rations indicated that the capacity of tubulin to bind additional amino acids at the carboxyl terminal end was about 0.3 mole per mole of dimer for both control and experimental animals. This result, besides strengthening the assumption of the unchangeability in size of the pool of modified tubulin molecules, establishes that the molecules unmodified in vivo in control and experimental animals maintain the same reactivity toward tyrosine in vitro. It also demonstrates that a certain proportion of the rat brain tubulin neither contained nor accepted aromatic amino acids at the carboxyl terminal position.

We attempted to compare the capacity of tubulin for assembling at 37°C in the brain extracts of normal and experimental animals by following the kinetics of the development of viscosity. Extracts were prepared from 7-day-old rats (9 to 11 mg/ml) as described in Table 1 and viscometry was monitored as described by Olmsted and Borisy (*14*). Although the analysis of viscosity development in the extracts was complicated by low initial rates and high values of viscosity after the first 10 or 15 minutes of incubation at 37°C, apparently due to gelation, no difference was observed between the samples from experimental and control animals. We conclude that the kinetics of polymerization as measured by viscometry did not substantially change after the induction of hyperphenylalaninemia. By using a sedimentation assay, it was recently demonstrated (*15*) that tubulinyl-[^{3}H]phenylalanine and tubulinyl-[^{14}C]tyrosine prepared in vitro were randomly assembled into microtubules when the mixture was incubated at 37°C. Those results agree with the idea that neither the removal nor the addition of an aromatic amino acid to the carboxyl terminal end of α-tubulin affects the polymerizability of the microtubule protein (*15, 16*).

The results of this study indicate that cytoplasmic brain tubulin from animals in which the biochemical characteristics of phenylketonuria were induced unambiguously differed from tubulin of control animals in the nature of the noncoded aromatic amino acid residue that could be released by carboxypeptidase digestion. Hyperphenylalaninemia resulted in enhanced substitution of phenylalanine for tyrosine. Although the addition of an aromatic residue to the carboxyl terminal glutamate of the α chain of tubulin seemingly does not affect the assembly of microtubules in vitro, the presence of such a residue could alter the configuration of that region of the polypeptide. Moreover, the chemical difference between tyrosine and phenylalanine could affect the capacity of the protein or of the resultant microtubules to interact with other cellular elements. Our results do not permit us to say whether the pathogenesis of brain dysfunction in phenylketonuric individuals is dependent on the modification of tubulin; however, the existence of this modification suggests that the possibility of a link between the phenylalanination of tubulin and phenylketonuric condition needs to be investigated further.

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Classification of Opioids on the Basis of Change in Seizure Threshold in Rats

Abstract. *Twenty opioids have been subdivided into four classes by using flurothyl-induced seizures in rats to measure dose-response relationships, stereospecificity, naloxone sensitivity, and tolerance-cross-tolerance. The data support current theories of multiple opiate receptor types. Since the receptors involved mediate effects that are antagonized, enhanced, or unaffected by naloxone, the model is uniquely suitable for detecting novel narcotic antagonists that can then be used to differentiate opiate receptors in other systems.*

Morphine and related compounds have recently been subdivided into at least three different classes on the basis of subjective effects in humans (*1*), different sensitivities towards the narcotic antagonist naloxone (*2*), and dissimilar pharmacological profiles both in vitro (*3*) and in vivo (*4, 5*). We have extended the in vivo profile approach to include altered seizure threshold as a measure and have classified 20 opioids by comparing qualitative effects, dose-response curves of enantiomers, sensitivities toward naloxone, and tolerance-cross-tolerance properties. In light of these experiments, we now report that opioids can be classified into at least four groups in vivo. Three of the groups show good correspondence to a classification obtained from the chronic spinal dog preparation (*4*). The fourth group represents a new category; meperidine and pentazocine are the prototype analgesics.

Groups of 10 to 20 male Sprague-Dawley albino rats (300 to 350 g; Zivic-Miller) were studied. Rats were given only one injection; they received the vehicle or one of at least three doses of test agent (*6*) subcutaneously 30 minutes before being exposed to flurothyl (Indoklon), a volatile convulsant. The flurothyl was given as a 10 percent solution in 95 percent ethanol (volume to volume) to rats placed individually in 1-gallon glass jars (*7*). A constant rate of infusion of 0.10 ml per minute was maintained by a Harvard pump. The time interval between the start of the infusion and the onset of a clonic convulsion (almost invariably with loss of posture) represented the seizure threshold. Testing took place between 1000 and 1300 hours. Mean seizure thresholds for rats injected with saline were routinely in the range of 350 to 380 seconds.

Dose-related anticonvulsant effects were associated with the so-called sigma (σ) receptor agonists (*4*) *N*-allylnormetazocine (SK & F 10,047) and cyclazocine (Table 1). Both of these psychotomimetic benzomorphans caused behavioral activation; specific features seemed to be determined by the environment. In the novel and restricted envi-

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ronment of the glass jars, the drugs caused circling, rearing, and side-to-side head movements. This activation was unrelated to the flurothyl infusion. In contrast, behavioral depression was associated with the dose-related anticonvulsant effects of morphine and other typical mu (μ) receptor agonists (*4*): etorphine, (−)-methadone, phenazocine, levorphanol, and buprenorphine. The third group of compounds had no clear (or dose-related) effect on seizure threshold. The kappa (κ) receptor agonists (*4*) ethylketocyclazocine, ketazocine, nalorphine, and nalbuphine were included in this group. Dose-related proconvulsant effects were obtained with compounds in the fourth category: pentazocine, meperidine, and the *N*-demethylated metabolite, normeperidine. Note the presence of a benzomorphan analgesic in each group (for example, cyclazocine, phenazocine, ketazocine, and pentazocine) and the opposite effects of the classical benzomorphans, cyclazocine and pentazocine.

The pharmacological properties of opioids are mainly associated with the (−) enantiomer (*8*). We addressed the question of stereospecificity by comparing the effects of three pairs of enantiomers on seizure threshold (Fig. 1). Both (+)- and (−)-cyclazocine had anticonvulsant properties; (−)-cyclazocine was 1.6 times more potent than (+)-cyclazocine. Methadone differed from cyclazocine in that its anticonvulsant properties were associated with the (−) enantiomer. An example of pharmacological activity associated mainly with the analgesically inactive (+) enantiomer of an opioid is provided by pentazocine. The regression lines for (+)- and (−)-pentazocine did not differ significantly in slope; (+)-pentazocine was 5.6 times more potent than (−)-pentazocine as a proconvulsant.

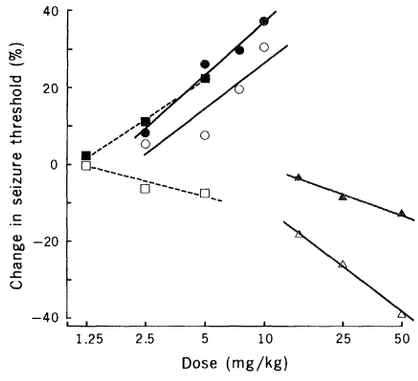

Fig. 1. Effects of enantiomers of analgesics on seizure threshold in rats. Each compound was injected subcutaneously 30 minutes before the rats were exposed to flurothyl. Key: ■, (−)-methadone; □, (+)-methadone; ●, (−)-cyclazocine; ○, (+)-cyclazocine; ▲, (−)-pentazocine; and △, (+)-pentazocine. Ordinate gives the mean percentage of change in seizure threshold relative to controls.

The groups could be further differentiated when naloxone HCl was injected subcutaneously at the same time as the opioid. Whereas even moderate to large doses of naloxone (1 to 10 mg per kilogram of body weight) had no significant influence on the anticonvulsant effects of SK & F 10,047 or cyclazocine, very low doses of the antagonist (0.01 to 0.10 mg/kg) could attenuate the anticonvulsant effects of the three μ receptor agonists tested: etorphine, morphine, and (−)-methadone. The (−)-naloxone (0.10 mg/kg) attenuated the anticonvulsant effects of etorphine, whereas (+)-naloxone (0.10 mg/kg) was without effect. It is therefore highly likely that the anticonvulsant effects of μ receptor agonists are mediated, in a stereospecific manner, by opiate receptors.

Naloxone potentiated the proconvulsant effects of meperidine and (−)-pentazocine. At 10 mg/kg (but not at 1 mg/kg) naloxone displaced, in a parallel, downward direction, the dose-response lines of both analgesics. Naloxone (10 mg/kg) had no marked influence on the already substantial proconvulsant effects of (+)-pentazocine. Finally, although the naloxone-normeperidine interactions produced a trend toward potentiation, no statistically significant differences were obtained.

Our standard procedure in tolerance-cross-tolerance studies was to inject groups of 12 to 16 rats subcutaneously at 0800 and 1700 hours daily for 11 consecutive days with vehicle or ascending doses of test compound. The last injections took place at 2400 on day 11. At 0900 on day 12, the rats received vehicle, the same test compound (tolerance studies), or a second test compound (cross-tolerance studies) and were tested 30 minutes later. Tolerance did not develop to the anticonvulsant effects of cyclazocine (5 mg/kg) or to the proconvulsant effects of either pentazocine (30 mg/kg) or meperidine (40 mg/kg) in rats earlier treated with cyclazocine (1.25 to 5 mg/kg), pentazocine (6.25 to 25 mg/kg), or meperidine (6.25 to 12.5 mg/kg), respectively. There was no cross-tolerance between the following pairs of compounds: cyclazocine and SK & F (40 mg/kg); cyclazocine and pentazocine (30 mg/kg); cyclazocine and normeperidine (25 mg/kg); pentazocine and meperidine (50 mg/kg); pentazocine and cyclazocine (5 mg/kg); and, perhaps surprisingly, meperidine and etorphine (0.02 mg/kg). In contrast, tolerance developed to the anticonvulsant effects of levorphanol (20 mg/

Table 1. Classification of opioids based on changes in seizure threshold (ST), sensitivity toward naloxone, and development of tolerance.

Compound	Dose range (mg/kg)	Maximum change in ST relative to controls (%)
Group 1 (effect not attenuated by naloxone at 10 mg/kg) (no tolerance)		
SK & F 10,047	10 to 40	+42.0
Cyclazocine	1 to 5	+28.5
Group 2 (effect attenuated by naloxone at 0.10 mg/kg) (tolerance develops)		
Etorphine hydrochloride	0.005 to 0.02	+28.6
Morphine sulfate	12.5 to 64	+25.6
(−)-Methadone hydrochloride	1.25 to 5	+22.8
Phenazocine hydrobromide	0.5 to 5	+18.0
Levorphanol tartrate	2.5 to 20	+17.5
Buprenorphine hydrochloride	0.004 to 12.5	+15.2
Group 3 (no consistent dose-related effect) (interaction with naloxone not tested)		
Moxazocine tartrate	12.5 to 50	+7.0
Cyclorphan hydrochloride	1 to 80	+5.1
Normorphine hydrochloride	50 to 100	+3.7
Norcyclazocine	6.25 to 25	+2.5
Nalbuphine hydrochloride	5 to 20	+2.4
Naloxone hydrochloride	1 to 10	−5.0
Nalorphine hydrochloride	25 to 100	−6.2
Ethylketocyclazocine methanesulfonate	0.5 to 50	−6.4
Ketazocine methanesulfonate	0.5 to 20	−7.0
Group 4 (effect potentiated by naloxone at 10 mg/kg) (no tolerance)		
Meperidine hydrochloride	12.5 to 50	−19.2
Pentazocine hydrochloride	12.5 to 50	−31.5
Normeperidine hydrochloride	1.56 to 50	−46.8

kg) in rats earlier treated with levorphanol (2.5 to 20 mg/kg). Rats treated chronically with levorphanol were cross-tolerant to the anticonvulsant effect of morphine (50 mg/kg).

Our experiments have provided, for what we believe to be the first time, comprehensive data on the relative effects of key analgesics on a novel measure in opiate research—the seizure susceptibility of rats exposed to flurothyl. It seems that analgesics classified as morphine-like (μ) receptor agonists in the chronic spinal dog preparation (*4*) are likely to raise seizure threshold (*9*). The receptor mediating this effect has the classical features of stereospecificity, sensitivity toward naloxone, and susceptibility to tolerance. A second receptor mediates the anticonvulsant actions of SK & F 10,047 and cyclazocine, two σ receptor agonists that cause bizarre behavior in rats. This receptor is only weakly stereospecific, the levorotatory enantiomer of cyclazocine being the preferred ligand. Naloxone insensitivity (*10*) and resistance to tolerance are further features of this receptor.

Several compounds have no pronounced effect on the flurothyl-induced seizure threshold. Present in this group are four analgesics (ethylketocyclazocine, nalbuphine, ketazocine, and nalorphine) that do not suppress signs of abstinence in withdrawn, morphine-dependent monkeys. It remains to be seen if this classification is fortuitous or is indeed an additional characteristic of certain κ receptor agonists. The presence of normorphine and norcyclazocine in this group indicates that anticonvulsant activity is associated with the respective parent molecules rather than with these metabolites.

Our finding that meperidine and pentazocine are not grouped with the prototype μ, κ, and σ agonists is in keeping with the difficulties experienced by others in trying to define the position of these analgesics relative to standard opioids (*5, 11*); for example, differences between meperidine and morphine have been noted in neurochemical, psychopharmacological, and physiological studies of mice, rats, pigeons, and dogs (*12*). We are unable to state unequivocally that the proconvulsant actions of meperidine and pentazocine are mediated by a subclass of opioid receptors as opposed to being merely a reflection of nonspecific stimulant (and perhaps local anesthetic) effects. Although tolerance could not be demonstrated with either meperidine or pentazocine, the involvement of receptors specific to opioids is not necessarily precluded. Indeed, seizure threshold was lowered as a function of dosage by the agonists, and stereospecificity was present, although activity was mainly associated with the (+) enantiomer. There seems to be a naloxone-sensitive link in the mechanism of action of (−)-pentazocine and meperidine since the proconvulsant effects of these analgesics were enhanced. That such enhancement was not obtained with compounds having minimal narcotic analgesic activity [normeperidine and (+)-pentazocine] may indicate the possibility of subdivision within group 4. In this regard, Gilbert and Martin (*13*) view meperidine and normeperidine as having different modes of action in producing convulsions in mice.

Our results with conventional analgesics have laid the foundation for future in vivo comparisons of novel synthetic endorphin analogs. The influence of various endorphins on chemically induced convulsions in rats has not yet been reported (*14*). Our model also offers an opportunity to define the specificity of new narcotic antagonists (*15*) in a simple but unique system in which receptors mediate effects that are (i) antagonized, (ii) enhanced, or (iii) unaffected by naloxone. Antagonists with profiles different from naloxone have been a recurring need; their availability will probably be critical for discriminating opiate receptors just as the advent of specific antagonists was associated with major advances in the areas of, for example, adrenergic and histaminergic pharmacology (*16*).

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7. The flurothyl solution was infused onto a gauze pad positioned in a perforated metal basket attached to the underside of the screw cap. M. W. Adler has reviewed the pharmacology of flurothyl [in *Current Concepts in Psychopharmacology*, W. Essman and L. Valzelli, Eds. (Spectrum, New York, 1975), vol. 2, pp. 31–61].
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