example by radioiodination. It is thus unwise to place too much reliance on the current procedures for assessing the equivalence of a radioligand to its native counterpart, particularly in the case of the more complex ligands.

The purity assessments of the present study revealed neither of the radiolabelled forms of fMet-Leu-Phe, on

receipt from the manufacturer, to be adequately pure for detailed receptor studies; some batches, particularly in the case of f[^{35}S]Met-Leu-Phe, were of such low purity as to preclude their use entirely. Since the major impurity, which appeared to be the sulfoxide, was itself capable of interacting with the formylpeptide receptors, a substantial distortion of the receptor-binding pattern could arise. Removal of the impurity by t.l.c., however, yielded a radioligand of much greater and more consistent purity; its estimated receptor-reactivity was then sufficiently high to lend confidence to the small 'bindability' correction in analysis with the use of the LIGAND program. Furthermore, aside from the misleading data that arose from the erroneous specific-radioactivity estimates, each repurified radioligand appeared to be closely equivalent to non-radioactive fMet-Leu-Phe in both biological and receptor-binding activity (Figs. 2 and 3). The repurified radioligands, both tritiated and ^{35}S -labelled, thus meet the criteria required of a suitable ligand for detailed analysis of the interaction between fMet-Leu-Phe and the formylpeptide receptors. The heterogeneous receptor-binding pattern previously reported for fMet-Leu-Phe [20,40] persisted in studies with the repurified radioligands [24]; this heterogeneity thus appears to be a genuine feature of the formylpeptide-receptor interaction.

Each of the radioligands used for other receptor studies needs to be subjected to an equally rigorous appraisal of its purity and equivalence to the native ligand. Like fMet-Leu-Phe, many of these radioligands may require repurification, either by standard biochemical procedures or by receptor adsorption [7,26,41,42]. Only after such appraisal is it possible to exclude unsuitability of the radioligand as an artifactual explanation for apparent heterogeneity in the ligand-receptor interaction. Validation of the radioligand becomes even more crucial when the incorporated radionuclide is a foreign atom whose presence must inevitably make the radioligand chemically distinct from the native compound. A further consideration with radioiodinated ligands, in particular, is the possibility of variability in the site of incorporation of the radionuclide. In the case of thyroid-stimulating hormone, for example, receptor-binding heterogeneity can be seen with some unsatisfactory radioligands but not with carefully prepared ^{125}I -labelled thyroid-stimulating hormone of proven quality and equivalence to the native hormone [19,34]. Furthermore, for any radioligand where a precise value for the specific radioactivity cannot be guaranteed it seems advisable to perform only competition ('labelled plus unlabelled') studies with a low radioligand concentration, since receptor affinity and capacity estimates would then be little affected by an erroneous value for specific radioactivity [18].

Although the present paper has focused on the need for a quality radioligand, this does not imply that other factors are unimportant in the design of a biologically meaningful receptor assay. In practice, many other factors are equally relevant: these include the nature of the receptor preparation, the choice of incubation conditions, reversibility of the binding reaction and absence of ligand internalization, lack of degradation of radioligand and receptor during the assay, and the choice of procedure to separate receptor-bound from free radioligand [18,24].

In conclusion, thorough validation of the chosen

radioligand should be considered an essential prerequisite for all receptor-binding studies. This is particularly important in those studies where a complex receptor-binding pattern is envisaged. The methods of validation described here for fMet-Leu-Phe should be widely applicable. Although commercially available fMet-Leu-[³H]Phe and f[³⁵S]Met-Leu-Phe are of inadequate purity for receptor-binding studies, repurification by t.l.c. results in radioligands of high purity and close equivalence to the non-radioactive formylpeptide. The repurified radioligands should be well suited to the study of the complex nature of the formylpeptide-receptor interaction.

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