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CORRELATION OF HYDROPHOBIC CHARACTER OF OPIOID PEPTIDES WITH THEIR BIOLOGICAL ACTIVITY MEASURED IN VARIOUS BIOASSAY SYSTEMS

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Abstract—Relative hydrophobicity of a series of enkephalin-like peptides has been studied by partitioning in the aqueous polymeric Ficoll–dextran biphasic system. The activities of the peptides in the guinea pig ileum, mouse vas deferens and rat brain receptor binding assay systems have been examined. The biological activities of the peptides in the two latter assays were correlated with their hydrophobic character. No correlation between the relative hydrophobicity of the compounds and their activity in the guinea pig ileum could be established. The correlations obtained appear to indicate that the drug–receptor interactions in the two bioassays occur under various conditions in regard to the local ionic composition at the membrane and/or the ionizable state of the receptor site. The activities of morphine and a number of similar drugs are described by a relationship different from that found for the peptides, which seems to support the multiple opiate receptor hypothesis.

Since the discovery of endogenous morphine-like peptides, the enkephalins [1, 2] and the endorphins [3–5], quite a few studies of the structure–activity relationships (SAR) of these peptides have been undertaken [6–10].

The importance of the hydrophobic nature of drugs in the use of the quantitative SAR has been proved beyond any doubt [11–13]. Partition coefficients of drugs in the 1-octanol–water biphasic system are a favorite measure of hydrophobicity used in the correlations of drug effects [11–13]. Biphasic organic solvent–water systems, however, are of limited usefulness for the study of ionizable labile naturally occurring substances as well as for the comparative estimation of the relative hydrophobicity of compounds of different chemical natures [14].

A few attempts to correlate biological activity of peptides with their hydrophobic character have been reported recently [10, 15]. The relative hydrophobicity of the peptides has been estimated by the authors [10, 15] in terms of the hydrophobic parameter π [11–13] and increments in the logarithm of the partition coefficient between *n*-octanol and water specific for the side chains of the substituent amino acids residues in certain positions in the peptide molecule. This approach seems, however, to be rather limited in comparison with the experimental one described later.

One of the most suitable biphasic systems providing the possibility of estimating the relative hydrophobicity of biological solutes appears to be the aqueous polymeric Ficoll–dextran biphasic system [14, 16–21]. This system has been successfully employed to estimate the hydrophobic character of amino acid side chains [18], proteins [16, 19], mononucleosides [21] etc. The Ficoll–dextran biphasic system was used in this study to evaluate the relative hydrophobicity of a series of enkephalin-like peptides and several morphine-like compounds.

In a study of the affinity of endogenous morphine-like peptides and their analogues for opiate receptors Lord *et al.* [22] have demonstrated the multiplicity of receptors for the opioid peptides. Lemaire *et al.* [23] have confirmed the existence of multiple heterogenous receptors for the peptides, and have shown that the biological effects produced by the drugs depend upon the choice of the bioassay system. Therefore the opiate activities of the peptides in this work were measured using three different bioassay systems—the depression of the electrically-induced contractions of the mouse vas deferens and the guinea pig ileum and the inhibition of the binding of [^{3}H]naloxone to rat brain homogenates.

The biological activity of the peptides determined in the earlier tests was correlated with their relative hydrophobicity. The results, herein reported, indicate the differences in the conditions of the drug–receptor interactions in various bioassay systems and appear to be explained by the multiplicity of receptors for opioid peptides and for morphine and similar synthetic drug compounds.

MATERIALS AND METHODS

Materials. Ficoll-400 (lot 11069) was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), dextran of mol. wt *ca.* 70,000 under the trade-name Polyglucinum (lot 580870) was obtained from Minmedprom (Moscow, U.S.S.R.). [^{3}H]Naloxone (20 Ci/mmole) purchased from the Radiochemical Center (Amersham, U.K.) was purified by TLC. 1-Octanol, all salts and chemicals of analytical reagent grade were used without further purification.

Peptides. Enkephalins and their analogues were synthesized by fragment condensation as described elsewhere [24, 25]. The purity of all the peptides was verified by TLC in four solvent systems and amino acid analysis of acid hydrolysates.

Animals. Male guinea pigs, 400–600 g, were used. The mice were of the white TO inbred strain, weighing 25–30 g. Male inbred albino rats, 180–200 g, were used in the work.

Methods

Preparation of aqueous polymeric biphasic systems. A mixture containing the amounts of polymers required to achieve the polymer composition indicated later was prepared by weighing appropriate amounts of the stock Ficoll and dextran solutions. The appropriate amounts of 0.44 M Na-phosphate buffer (pH 7.4) and 0.6 M NaCl in 0.04 M Na-phosphate buffer (pH 7.4) were added so as to give the required ionic composition (ionic strength) and polymer compositions of 12.5% (w/w) Ficoll and 10.8% (w/w) dextran. The amounts of NaCl and Na-phosphate buffer, pH 7.4, in a given biphasic system can be calculated from the equations:

$$C_{NaCl} = (0.288 - I)/0.75 \text{ and}$$

$$C_{buffer} = 0.11 - 0.67C_{NaCl},$$

where C_{NaCl} and C_{buffer} are the NaCl and Na-phosphate buffer concentrations, respectively, and I is the ionic strength of the biphasic system.

Partition experiments. The partition experiments were carried out as described elsewhere [16–21]. The phases were allowed to settle at room temperature for 21–24 hr. After that aliquots (0.1–0.2 ml) of both phases were carefully pipetted from the biphasic system, each was diluted by addition of an appropriate volume of water and the concentration of the partitioned solute was determined.

The concentrations of peptides with the free terminal amino group were measured using the fluorescamine (Fluram) technique [26]. The concentrations of the peptides with a blocked *N*-terminal NH_2-group and those of morphine and similar compounds were determined by absorbance measurements. In all instances the correspondingly diluted pure phases were used as blank solutions.

The partition coefficient, K, is defined as the ratio of the sample concentration in the Ficoll-rich phase to that in the dextran-rich phase.

The octanol-buffer systems were formed by equal volumes (1–2 ml) of *n*-octanol and a solution of morphine or another drug in 0.11 M Na-phosphate buffer, pH 7.4, or in 0.15 M NaCl in 0.01 M Na-phosphate buffer, pH 7.4. The systems were vigorously mixed for 2–5 min at room temperature, centrifuged for 20–30 min at 1200 *g* to speed phase settling, and concentrations of the solutes in both phases were determined by absorbance measurements. The distribution coefficient, P, is defined as the ratio of the solute concentration in the octanol phase to that in the aqueous phase.

The partition coefficients were measured for each solute over approximately 10-fold concentration ranges and were found to be independent of the solute concentration in the Ficoll–dextran phase systems as well as in the octanol-buffer systems. The partition coefficient for each solute was determined as the mean of two measurements on three dilutions from each partition experiment carried out 2–3 times at a given ionic strength. The deviation from the average K-value did not exceed 3% for all the compounds under study.

Biological assays. Two of the assays were based on pharmacological responses: the depression of the electrically evoked contraction of the myenteric plexus–longitudinal muscle preparation of the guinea pig ileum according to the method of Kosterlitz *et al.* [27] and of the mouse vas deferens as reported by Hughes *et al.* [28].

Activities of the agents expressed as the molar concentrations which elicited a 50% reduction in the stength of contraction of the muscle preparation (IC_{50}) were calculated from concentration–response plots and each IC_{50} value given later is the mean of five to seven determinations.

Binding measurements. Opiate binding assays were carried out on the rat brain homogenate according to the method reported in Ref. 29. All determinations were performed in triplicate.

RESULTS

Activity of the peptides in various bioassay systems

The activities of the peptides under study displayed in three different bioassay systems are presented in Table 1 together with those manifested by morphine and similar drugs. It can be seen from the data given that the effect of a structural alteration on the activity of a peptide varied greatly depending on the particular bioassay system used.

It should be noted that this paper deals with the results obtained separately by several research groups. By the time the data had been gathered it was found that some of the peptides were not tested in all the assay systems. As up to that moment the peptides had been unavailable it was impossible to fill up the gaps observed in Table 1.

Partition of the drugs in Ficoll–dextran biphasic systems

Fig. 1 shows typical examples of the partition behavior of the peptides examined as a function of the ionic composition and/or ionic strength of the biphasic system. The relationships between the logarithm of the solute partition coefficient and the ionic strength of the system (under conditions indicated earlier) can be described as:

$$\ln K = A + BI, \quad (1)$$

where K is the partition coefficient, I is the ionic strength of the system (varied as indicated earlier

Table 1. Activity of opioid peptides, morphine and naloxone in the guinea pig ileum (GPI), mouse vas deferens (MVD) and rat brain receptor binding (RBR) assay systems

Compound	GPI $IC_{50}10^7$M	MVD $IC_{50}10^7$M	RBR $IC_{50}10^9$M
Naloxone	—	—	1.87 ± 0.73
Morphine	—	3.36 ± 2.0	0.87 ± 0.15
Lys-Tyr-D-Ala-Gly-Phe-NH_2	1.7 ± 1.2	1.20 ± 0.5	3.72 ± 0.90
Pro-Tyr-D-Ala-Gly-Phe-NH_2	10.2 ± 2.2	7.27 ± 3.6	30.0 ± 15.0
Arg-Tyr-D-Ala-Gly-Phe-NH_2	—	—	5.80 ± 1.50
Tyr-D-Ala-Gly-Phe-NH_2	1.2 ± 0.7	1.17 ± 3.1	3.20 ± 0.10
Tyr-D-Ala-Gly-Phe(NO_2)-NH_2	0.069 ± 0.06	0.36 ± 0.7	1.14 ± 0.27
Tyr-D-Ala-Gly-Phe(NO_2)-Leu-OH	0.074 ± 0.053	$(7.8 \pm 1.4) \times 10^{-4}$	7.43 ± 3.0
Tyr-Gly-Gly-Phe-Leu-OH	3.03 ± 1.1	0.13 ± 0.24	47.0 ± 3.4
Try-Gly-Gly-Phe-Met-OH	1.72 ± 0.8	0.15 ± 0.10	33.0 ± 7.0
Tyr-Gly-Gly-Phe(NO_2)-Leu-OH	—	—	16.46 ± 1.0
Tyr-D-Ala-Gly-Phe(NO_2)-OH	0.94 ± 0.55	0.14 ± 0.70	5.82 ± 2.2
Tyr-D-Ala-Gly-Phe-N_2H_2-Leu	1.00 ± 2.20	0.18 ± 0.63	—
Tyr-D-Ala-Gly-Phe-N_2H_2-His	1.90 ± 3.24	0.35 ± 0.71	—
α-Endorphin	8.51 ± 7.20	3.31 ± 0.84	13.5 ± 12.0
γ-Endorphin	9.3 ± 7.20	3.90 ± 1.50	14.8 ± 10.0
Des-Tyr-γ-endorphin	17.0 ± 5.00	3.58 ± 1.50	—

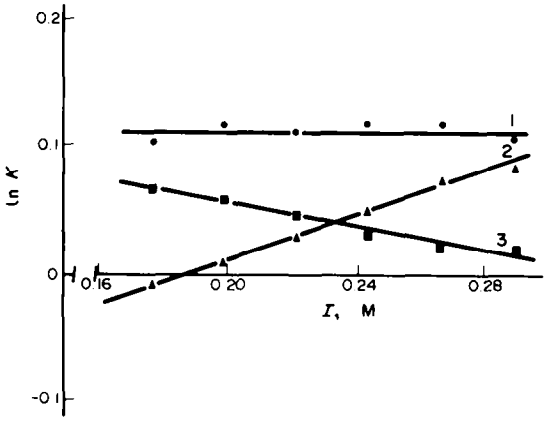

Fig. 1. Logarithm of the partition coefficient for some peptides as a function of ionic strength (I) in the Ficoll–dextran biphasic system containing different amounts of NaCl and sodium phosphate buffer (pH 7.4) as indicated in the text: (1) Tyr-D-Ala-Gly-Phe-NH_2, (2) Tyr-Gly-Gly-Phe-Met-OH, (3) Lys-Tyr-D-Ala-Gly-Phe-NH_2.

from 0.176 to 0.288 M), and A and B are constants.

The physical meaning of parameters A and B has been discussed earlier [16, 17, 19–21] and it has been shown that parameter B reflects the effect of the ionic composition and/or ionic strength of the medium on the hydrophobic character of a given substance's ionizable group. Parameter A represents the relative hydrophobicity of the compound under study at zero ionic strength in the medium.

A least-squares treatment of the experimental data according to equation (1) led to the values of A and B for the peptides and morphine-like drugs listed in Table 2.

Distribution of morphine · HCl and similar drugs in octanol-buffer biphasic systems

It is known that the distribution coefficients of solutes under study are sensitive to the pH value of the aqueous phase of the octanol-buffer biphasic system [30]. Therefore the experiments were performed in the system containing 0.11 M Na-phosphate buffer, pH 7.4, instead of pure water, and in that containing 0.15 M NaCl in 0.01 M Na-phosphate buffer, pH 7.4. The employment of the two variants of the system was suggested also by the data reported by Wang *et al.* [31] about the influence of different buffers on the distribution coefficients of solutes in the octanol-buffer systems. Our results indicate that the distribution coefficients of the drugs examined are independent of the ionic composition of the aqueous phase of the octanol-buffer system in the range used. The averaged P-values are 1.133 ± 0.040 for morphine · HCl, 2.676 ± 0.025 for naloxone, and 3.9 ± 0.075 for nalorphine · HCl.

Comparison of the P-values with the corresponding K-values (listed in Table 2) indicates that the partition coefficients determined in the Ficoll–dextran biphasic system are related to those measured in the octanol-buffer system for the morphine-like drugs examined according to the solvent regression equation [11, 12, 14, 32]:

$$\ln P = 29.43 \ln K - 1.582. \quad (2)$$

The constants in equation (2) do not appear to follow the relationship established earlier [14] for the relative hydrophobicities of the biphasic systems compared. The reasons for that are unclear at present. Equation (2), however, allows one to calculate the K-values for the drugs for which the distribution coefficients in the octanol-buffer system have been reported in the literature [30]. These drugs include codeine (P = 42.20 [30], i.e. K = 1.20), *d*-methadone (P = 28.3 [30], i.e. K = 1.182) and levorphanol (P = 8.7 [30], i.e. K = 1.136).

DISCUSSION

The data presented in Table 1 indicate that there is no direct correlation between the potencies of the enkephalin analogues examined in the guinea pig ileum, mouse vas deferens or rat brain receptor binding assays. There is a general agreement among investigators as to the main tendencies of SAR in

Table 2. Characteristics of the partition behaviour of morphine, nalorphine, naloxone and opioid peptides in the Ficoll–dextran biphasic system*

Compound	A	B
Naloxone	0.087 ± 0.066	0
Morphine	0.058 ± 0.020	0
Nalorphine	0.100 ± 0.049	0
Lys-Tyr-D-Ala-Gly-Phe-NH_2	0.203 ± 0.031	−0.544 ± 0.054
Pro-Tyr-D-Ala-Gly-Phe-NH_2	0.037 ± 0.027	0
Arg-Tyr-D-Ala-Gly-Phe-NH_2	0.210 ± 0.020	−0.679 ± 0.141
Tyr-D-Ala-Gly-Phe-NH_2	0.104 ± 0.020	0
Tyr-D-Ala-Gly-Phe(NO_2)-NH_2	0.144 ± 0.026	0
Tyr-D-Ala-Gly-Phe(NO_2)-Leu-OH	−0.097 ± 0.019	1.075 ± 0.079
Tyr-Gly-Gly-Phe-Leu-OH	−0.169 ± 0.015	1.107 ± 0.062
Tyr-Gly-Gly-Phe-Met-OH	−0.141 ± 0.012	1.000 ± 0.047
Tyr-Gly-Gly-Phe(NO_2)-Leu-OH	−0.126 ± 0.042	1.081 ± 0.170
Tyr-D-Ala-Gly-Phe(NO_2)-OH	−0.077 ± 0.015	1.009 ± 0.061
Tyr-D-Ala-Gly-Phe-N_2H_2-Leu	0.392 ± 0.009	−0.711 ± 0.034
Tyr-D-Ala-Gly-Phe-N_2H_2-His	0.338 ± 0.048	−0.610 ± 0.093
α-Endorphin	0.060 ± 0.028	0
γ-Endorphin	0.064 ± 0.022	0
Des-Tyr-γ-endorphin	0.062 ± 0.043	0

* The logarithm of the partition coefficient of each compound indicated is described as $\ln K = A + BI$, where I is the ionic strength of the Ficoll–dextran biphasic system. (For details see text.)

these assay systems [6–9]. A good correlation is usually found between the activity of enkephalin analogues in the mouse vas deferens and the [^{3}H]naloxone (or other opiate) receptor binding assay [9]. The correlation seems to be less good and sometimes to be absent with the guinea pig ileum [6]. There are a lot of conflicting results, however, in regard to the existence or lack of correlation between activities of various opioid peptides measured in different bioassays. Audigier *et al.* [7] have established a strong correlation between activity of opioid peptides in the guinea pig ileum and their affinity for [^{3}H]etorphine binding sites in the mouse brain. Similar correlations have been found for the same peptides [7] between the relative affinities for [^{3}H](D-Ala)^2-Leu^5-enkephalin amide binding sites in the mouse brain and the agonist potency on the mouse vas deferens. For some of the enkephalin analogues studied so far [9] a good correlation can be observed between the activities in the guinea pig ileum and those observed in the mouse vas deferens assay.

The data given in Table 1 are far from being conclusive but there are some points worthy of notice. First of all, the activities of Leu-enkephalin, Met-enkephalin, their pentapeptide analogues examined, and α- and γ-endorphins measured in the mouse vas deferens assay appear to be approximately 10-fold that observed in the guinea pig ileum assay system. Secondly, it seems that amidation of the carboxyl group of the Phe^4-residue with (D-Ala)^2-derivatives leads to a drop in the potency displayed in the mouse vas deferens test followed by an increase in the activity in the opiate receptor binding and in the guinea pig ileum assay systems. The activities of Tyr-D-Ala-Gly-Phe-NH_2 observed in the mouse vas deferens and guinea pig ileum assays appear to level off as the result of the *N*-extension by an amino acid with ionogenic side chain (Lys or Arg). All these findings seem to be in line with the data reported in the literature [9].

The differential effects of some enkephalin analogues in the guinea pig ileum and mouse vas deferens are usually explained by the existence of multiple opiate receptors [22, 23]. It has been demonstrated by Lord *et al.* [22] that the enkephalins' and endorphins' action in the mouse vas deferens occurs on receptors different from those on which morphine and its classical surrogates act. These receptors, called by Lord *et al.* [22] σ-receptors, are not responsible for the action of the enkephalins in the guinea pig ileum. In this preparation the peptides are assumed to interact mainly with the μ-receptors which mediate the action of the classical morphine-like compounds. The evidence available at present is insufficient to allocate different receptors to different physiological functions. A physico-chemical theory of the mode of narcotic–opiate receptor interaction, however, has been proposed [33]. According to the theory the hydrophilic–hydrophobic balance of the opiate receptor and its complex with a drug is of fundamental importance for the pharmacological action of the opiates. One of the key factors essential for the expression of opiate action taken into consideration when designing the theory [33] was the relative hydrophobicity of the drug.

It has been shown recently [18–21] that the hydrophobic character of a solute measured by its partitioning in a biphasic system should be estimated in terms of so-called equivalent number of CH_2 groups, n^{CH_2}. The number, n^{CH_2}, is related to the logarithm of the partition coefficient value for the solute in question according to equation (3):

$$n^{CH_2} = (RT \ln K)\Delta G^{CH_2}, \qquad (3)$$

where ΔG^{CH_2} is the free energy of transfer of a CH_2 group from one phase to the other in a given biphasic system. A positive n^{CH_2}-value means that the relative hydrophobicity of a given molecule (or moiety) is equal to that produced by n CH_2 groups, and a negative n^{CH_2}-value means that the molecule (or

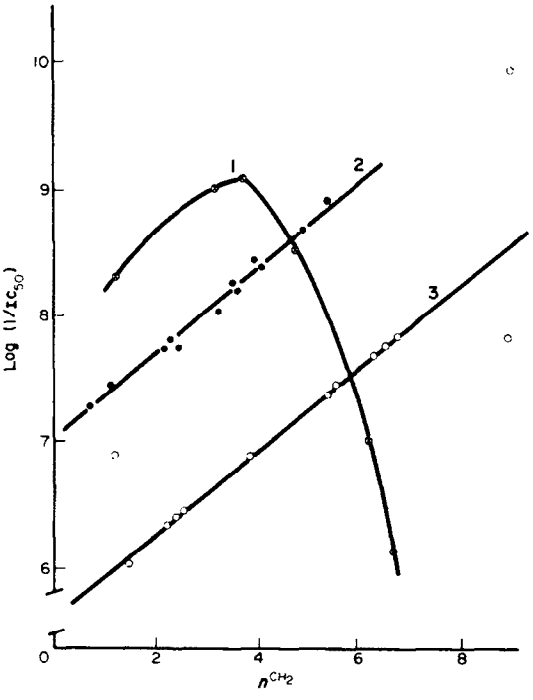

Fig. 2. Relationships between the activity of morphine-like drugs (1) and opioid peptides in rat brain receptor binding (2) and mouse vas deferens (3) assay systems [expressed as log ($1/IC_{50}$)] and the relative hydrophobicity of the compounds (expressed in n^{CH_2}-terms at the corresponding ionic strengths). (For details see text.)

moiety) is hydrophilic and its hydrophobicity is the reverse of that produced by n CH_2 groups. (It should be noted that the ΔG^{CH_2} value in the Ficoll–dextran biphasic system used in this work amounts to 16 cal/mole CH_2 [16, 20].)

We have attempted to correlate the relative hydrophobicity of the opioid peptides under study with their activities measured in the three different bioassay systems. It can be seen from the data in Fig. 2, however, that the relative hydrophobicity of most of the peptides examined depends on the ionic composition and/or ionic strength of the medium. It should be particularly noted that the influence of the ionic composition (ionic strength) on the relative hydrophobicity of some of the peptides under study appears to be almost diametrically opposite depending on the structure of the peptide in question. In looking for some correlation between the hydrophobic character of the peptides and their biological activity we have established two correlations presented in Fig. 2.

There is a strong correlation between the affinity of the opioid peptides for [^{3}H]naloxone binding sites in rat brain homogenate and the relative hydrophobicity of the peptides at an ionic strength of 0.170 M. This correlation presented in Fig. 2 (2) is described as:

$$\log (1/IC_{50})_{RBR} = 7.065 + 0.345 n^{CH_2}_{(at\ 0.170\ M)}, \quad (4)$$

$$n = 12,\ r = 0.996,\ s = 0.044.$$

The activities of morphine, nalorphine, *d*-methadone, levorphanol, codeine and naxolone in the rat brain receptors binding assay as reported by Terenius [34] are plotted in Fig. 2 vs the relative hydrophobicity of the drugs expressed in the n^{CH_2}-terms calculated from the corresponding K-values given earlier. The number of drugs is insufficient to claim that the relationship presented in Fig. 2 is reliably significant but it seems evident that curves 1 and 2 in Fig. 2 differ considerably.

A quite good correlation was also found between the potency of the peptides in the mouse vas deferens assay and the relative hydrophobicity of the peptides at an ionic strength of 0.315 M. The correlation presented in Fig. 2 (3) is described as:

$$\log (1/IC_{50})_{MVD} = 5.806 + 0.329 n^{CH_2}_{(at\ 0.315\ M)} \quad (5)$$

$$n = 13,\ r = 0.863,\ s = 0.550.$$

No correlation could be found between the activity of the peptides on the guinea pig ileum and their relative hydrophobicity independent of the ionic strength value. (It should be emphasized that the term "ionic strength" used throughout this paper must be considered as the parameter representing the ionic composition of the medium containing NaCl and Na-phosphate buffer.)

It should be noted, first of all, that the activities of α- and γ-endorphins are described by both of equations (4) and (5). It is particularly interesting as the size of these peptides is approximately 3 times that of Leu-enkephalin. The relative hydrophobicity of these peptides, however, is of the same order of magnitude which supports the view [19, 20] that the hydrophobic character of conformationally flexible compounds is governed not by the total structure of the molecule but solely by the nature of groups accessible to water.

Secondly, it should be noted that it follows from the relationships established that: (i) the σ-receptor characterized in the mouse vas deferens interacts with opioid peptides under conditions unlike those which exist in the rat brain homogenate (by "conditions" is meant the local membrane environment—ionic composition, and probably the state of the ionizable groups of the receptor); (ii) hydrophobic interactions are an important part of the peptide–receptor interactions in both assay systems, and it seems that this part is independent of the specific features of the particular bioassay system (as an increase in the relative hydrophobicity of a peptide by one equivalent CH_2 group is followed by an increase in the potency of the peptide by 0.34 in both bioassays).

Thirdly, the fact that the activities of the morphine-like drugs fit a different relationship from that established for the peptides seems to be in line with the hypothesis [22] that opioid peptides and opiates interact with different receptors.

The results obtained in this work are clearly insufficient for any far reaching generalizations except that the study of correlations of different biological activities of drugs and their relative hydrophobicity appears to be promising for more insight into mechanisms of drug–receptor interactions. The closer study of such correlations for opiates is evidently called for and is in progress in our laboratories at present.

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