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THE INTERACTION OF ^{14}C-MORPHINE WITH SERA FROM IMMUNIZED RABBITS AND FROM PATIENTS ADDICTED TO HEROIN

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SUMMARY

An intravenous injection of morphine reduced the binding of ^{14}C-morphine by sera from rabbits immunized with morphine-6-hemisuccinated bovine serum albumin. Treatment of the sera with dialysis against glycine buffer (pH 3) followed by dialysis against phosphate buffered saline (PBS), conditions known to dissociate antigen–antibody complexes, restored approximately 76% of the original binding capacity. The heterogeneity of the antibody affinities was shown in both early 'nonavid' and hyperimmune 'avid' antisera by the demonstration of at least two distinct populations of antibodies. One population of antibodies formed loosely bonded antigen–antibody complexes and these complexes completely dissociated within 30 min. The second population had different dissociation times in the 'nonavid' and 'avid' antisera (15 and >72 hr respectively). The presence of the low affinity antibody resulted in different degrees of reduction of detectable binding by the standard washing procedures usually employed in the radioimmunoassay used in these studies. Washing caused less reduction in the amount of antigen bound by the more 'avid' antisera.

Seventy-three per cent of sixty-three serum samples from heroin addicts studied, contained opioid capable of inhibiting the binding of morphine in the radioimmunoassay employed. Methadone at concentrations likely to be present in sera did not interfere with the binding of ^{14}C-morphine. Sera from thirty-one of the patients were treated by dialysis against glycine buffer and PBS and then studied for the capacity to bind morphine. Only one of these thirty-one sera and none of the thirty-two sera that were not pretreated bound ^{14}C-morphine suggesting that an immune response to heroin is not a significant contributing factor to opioid tolerance or the development of complications, such as pulmonary oedema, following opioid administration.

INTRODUCTION

The development of sensitive radioimmunoassay methods to detect opioids or the presence

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of antibody that binds opioids (Spector & Parker, 1970; Spector, 1971; van Vunakis, Wisserman & Levine, 1972; Wainer *et al.*, 1972a, b, 1973; Catlin, Cleeland & Grunberg, 1973) has prompted the re-examination of past suggestions that chronic opioid administration can lead to an altered interaction of these drugs with γ-globulin (Cochin, 1970). This possible immune reaction may be related to adverse reactions to opioids such as pulmonary oedema (Helpern & Rho, 1966) or to opioid tolerance (Cochin, 1970). Recently, it has been reported that sera from heroin users (Ryan, Parker & Williams, 1972) and sera from rabbits given repeated intravenous injections of morphine salts (Ringle & Herndon, 1972) bind more morphine than normal sera. In both studies, the suggestion was made that the apparent increase in morphine binding to globulin was due to the presence of specific antibody.

If antibody were present in sera from patients addicted to heroin, a recent heroin injection might saturate some or all of the drug-binding sites in the serum and result in a partial or complete failure to detect the binding of opioid. It is also not known whether the present radioimmunoassay methods can detect unlabelled opioid bound to circulating antibody. The present experiments were undertaken to determine: (a) the effect of a recent opioid injection on detectable antibody concentrations in immunized rabbits, (b) some of the characteristics of the dissociation of morphine–antimorphine complexes and (c) whether serum samples obtained from patients chronically addicted to heroin had detectable binding of ^{14}C-morphine by globulin.

MATERIALS AND METHODS

Rabbit antisera to morphine

Six adult male New Zealand White rabbits were immunized by repeated subcutaneous injections of morphine-6-hemisuccinate-bovine serum albumin (M-6-HS-BSA) emulsified in Freund's adjuvant. The immunization schedule, the kinetics of the immune response, and the specificity of the antisera obtained have been described elsewhere (Wainer *et al.*, 1973). The sera used in this study were obtained approximately 10 weeks after the initiation of immunization and 6 weeks after the last injection of antigen.

Human sera

Sera were obtained from sixty-three heroin users seeking admission to the Illinois or Indiana Drug Abuse Methadone programs. Twenty-three were from Chicago and the remaining forty from Gary, Indiana. The samples were obtained at the initial physical examination and at that time all but twelve had morphine in their urine as determined by thin layer chromatography at the Illinois Drug Abuse Laboratory. The patients ages ranged from 17 to 53 years with a mean of 25·3 years and nine were female. Information on the length of addiction was available for only forty of these patients and showed a range of 8 months to 24 years with a mean of 4·2 years. Among the sixty-three samples, eighteen had a positive serologic test for syphilis (RPR card test; Hynson, Wescott and Dunning, Inc., Baltimore, Md.) and only one was Australia antigen positive. The normal control serum samples were obtained from twelve non-addicted subjects with no recent history of opioid exposure.

Elution procedure

Sera from immunized rabbits that were injected intravenously with 1·5 mg morphine hydrochloride/kg body weight and selected human serum samples were treated to elute hapten that might be bound to antibody. Aliquots of whole serum or serum globulin, prepared by ammonium sulphate fractionation, were dialysed at 4°C against phosphate buffered saline (PBS; 0·01 M Na_2PO_4, 0·15 M NaCl, pH 7·2) followed by glycine buffer (0·56 M glycine, 0·1 M HCl, 0·15 M NaCl, pH 3·0) for 36 hr. The samples were then dialysed against PBS for 24 hr to restore neutral pH. There was a minimal increase in the volumes of the eluted serum samples and the final volumes of the globulin fractions that were similarly dialysed were between 0·67 and 1·2 times the starting volumes.

Assay for ^{14}C-morphine binding

Whole sera, dialysed sera and dialysed globulin fractions from both patients and rabbits were assayed for morphine-binding capacity by a radioimmunoassay (Wainer *et al.*, 1973) employing a modification of the ammonium sulphate method (Farr, 1958; Minden & Farr, 1967). One millilitre of a serum dilution in PBS was incubated overnight with 0·5 ml of PBS containing 100 pmol ^{14}C-morphine/ml (12,000 cpm/100 pmol morphine; Amersham Searle, Des Plaines, Ill.). One and one-half millilitres of saturated ammonium sulphate was then added and the mixture incubated for 30 min at 4°C. The tubes were then centrifuged in an International PR-6 centrifuge at 2500 rpm for 30 min. The supernatant fluid was decanted and, except where noted, the precipitate washed twice with 3·0 ml 50% saturated ammonium sulphate (SAS). The final precipitate was dissolved in 0·8 ml NCS (Amersham/Searle), added to 10 ml of scintillation fluid (Liquifluor; New England Nuclear, Boston, Mass.) and counted in a Packard Tri-carb liquid scintillation counter for 5 min. To determine the binding of ^{14}C-morphine by dialysed sera and dialysed globulin samples, 0·5 ml of the sample was added to an equal volume of PBS and then incubated overnight with 0·5 ml of PBS containing 100 pmol ^{14}C-morphine/ml.

Under these conditions, the eluted and noneluted serum samples from nonopioid exposed persons bound $<1{\cdot}0\%$ of the ^{14}C-morphine. However, the cpm bound by the dialysed globulin fraction of normal human sera increased to 160 cpm (2·6% of the cpm ^{14}C-morphine added) and all data derived from such samples were corrected for this nonspecific binding using the following equation:

$$100 - \left(\frac{AA - Exp}{AA - NS} \times 100\right) = \% \text{ bound}$$

where AA is cpm of added antigen, Exp is cpm of the precipitate of experimental serum, and NS is cpm in the precipitate of normal serum. The derivation of this correction factor is described elsewhere (Minden & Farr, 1967).

Morphine binding was considered present when more than 5% of the added ^{14}C-morphine was precipitated (McCleery, Kraft & Rothberg, 1970). An antigen-binding capacity (ABC-33) at 10 and 100 pmol/ml ^{14}C-morphine was determined for all rabbit sera. The ABC-33s were expressed as the pmol ^{14}C-morphine bound/ml of serum at each antigen concentration. The experimental procedures and calculations have been described previously (Farr, 1958; Minden & Farr, 1967).

Strength of antigen–antibody bond

The strength of the opioid–antibody bond was assessed by the effect of antigen dilution (Farr, 1958; Minden & Farr, 1967) using ABC-33 determined at two antigen concentrations according to the following equation:

$$\frac{\text{ABC-33 @ 10 pmol } ^{14}\text{C-morphine}}{\text{ABC-33 @ 100 pmol } ^{14}\text{C-morphine}} \times 100 = \text{'effect of dilution'}.$$

As the mean binding strength of the antibody populations present increases, the 'effect of dilution' value approaches 100.

The rate of dissociation of ^{14}C-morphine bound to antibody populations present in two selected rabbit sera was studied by determining the amount of ^{14}C-morphine bound to antibody at varying times after the addition of a large excess of unlabelled morphine to an equilibrium mixture (Farr, 1958; Minden & Farr, 1967). Each antisera was diluted so that it bound approximately 40% of the ^{14}C-morphine added. Following an 18-hr incubation, three aliquots were removed for determination of the % ^{14}C-morphine bound at equilibrium. A large excess of unlabelled morphine, 200 times the amount of ^{14}C-morphine added, was then added and aliquots removed after 5, 15, 30 and 60 min and 2, 4, 8, 16, 24, 48 and 72 hr. Ammonium sulphate precipitation of globulin was performed immediately and the amount of ^{14}C-morphine bound was determined as above. The % of the ^{14}C-morphine remaining bound to the antibody at time 'x' after the addition of unlabelled morphine to the equilibrium mixture was calculated according to the following equation:

$$\frac{\text{cpm } ^{14}\text{C-morphine bound at time 'x'}}{\text{cpm } ^{14}\text{C-morphine bound at equilibrium}} \times 100 = \begin{matrix}\%\ ^{14}\text{C-morphine remaining} \\ \text{bound to antibody at time 'x'}.\end{matrix}$$

Detection of the presence of unlabelled opioid

The presence of unlabelled opioid in a serum sample was studied employing a modification of the ammonium sulphate method (Rothberg & Farr, 1965; Minden & Farr, 1967). 'Reagent' morphine antiserum, from rabbits hyperimmunized with M-6-HS-BSA, was diluted so that it bound approximately 40–55 % of the ^{14}C-morphine added. The serum samples to be assayed were diluted 1:5 in PBS and 0·5 ml of these samples were incubated with 0·5 ml of the reagent antibodies for 5 hr at 20°C. One-half millilitre of a solution containing 100 pmol ^{14}C-morphine/ml was then added and the mixture incubated overnight at 4°C. One and one-half millilitre of saturated ammonium sulphate was then added to each tube and the tubes incubated at 4°C for an additional 30 min. Washing and scintillation counting were then carried out as described above. Inhibition due to the presence of opioid in the sample was compared to inhibition caused by known concentrations of unlabelled morphine added to normal serum diluted 1:5 in PBS. The results of these experiments were expressed as morphine equivalents since the 'reagent' antisera has been shown to cross-react to varying degrees with related opioids (Wainer *et al.*, 1973) and the specific structure of the opioid present in the sera of opioid users is not known. The extent to which methadone reacted with the 'reagent' antiserum also was ascertained by this method.

RESULTS

The effect of intravenously injected morphine on apparent circulating antibody concentrations was evaluated in six rabbits that had been actively immunized with M-6-HS-BSA

emulsified in Freund's complete adjuvant (Wainer *et al.*, 1973). Antigen-binding capacities of sera from these rabbits just before intravenous injection of unconjugated morphine ranged from trace binding to 2310 pmol ^{14}C-morphine/ml when 100 pmol ^{14}C-morphine/ml was the antigen concentration employed. Each rabbit was given a single intravenous injection of 1·5 mg/kg morphine hydrochloride and blood samples were obtained 5, 30 and 120 min and 24 hr after injection of morphine. Each serum isolated from the blood samples obtained before and 5 min after the morphine injection was divided into three aliquots. One aliquot was analysed for antibody content without further processing. The second was dialysed against glycine buffer (pH 3·0) and then PBS to elute any morphine bound to antibody. Globulin was isolated from the third aliquot by ammonium sulphate fractionation and dialysed against glycine buffer and then PBS. Morphine-binding capacities were determined for all samples.

The degree to which the elution procedure affected the quantification of the morphine-binding capacities was determined using the pre-injection sera. As shown in Table 1,

TABLE 1. The effect of elution procedures on the binding of ^{14}C-morphine by rabbit antisera

No treatment	Dialysis against glycine buffer		Globulin fractionation and dialysis against glycine buffer	
ABC-33*	ABC-33	% Reduction in ABC-33	ABC-33	% Reduction in ABC-33
2310	1914	17·1	2310	0
2277	2277	0	2145	5·8
1980	1254	36·7	1815	8·3
1683	1353	19·6	1589	5·6
1139	1122	1·4	1089	4·3
Trace†	Trace	0	Trace	0

* ABC-33: Antigen-binding capacity calculated from the serum dilution binding 33% of the antigen added when 100 pmol/ml ^{14}C-morphine was used, expressed as pmol ^{14}C-morphine bound/ml of undiluted serum.

† Trace: A 1:10 serum dilution bound 29% of the added antigen. Since this is < 33%, an ABC-33 could not be calculated.

dialysis against glycine buffer and PBS reduced the binding capacity approximately 12·5% (range 0–35%) and the loss of binding capacity after globulin fractionation and dialysis was 4% (range 0–8%). The serum that had only a trace amount of antibody activity showed no appreciable change in antibody activity following either procedure.

The effect of the morphine injection on detectable antibody concentrations in rabbits is shown in Table 2. Five minutes after morphine injection, antibody activity could not be detected in sera from five of these six rabbits. Treatment by the elution procedure restored a mean of 76% (range 47–99%) of the original antibody activity present in these sera before morphine injection. After globulin fractionation and elution, a mean recovery of 74·7% (range 67–98%) of the original antigen-binding capacity was obtained. These

TABLE 2. The ability of elution procedures to remove morphine from rabbit antisera*

ABC-33† of antisera prior to morphine injection	Percentage of original ABC-33 detected in sera 5 min after injection of 1·5 mg/kg morphine · HCl		
	No treatment	Dialysis against glycine buffer	Globulin fractionation and dialysis against glycine buffer
2310	0	47	69
2277	57	71	69
1980	0	76	73
1683	0	84	72
1138	0	81	67
Trace	0	99	98
Mean	9	76	74

* Rabbits, previously immunized against M-6-HS-BSA, received an intravenous injection of 1·5 mg/kg morphine · HCl and 5 min later blood samples were obtained.

† ABC-33: Antigen-binding capacity calculated from the serum dilution binding 33% of the antigen added when 100 pmol/ml ^{14}C-morphine was used, expressed as pmol ^{14}C-morphine bound/ml of undiluted serum.

procedures were most effective with the serum that had the lowest level of antibody activity. These data suggest that the procedures employed could be utilized to dissociate and remove antibody-bound opioid and permit detection of serum binding of morphine. At various times following intravenous injection, the concentration of unlabelled morphine in the serum was quantitated by radioimmunoassay. Morphine was detected 5 min after injection but not at 30 and 60 min after injection. In samples obtained at 30 and 60 min, the expected binding of the ^{14}C-morphine by reagent antisera was increased rather than reduced.

The characteristics of antigen binding by these rabbit antisera were studied by measuring the dissociation of ^{14}C-morphine–antibody complexes at equilibrium. Two antisera were employed, one with a high 'effect of dilution' ('avid') and one with a low 'effect of dilution' ('nonavid'). The results shown in Fig. 1 indicate that at least two populations of antibody were present in both sera. Dissociation of the complexes formed by antibodies that weakly bound hapten in both sera was observed within 5 min and appeared to be complete after 30 min. This dissociation is more rapid than that observed with complexes formed between protein antigens and weakly binding antibodies (Farr, 1958). The population of antibodies that strongly bound morphine in the 'avid' serum (curve A) comprised approximately 50% of the antibody activity present and resembled similar antibodies found in protein–anti-protein systems. These antibodies have a half dissociation time >72 hr. In the 'nonavid' sera (curve B), the antibody populations forming stronger bonds with morphine comprised < 20% of the total antibody present and had an approximate half-dissociation time of 15 hr.

The finding of rapidly dissociating antibodies in both 'avid' and 'nonavid' rabbit antisera suggested that the washing procedures conventionally employed in the ammonium sulphate

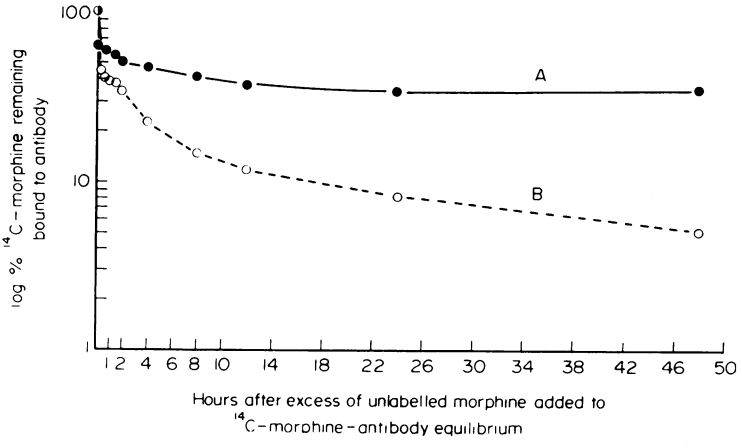

FIG. 1. Dissociation of soluble ^{14}C-morphine-antibody complexes.

assay might result in an appreciable reduction in detectable hapten binding. This possibility was studied in seven rabbit antisera, three 'nonavid' and four 'avid', which were diluted to bind approximately 55–80% of the antigen added. Nine aliquots of each antisera dilution were incubated with 0·5 ml of 100 pmol/ml of ^{14}C-morphine and the globulin precipitated with 50% ammonium sulphate. The amount of radioactivity in the precipitate was measured in triplicate samples prepared without washing, or after one or two washes of the globulin precipitates with 50% saturated ammonium sulphate. Nonspecific precipitation of morphine was corrected for by appropriately treated normal rabbit serum controls. When the assay was carried out without washing, six normal sera bound a mean of 257 cpm (4·2% of the added antigen). This was reduced to a mean of 60 cpm with one washing and 46 cpm with two washings, i.e. the cpm nonspecifically bound by normal serum was reduced 76 and 82% respectively. As shown in Table 3, washing twice resulted in approximately 30% reduction of specific binding of ^{14}C-morphine by 'nonavid' sera while binding by 'avid' sera was reduced approximately 18%. These data indicate that reduction in detectable hapten binding as a result of washing would not be sufficient to prevent detection of morphine binding by sera from heroin users if these sera contain sufficient antibody to bind more than trace amounts of morphine.

Opioid concentrations in sera from patients chronically using heroin was determined using a competitive inhibition assay. Inhibition of the binding of ^{14}C-morphine by rabbit antibody produced by patient serum was compared with that produced by known concentrations of unlabelled morphine. Since some of the serum samples from patients might contain methadone (Bazell, 1973), the capacity of methadone to inhibit ^{14}C-morphine binding by 'reagent' antisera was determined. The results shown in Fig. 2 confirm the findings of others (Catlin *et al.*, 1973) that methadone at concentrations likely to be present in serum did not significantly interfere with the binding of morphine.

Sera from twelve normal subjects without recent opioid exposure were assayed for morphine-binding capacity. These serum samples from normal subjects bound only 5–24 cpm or about 0·3% of the 6000 cpm added. Sera from sixty-three heroin-addicted patients were assayed both for the presence of opioid and for capacity to bind morphine. The results

TABLE 3. Effect of washing ^{14}C-morphine–antimorphine complexes precipitated in half saturated ammonium sulphate*

Effect of dilution†	% added antigen bound by antibody‡ following			% reduction in the amount of antigen bound§ after:	
	No wash	1 wash	2 washes	1 wash	2 washes
8·2	71·0	57·5	47·9	20·0	33·4
10·0	71·4	62·3	53·6	13·8	25·9
21·9	70·3	57·6	48·7	19·2	31·7
51·3	80·6	75·0	71·8	7·5	11·5
60·0	60·3	53·4	47·5	12·8	22·5
65·7	78·2	70·7	65·1	10·1	17·2
78·2	56·9	51·8	47·2	10·6	18·6

* Precipitates washed with 3·0 ml aliquots of 50% saturated ammonium sulphate.

† A semi-quantitative measure of antibody affinity (see text).

‡ Calculated by the relation:

$$100 - \left(\frac{AA - Exp}{AA - NRS} \times 100\right) = \% \text{ bound}$$

where AA is total antigen added radioactivity, Exp is radioactivity precipitated in tubes containing antisera, and NRS is radioactivity precipitated in tubes containing normal rabbit sera. The NRS values used to calculate the amount of antigen bound underwent identical washing conditions.

§ Calculated by the relation:

$$100 - \left(\frac{\text{cpm after 1 or 2 washes}}{\text{cpm after no washes}} \times 100\right) = \% \text{ reduction in amount of antigen bound.}$$

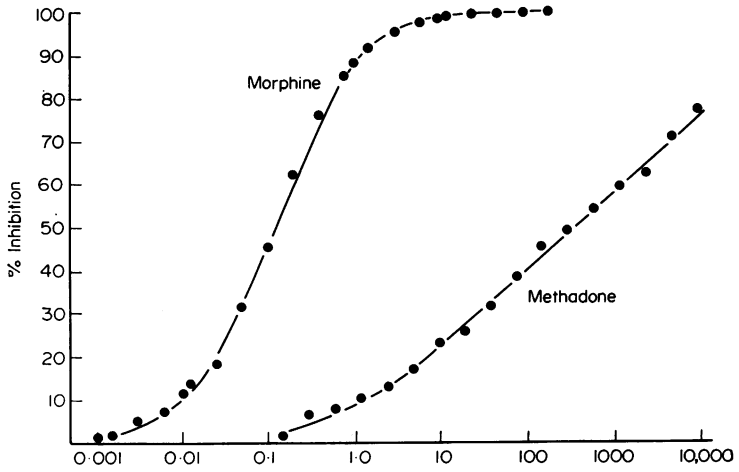

FIG. 2. Competitive inhibition of ^{14}C-morphine binding by the prior addition of increasing concentrations of morphine or methadone.

obtained are shown in Table 4. Of the sixty-three serum samples, seventeen contained no detectable opioids (<2·0 ng/ml) and forty-six had detectable levels of opioids with a mean concentration of 163 ng morphine equivalents/ml (4·6 ng/ml to 11,412 ng/ml). Dialysis of the serum against glycine buffer and PBS removed detectable opioid.

No significant binding of ^{14}C-morphine was detected in thirty-two serum samples studied without further treatment (mean, 14 cpm, range 8–24 cpm), and the absence of binding could not be related to the presence or absence of detectable circulating opioid. The twenty-five opioid containing sera bound between 5–21 cpm (mean, 14 cpm) as compared to 12–24 cpm (mean, 15 cpm) for the seven sera that contained no detectable opioids.

TABLE 4. The capacity of sera from patients addicted to heroin to bind ^{14}C-morphine

Serum treatment	Number of sera	Opioid concentration in serum		Number of sera with significant* binding of ^{14}C-morphine
		Before treatment	After treatment	
None	7	0†		0
	25	470 (4·6–11412)‡	0	0
Dialysed	5	0		0
	14	26·3 (5·1–95·6)	0	0
Globulin isolated and then dialysed	5	0		1§
	7	15 (3·4–28·5)	0	0

* >5% binding of added antigen.
† <2·1 ng/ml.
‡ Mean (range) values in ng morphine equivalents/ml.
§ Sample bound 10·6% of 100 pmol ^{14}C-morphine added/ml (see text).

Since the presence of opioids had been shown to reduce the amount of detectable ^{14}C-morphine binding by rabbit antisera, sera from nineteen additional patients were dialysed against glycine buffer to remove any unlabelled opioid. As shown above, this procedure is effective in restoring detectable ^{14}C-morphine binding in sera from immunized rabbits injected with unlabelled morphine. Opioid that could be detected in untreated serum was removed by the elution procedure (Table 4), however, none of these serum samples had a significant capacity to bind ^{14}C-morphine (mean, 19 cpm, range 15–33 cpm). In an attempt to increase the sensitivity of the assay, globulin was isolated and concentrated from serum of twelve additional patients by precipitation with 50% ammonium sulphate. This material was dialysed against glycine buffer to elute bound opioid. The opioids detected in five of the twelve sera before fractionation and dialysis could no longer be detected following these procedures. After correction for the increased nonspecific binding of normal serum globulin treated in the same manner, only one globulin fraction had the capacity to bind more than 5% (10·6%) of the antigen added which is the lower limit of significant antibody binding in this system (McCleery *et al.*, 1970). After correcting for the concentration that was accomplished during fractionation and dialysis, the binding of 10·6% of the added antigen corresponds to the binding of 6 pmol ^{14}C-morphine/ml by this patient's serum. Globulin from the eleven other serum samples, under these conditions, bound <2% of the ^{14}C-morphine added (mean, 14 cpm, range 0–91 cpm). Due to the small amount of serum

available from each patient, each patient's serum was studied by only one of the above techniques.

DISCUSSION

The results of the present experiments show that the intravenous injection of morphine can reduce detectable binding of ^{14}C-morphine by circulating rabbit antibodies and that dialysis under conditions known to dissociate antigen–antibody complexes restores almost all of the binding activity (Table 2). These studies also demonstrate the heterogeneity of antibody affinities in both early 'nonavid' and hyperimmune 'avid' rabbit anti-morphine antisera (Fig. 1). At least two distinct populations of antibodies were observed in both antisera. A population of loosely bound antigen–antibody complexes appeared to be completely dissociated within 30 min while the second population had quite different dissociation times in the 'nonavid' and 'avid' antisera (15 and >72 hr respectively). Some indication of the presence of low affinity antibodies in the antisera could also be inferred from the effect of washing on the precipitated hapten–antibody complexes. As shown in Table 3, the reduction of detectable binding by the washing procedures was related to the value for 'effect dilution', that is, the more 'avid' antisera lost less bound antigen with washing than did 'nonavid' antisera. Heterogeneity of antibody binding may be important to consider when establishing optimal conditions for a radioimmunoassay procedure. The sensitivity of the technique depends on average binding affinities of antisera employed and selective purification of high affinity antibody populations may be advantageous.

The presence of rapidly dissociating populations of antibodies may also explain the prolonged half-life of injected ^{3}H-digoxin in sera of immunized rabbits (Butler, 1973). A constant exchange of the ^{3}H-digoxin between old and newly synthesized low affinity antibody populations could explain this prolonged circulation of the hapten, in contrast to the phenomenon of immune clearance in protein antigen systems.

Standard radioimmunoassay methods employ high avidity antisera to determine the amount of antigen present in samples that do not contain specific antibodies. In the present studies, this method was used in an attempt to detect free morphine in serum of six immunized rabbits at various times after intravenous injection of morphine hydrochloride. Unlabelled morphine could be detected only in the serum samples obtained 5 min after injection indicating that these samples contained an excess of unlabelled antigen. In samples obtained at 30 and 60 min, the expected binding of the ^{14}C-morphine by reagent antisera was increased rather than reduced suggesting that ^{14}C-morphine competes with the reduced circulating quantities of unlabelled morphine for the binding sites of low avidity antibody in the sera of these immunized rabbits. Under these conditions, circulating antigen and antibody can not simultaneously be quantified directly.

In the present experiments, forty-six of the sixty-three human serum samples contained nanogram amounts of material capable of competitively inhibiting the binding of morphine to antibody. Since the presence of opioid would prevent accurate quantitation of morphine binding, some of the serum samples were treated with the procedures shown to be effective in removing morphine from rabbit antimorphine antibody. As shown in Table 2, the elution procedures were most effective in removing morphine from the antisera that contained a trace amount of antibody suggesting that trace amounts of antibody binding of opioid could be detected following these procedures. Since the lower limit of sensitivity for the detection of antibody in the ammonium sulphate technique is approximately 5% of the

added antigen, or twice the expected variation due to the experimental error of the technique (McCleery *et al.*, 1970), only the one of thirty-one eluted serum or globulin fractions which bound $> 5\%$ of the added antigen was considered to contain specific antibody. These results are not consistent with the previously reported finding (Ryan *et al.*, 1972) of an increased binding of tritiated dihydromorphine by sera from approximately 40% of the fifty-three heroin users studied. The differences between the experimental and control populations noted by these investigators were extremely small and sera from only two or three patients bound 5% more of the added ^{3}H-dihydromorphine than was bound by normal serum. In the present experiments, no differences were found in the absolute amount of ^{14}C-morphine bound by serum from normal subjects and sera from sixty-two of sixty-three addicted patients. The reasons for the differences in the findings of the two studies are not clear. However, it is unlikely that the minimal amounts of binding found by Ryan *et al.* (1972) and in this study are a significant contributing factor to opioid tolerance or to the development of complications following opioid administration. Further, a recent report on the occurrence of heroin induced pulmonary oedema in adolescent patients, who occasionally use heroin but who have not become addicted to the drug (Kaufman, Hegyi & Duberstein, 1972) suggests that this process is a direct opioid effect rather than the result of an immune reaction to heroin.

The present experiments do not preclude the possibility of an immunologic reaction associated with opioid administration. However, the incidence of such phenomena appears to be low and further studies are required to determine their significance.

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