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A Photochemical Affinity-Labelling Reagent for the Opiate Receptor(s)

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SUMMARY

A radioactive analogue of levorphanol, [^{3}H]*N*-β-(*p*-azidophenyl)ethylnorlevorphanol (compound **6**), has been synthesized as a photochemical affinity label for the opiate receptor site(s). It has potent opiate-like pharmacological activity in whole mice and isolated intact guinea pig ileum. Upon photolysis of the analogue in the presence of bovine serum albumin as a test protein, radioactivity is incorporated into the protein. Photolysis of the analogue in the presence of total particulate matter from whole mouse brain results in incorporation of radioactivity into a pellet insoluble in water and organic solvent. This incorporation is significantly but incompletely blocked by both levorphanol and dextrorphan. Incorporation into guinea pig ileum longitudinal muscle strips is also observed but is not significantly blocked by levorphanol or dextrorphan. The implications of the results are discussed.

INTRODUCTION

The location and isolation of drug receptors from highly complex biological systems is one of the most formidable problems of current molecular pharmacology. Conventional affinity site-labeling methods (1–3) used for purified enzyme systems are generally inadequate for the "intact receptor system" for two major reasons. First of all, the formation of an irreversible complex or covalent linkage to the receptor disrupts the normal receptor function. Although this altered biological response in itself indicates probable receptor site occupation, a reactive affinity label when presented with an organized multicomponent system may, in fact, bind to many substituents, any of which could interfere with the normal receptor response. Confirmation is needed that the label is capable of interacting at the site of interest and the biological response is generally the only available assay.

Second, even if it were assured that an affinity label could reach a specific receptor, it might simultaneously interact and bind to "nonspecific sites," so that attempts to isolate the true receptor would be complicated by nonspecifically bound material. In addition, binding to the true receptor may not occur at all if there are no functional groups in the site to which the affinity label can chemically bind.

In order to circumvent these difficulties, a method of affinity labeling for receptors is needed in which a proposed label can first be tested for biological activity and subsequently be bound to specific receptor sites. Such a method using compounds which are

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chemically inactive until irradiated by ultraviolet light has been introduced for active site labeling of enzymes by Westheimer and co-workers (4) and is described as "photochemical labeling." Photolysis converts an unreactive compound into a highly reactive intermediate which can covalently bind to nearby substituents, including insertion into the C—H bonds of alkyl groups. Several studies utilizing photochemical labeling have been reported.

Westheimer and co-workers (4) used diazoacyl compounds to photolytically generate reactive carbenes as probes for the active sites of chymotrypsin, trypsin, and alcohol dehydrogenase. Aryl azides were introduced by Fleet, Knowles, and Porter (5) to study binding to a specific antibody against a phenyl azide derivative. Antibody-combining sites were also investigated by Converse and Richards (6), using diazoacyl compounds. Recently Brunswick and Cooperman (7) prepared photochemical labels related to cyclic AMP and extensively examined binding to purified phosphofructokinase as a preliminary study to binding in more complex systems. Only Singer and co-workers (8) have reported the use of this approach to investigate more complex systems of macromolecules (acetylcholinesterase and acetylcholine receptor) in intact membranes.

The earlier reports encouraged the utilization of this technique to investigate a receptor which is as yet not located, isolated, or even functionally understood—the opiate receptor(s).[2] Other approaches to irreversible binding of the opiate receptor have been reported by May *et al.* (9) and Portoghese *et al.* (10). In addition, the reversible stereospecific interaction of levorphanol is being utilized by Goldstein *et al.* (11). The application of photochemical labeling to the complex systems required to study the opiate receptor was made attractive by the following possibilities. (a) A potential photochemical label could be tested for pharmacological activity by established standards for opiate-like compounds. This would verify the ability of the compound to occupy the desired receptor sites *in vivo*. (b) Subsequent irradiation of a suitable tissue preparation *in vitro* could result in covalent binding of the label largely to those macromolecules with which it is in contact. Nonspecifically, loosely bound label, when activated, could react with solvent and be easily separated. Since the chemical groups in the opiate receptor sites are unknown and very possibly hydrophobic, a photochemical label which can bind to a wide variety of substituents is suitable.

This paper reports the synthesis of a radioactive opiate-like photochemical label, its pharmacological testing, and its incorporation upon photolysis into biological material.

[2] The opiate receptor will henceforth be referred to in the singular, although there certainly may be more than one chemical species.

METHODS

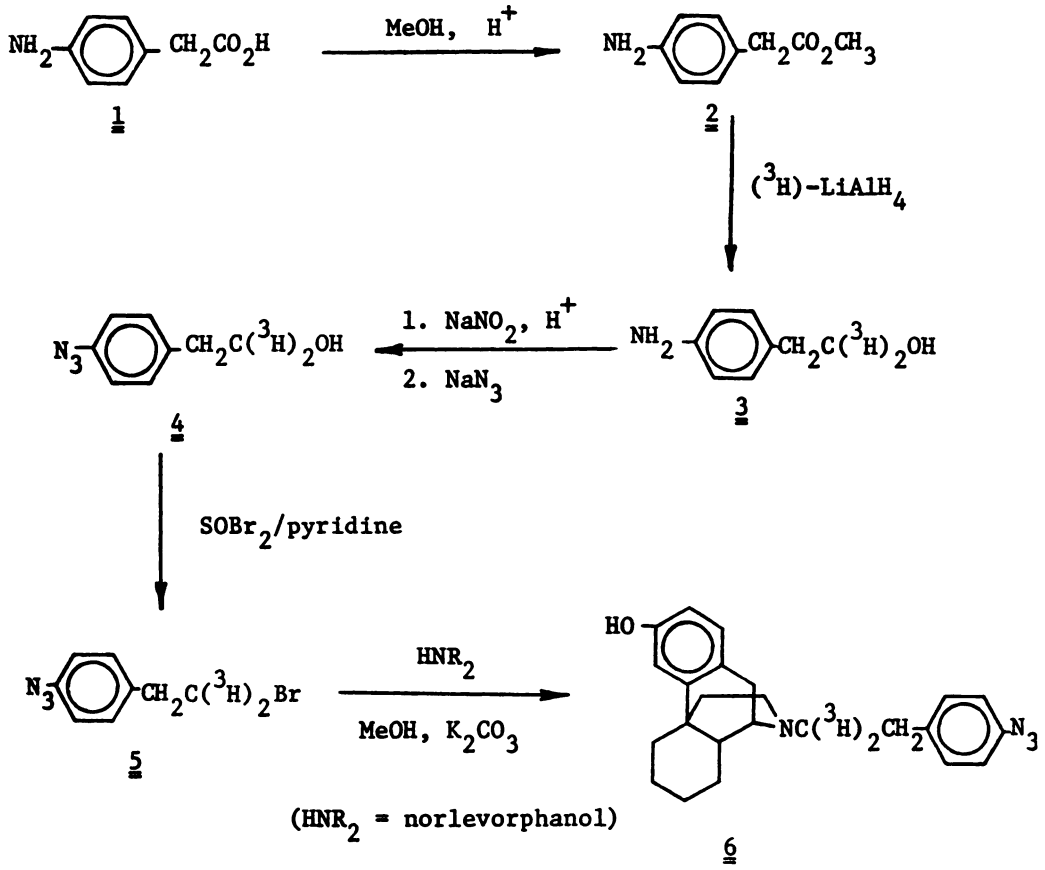
General

Radioactivity was determined by liquid scintillation counting (Packard Tri-Carb scintillation counter) in naphthalene-xylene-dioxane mixture (12) with Hyamine hydroxide (Packard) or ethanol as appropriate for solubilization. Counting efficiencies were determined with [^{3}H]water or [^{3}H]toluene as internal standard.

Levorphanol tartrate, dextrorphan tartrate and norlevorphanol hydrochloride were generously furnished by Hoffmann–La Roche, Inc. Morphine sulfate was purchased from Mallinckrodt Chemicals, and naloxone hydrochloride was a gift from Endo Laboratories. Elemental analyses were determined by the Microanalytical Laboratory, Department of Chemistry, Stanford University.

Synthesis

The synthesis was approached with the following desired characteristics in mind: high probability of pharmacological activity, potential extension to a range of opiate-like compounds, possible incorporation of radioactivity, chemical stability prior to irradiation, and synthetic simplicity. These characteristics were found to be incorporated into the synthetic sequence shown in Scheme 1 and the resulting photochemical label, compound **6**, which is an analogue of levorphanol.



SCHEME I

The choice of a phenethyl moiety was based on structure-activity relationships (13) which demonstrate the high analgesic potency of similar *N*-substituted norlevorphanols. Although the β-(*p*-azidophenyl)-ethyl moiety was only added to norlevorphanol, additional potential photochemical labels could be prepared simply by using other dealkylated opiates. Radioactivity was incorporated using tritium-enriched lithium aluminum hydride in the preparation of **3**. Aryl azides are stable in acid so that water-soluble amine salts can be prepared and stored. Other desirable features of aryl azides are discussed by Fleet, Knowles, and Porter (5). Photolysis of aryl azides produces the very reactive nitrene group which forms covalent linkages by insertion into single bonds (including C—H), hydrogen abstraction, and addition to double bonds (14).

Methyl-p-aminophenylacetate. *p*-Aminophenylacetic acid (4 g, 0.026 mole, Baker Chemicals, recrystallized from ethanol), sulfuric acid (5 ml, concentrated), and absolute methanol (100 ml) were refluxed with stirring for 14 hr. The reaction mixture was cooled and concentrated by rotary evaporation to approximately one-third its original volume. The reaction mixture was adjusted to pH 7–8 by slow addition of saturated $NaHCO_3$. The weakly basic solution was extracted with ether (three times), and the combined ether extracts were washed (NaCl, saturated; two times). The organic layer was dried ($MgSO_4$) and filtered, and the ether was removed by rotary evaporation. A liquid remained (3.8 g, 85% crude yield) which was purified by bulb-to-bulb distillation under vacuum (1 torr): NMR ($CDCl_3$), 3.46 (CH_2) (s, 2H), 3.55 (variable, NH_2) (s, 2H), 3.62 (OCH_3) (s, 3H), 6.60 (aromatic) (d, J = 8 Hz, 2H), and 6.97 δ (aromatic) (d,

J = 8 Hz, 2H); infrared spectrum (film), 1725 (CO_2R), 3375, and 3450 cm^{-1} (ϕNH_2).

p-Aminophenylethanol. Methyl-*p*-aminophenylacetate (2 g, 0.012 mole) was diluted with anhydrous ether (10 ml) and added dropwise to a cooled (0–5°), stirred solution of excess lithium aluminum hydride (1 g, 0.027 mole) in ether (50 ml). The reaction mixture was stirred overnight and then hydrolyzed (H_2O, 10% KOH, H_2O) (15); the ether layer was dried (K_2CO_3) and filtered, and the ether was removed by rotary evaporation. A pale yellow-orange solid remained (1.4 g, 85% yield): m.p. 105–107° [reported (16), 108°]; thin-layer chromatography (silica gel G; $CHCl_3$–CH_3OH, 3:1; I_2), one spot with R_F 0.50; NMR ($CDCl_3$), 2.73 (ϕCH_2) (t, J = 6 Hz), 2.4–3.0 (NH_2, OH) (broad; with 2.73, 5H), 3.80 (CH_2OH)(t, J = 6 Hz, 2H), 6.60 (aromatic) (d, J = 8 Hz, 2H), and 7.00 δ (aromatic) (d, J = 8 Hz, 2H).

p-Azidophenylethanol. *p*-Azidophenylethanol was prepared by the general method of Smith and Bayer (17) by diazotization of the corresponding amine (HCl, $NaNO_2$) followed by addition of NaN_3. However, as no precipitate formed upon addition of NaN_3, the mixture was extracted with ether, the combined ether extracts were shaken with water and dried ($MgSO_4$), and the solvent was removed by rotary evaporation. *p*-Aminophenylethanol (6.4 g, 0.044 mole) yielded a crude oil (5.9 g, 78% yield): thin-layer chromatography (silica gel G; $CHCl_3$; I_2), R_F 0.35; NMR ($CDCl_3$), 2.83 (ϕCH_2) (t, J = 6 Hz, 2H), 3.83 (CH_2OH) (t, J = 6 Hz, 2H), 6.93 (aromatic) (d, J = 8 Hz, 2H), and 7.20 δ (aromatic) (d, J = 8 Hz, 2H); infrared spectrum (film), 2125 (—N_3), and 1050 cm^{-1} (—CH_2OH); ultraviolet spectrum (methanol), λ_{max} 250 (13,000), 280 (2340), and 290 nm (1560) (18). As the liquid was pure by NMR, it was used directly in the subsequent reaction.

A solution of *p*-azidophenylethanol in methanol (34 mM) was photolyzed as described below, and nitrogen evolution was measured by a gas burette. The azide was at least 80% decomposed within 10 min, and the solution turned from pale yellow to dark orange.

The thermal (>80°) and photolytic decomposition of aryl azides made it necessary to employ appropriate precautions in all subsequent reaction procedures.

β-(p-Azidophenyl)ethyl bromide. Thionyl bromide (5 g, 0.024 mole), prepared as described by Frazer and Gerrard (19), was diluted with anhydrous pyridine (5 ml) and cooled (0–5°). It was added slowly dropwise to a cooled (0–5°) and stirred solution of *p*-azidophenylethanol (3 g, 0.018 mole) in anhydrous pyridine (10 ml). Pyridine hydrobromide precipitated almost immediately. After stirring for 30 min at room temperature, the reaction mixture was warmed to 40° for 2.5 hr to decompose the intermediate sulfonyl bromide. The solution was cooled and extracted with ether (four times), and the combined extracts were washed with dilute HCl until pH paper indicated that the aqueous layer was distinctly acid. The organic layer was washed ($NaHCO_3$, saturated, two times; NaCl, saturated, one time), dried ($MgSO_4$), and filtered, and the solvent was removed by rotary evaporation at room temperature. A crude liquid product remained (1.4 g, 34% crude yield). The product was contaminated with an unidentified yellow solid believed to be sulfur and a product tentatively identified as an ester of sulfurous acid. Reverse addition of reagents is suggested to eliminate this side product.

The crude product was purified by preparative thin-layer chromatography (silica gel HF_{254}; $CHCl_3$). The desired product as determined by NMR had an R_F of 0.83 and it was shown by infrared spectroscopy that the azide function did not decompose during the purification: NMR ($CDCl_3$), 3.13 (ϕCH_2) (t, J = 6 Hz, 2H), 3.53 (CH_2Br) (t, J = 6 Hz, 2H), 6.93 (aromatic) (d, J = 8 Hz, 2H), and 7.20 δ (aromatic) (d, J = 8 Hz, 2H); infrared spectrum (film), 2125 cm^{-1} (—N_3); ultraviolet spectrum (methanol), λ_{max} 250, 280, and 290 nm; bromine analysis: calculated 35.34%, found 35.59%.

N-β-(p-Azidophenyl)ethylnorlevorphanol (6). Norlevorphanol hydrochloride hemihydrate had the following properties: ultraviolet spectrum (ethanol), λ_{max} 219 (7820), 282 (2320), and inflection 288 nm (2120); infrared spectrum (KBr), 3200, 2920, 2500,

3500–2250 (broad), 1640–1440 (three bands), and 1240 cm^{-1}; partial NMR, 1.2–1.9 (alkyl, broad), 6.80, 6.95, and 7.10 δ (aromatic).

Anhydrous potassium carbonate (690 mg, 5 mmoles) was added with stirring to a solution of norlevorphanol hydrochloride hemihydrate (688 mg, 2.4 mmoles) which was dissolved in absolute methanol (10 ml) (20). β-(*p*-Azidophenyl)ethyl bromide (540 mg, 2.4 mmoles, purified by thin-layer chromatography) was then added. The reaction flask was protected from light and gently heated (42 ± 5°). The course of the reaction was conveniently monitored by thin-layer chromatography (silica gel GF, 2 × 8 inches; $CHCl_3$–methanol, 90:10): R_F (norlevorphanol) < 0.25, (azido-bromide) 0.90, (product) 0.55. The product which developed during the course of the reaction was intensely ultraviolet-absorbing on fluorescent silica gel, as was the azido-bromide but not norlevorphanol. After 90 hr, the reaction mixture was cooled, filtered, streaked directly onto a preparative thin-layer chromatography plate (silica gel HF_{254}), and eluted with $CHCl_3$–CH_3OH (90:10). The band corresponding to the product was collected and extracted with $CHCl_3$–CH_3OH (3:1). The major portion of the silica gel was removed by filtration through sintered glass, and the filtrate was taken to dryness by rotary evaporation at room temperature. To remove remaining traces of silica gel, $CHCl_3$ was added and the solution was filtered again. The filtrate was concentrated, and a solution of ether saturated with HCl gas was added to precipitate the amine hydrochloride salt. The precipitate was collected by filtration and shown by thin-layer chromatography to form one spot corresponding to the reaction product. The chromatographically purified product had the following properties which identified it as the desired product: m.p. 80° (decomposition); NMR ($CDCl_3$) (free base), ratio of aromatic to alkyl hydrogen, 8:20; aromatic—6.93 (d) and 7.20 (d) ($-\phi N_3$); 6.80, 6.95, and 7.10 δ(norlevorphanol); alkyl—2.8–3.6 (7H) (α to N; $CH_2\phi N_3$) and 1.0–2.6 δ (13H)(alkyl norlevorphanol); ultraviolet spectrum (methanol), λ_{max} 255 (14,580), 280 (4482), and 289 nm (3527); infrared spectrum (KBr), 2125 cm^{-1} ($-N_3$); low-resolution mass spectrum (Atlas), 360 (M^+-N_2) and 256 ($M^+-CH_2\phi N_3$, from cleavage β to N). The product was recrystallized with difficulty from water–methanol.

$C_{24}H_{29}N_4OCl$

Calculated: C 67.92, H 6.84, N 13.20

Found: C 67.09, H 6.89, N 13.10

Preparation of [^3H]Analogue **6**

^3H-Enriched $LiAlH_4$ (New England Nuclear, 100 μCi/μmole) was used to prepare ^3H-labeled *p*-aminophenylethanol with the following modification in addition to reagents: the ester was first added to a solution containing 2 eq. of $LiAlH_4$ and stirred for 30 min. [^3H]$LiAlH_4$ [1.9 mg (0.05 mmole) for 2 g (12 mmoles) of distilled ester] was then added, and the mixture was stirred overnight at room temperature. Remaining equivalents of $LiAlH_4$ were added, and the procedure was continued as described previously. The final product had a specific activity of 0.75 μCi/μmole.

Photolysis

Photolyses were carried out using a Hanovia medium-pressure mercury arc lamp with a Kimax sleeve in a quartz immersion well. Mixtures to be photolyzed were immersed in a water bath maintained at 20–24° surrounding the immersion well, and stirred when appropriate with a variable-speed mechanical stirrer. Photolysis mixtures were contained in Kimax or Pyrex test tubes. Times of exposure were 10 min for BSA experiments and 15 min for the total particulate and longitudinal strip experiments.

Determination of ED_{50} for Analogue **6**

Male Simonsen mice (25–40 g) were randomized into groups of nine; one group was used for each dose. Mice were weighed and doses within each group were adjusted to a constant milligram per kilogram value (free base concentration). Analgesia was tested by the hot plate method as described by Goldstein and Sheehan (21). The temperature of the hot plate was maintained at 57 ± 1°. Tests were performed 30 min after

intraperitoneal injection of the drug. A positive analgesic response was recorded for mice which failed to react by licking their paws within 30 sec. ED_{50} was calculated using the method of Litchfield and Wilcoxon (22); five dose values were used. Running activity was measured as described by Goldstein and Sheehan (21), but without previously screening the mice.

Transmurally Stimulated Guinea Pig Ileum

Experiments on intact isolated guinea pig ileum were performed as described by Kosterlitz and Watt (23).[3] Male guinea pigs (Martin Farms, 250–300 g) were decapitated, and the removed ileum was washed with Krebs' solution. The ileum was mounted with the oral end tied at the top of a platinum anode attached to a mechano-electrical transducer (Grass FT.03) and with the aboral end tied to a tube to allow drainage of mucus. Isometric contractions in response to electrical stimulation (Grass stimulator, 0.1 Hz, 0.5 msec, 20 V) were recorded (polygraph). Baseline tension was maintained at 1 g; twitch tensions were typically 2–3 g. The ileum was allowed to equilibrate for 30 min prior to stimulation and 30 min after starting stimulation before drugs were added. Bath contents (40 ml) were regularly changed at 10-min intervals except immediately after a dose of drug was added. In that case the bath was changed when maximum drug response was observed, 3–4 min later, and then at 10-min intervals.

Incorporation of **6** *Into Bovine Serum Albumin*

Solutions were prepared as described below. In all runs the pH was 7.4, the BSA concentration was 1 mg/ml, the concentration of **6** was 3 μg/ml (free base), and the buffer was at 50 mM. Photolyses were performed as previously described. *Dark run*: A solution of BSA and **6** in Tris buffer was kept in the dark. *Separate run*: A solution of **6** in Tris and a solution of BSA in Tris were separately but simultaneously photolyzed and added together immediately after cessation of photolysis. *Together, Tris*: A solution of BSA and **6** in Tris buffer was photolyzed. *Together, phosphate*: A solution of BSA and **6** in phosphate buffer was photolyzed. All runs were done in triplicate.

In all runs, protein was precipitated with an equal volume of 10% trichloracetic acid and filtered with suction on a glass filter. The precipitate was rinsed with trichloracetic acid (5%, 10 ml) and $CHCl_3$–CH_3OH (2:1, 50 ml), and the filter with precipitate was removed for scintillation counting. Counting efficiency was determined to be 13%. Based on a molecular weight for BSA of 69,000 and a specific activity for **6** of 0.75 μCi/μmole, approximately 1 molecule of **6** was bound per 26 molecules of BSA. This corresponds to about 7% of the total counts present during the photolysis.

Incorporation of **6** *into Mouse Brain Total Particulate Fraction*

Male Simonsen mice (30–35 g) were decapitated and their whole brains were homogenized in cold 0.32 M sucrose with a Dounce homogenizer, using six strokes with an A pestle. The total particulate fraction was obtained by centrifugation (93,000 × *g*, 1 hr) of the homogenate. Mixtures were prepared as described below. In all runs the pH was 7.4, the concentration of **6** was 7.7 μM, the buffer was 50 mM phosphate, and half the brain total particulate was used in each run. Photolyses were carried out as previously described. *Dark run*: a mixture of buffer, total particulate, and analogue **6** was kept in the dark. *Separate run*: A mixture of buffer and total particulate and a mixture of buffer and **6** were photolyzed separately but simultaneously, added together immediately after cessation of photolysis, and allowed to equilibrate for 10 min. *Together*: A mixture of buffer, **6**, and total particulate was photolyzed. *Together—levorphanol/dextrorphan*: A mixture of buffer, **6**, total particulate, and levorphanol or dextrorphan (3.8 mM) was photolyzed. The mole ratio of levorphanol or dextrorphan to **6** was 500:1. All runs were performed in triplicate; the total volume of each was 5 ml.

Identical work-ups were used in all runs. Centrifugations were identical (93,000 × *g*, 1 hr). Mixtures were centrifuged and the supernatants were retained. The pellet was washed with cold phosphate buffer (50 mM,

[3] H. W. Kosterlitz, personal communication.

5 ml) and centrifuged, and the supernatant was combined with aqueous supernatant from the previous centrifugation to form the "water-soluble" fraction. The pellet was then vigorously shaken with $CHCl_3$–CH_3OH (2:1, 5 ml) and centrifuged, and the process was repeated. The two organic supernatants were combined to form the "organic-soluble" fraction; the "pellet" remained. The percentage of total disintegrations per minute was calculated for each fraction. Recovery of radioactivity was greater than 90%.

Incorporation of **6** *into Guinea Pig Ileum Longitudinal Muscle Strips*

Guinea pig ileum longitudinal muscle strips were prepared as described by Paton and Zar (24). The presence of exposed myenteric plexus was verified by photomicroscopy of samples stained with methylene blue. In all runs the pH was 7.4, 50 mM phosphate buffer was used, the concentration of **6** was 3.8 μM, and five strips (1–2 cm long) were added per 5-ml volume. *Dark*, *separate*, and *together* runs were obtained as previously described. In *Together—levorphanol/dextrorphan* runs, the concentration of levorphanol or dextrorphan was 3.8 mM. The mole ratio of levorphanol or dextrorphan to **6** was thus 1000:1. Mixtures were photolyzed as previously described.

Strips were easily transferred with tweezers and were washed according to the following sequence: distilled water (10 ml/wash; two times for 15 min, one time for 1 hr), 10% alcoholic formalin (10 ml/wash; two times for 15 min, one time for 1 hr), and distilled water (10 ml; 15 min). Each strip was spread flat on a glass slide and its area was traced. It was then removed for scintillation counting. The traced area was measured by a planimeter (Keuffel & Esser). The area was chosen to normalize the radioactivity data on each strip in part to provide information useful for radioautography experiments.

RESULTS

Pharmacological Activity

Mice. The opiate activity of **6** was tested in male Simonsen mice. Mice displayed typical opiate-induced behavior: analgesia, running activity, and Straub tail phenomenon. Data on analgesia (hot plate method) were obtained using five groups of nine mice each. The ED_{50} for analgesia was 0.78 (0.62–0.97 mg/kg, free base) (95% confidence), as determined by the Litchfield-Wilcoxon method (22). This compares to an ED_{50} for levorphanol tartrate of 2.8 (1.8–4.4 mg/kg) (95% confidence) determined in the same manner (21). The potency ratio of analogue **6** to levorphanol was 3.6 (2.2–5.9) (95% confidence). The slopes of log dose-response curves for **6** and levorphanol were parallel within experimental error. The duration of analgesia at the ED_{50} dose was between 30 min and 1 hr. Running activity

TABLE 1

Effect of analogue **6** *on transmurally stimulated guinea pig ileum*

See METHODS and ref. 23; also text footnote 3.

Compound	Depression[a]	Onset[b]	$t_{1/2}$ for recovery[c]
	%	*sec*	*min*
Morphine (sulfate), 140 nM	21	30	10
Levorphanol (tartrate), 100 nM	37	60	15
6 (hydrochloride), 148 nM	47	60	>45[d]
Naloxone (hydrochloride), 380 nM, followed by **6**, 148 nM	0		
6, 148 nM, followed by naloxone (hydrochloride), 380nM	Reverses depression		

[a] Data for morphine, levorphanol, and **6** are from a single preparation. Similar results were obtained in repeated runs. Compounds were given in the order listed.

[b] Time to reach maximum depression.

[c] Half-time for recovery to full sensitivity to same dose of drug. Recovery to electrical stimulation in all cases occurred before recovery to drug sensitivity.

[d] When **6** (180 nM) was given, there was no response 1 hr later from morphine (140 nM). An equivalent dose of **6** (180 nM) given 1.5 hr after the original dose of **6** elicited only 19% of the previous effect.

was observed within 6 min after injection and at the ED_{50} continued for 1.6 hr.

In addition, one group of nine mice was treated with naloxone (2 mg/kg, subcutaneously) 10 min before injection of **6** (1.2 mg/kg, intraperitoneally). Whereas all mice given this dose of **6** without prior treatment showed analgesia, none of the naloxone-treated mice did 30 min after injection of **6**, and none showed running activity or Straub tail.

Guinea pig ileum. The pharmacological properties of analogue **6** on transmurally stimulated isolated guinea pig ileum were also determined; isometric contractions were recorded (23).[3] A summary of results is provided in Table 1. Analogue **6** depresses the twitch height of the transmurally stimulated ileum in concentrations comparable to morphine and levorphanol. It reaches its maximum effect more slowly than morphine. Although nearly full recovery to electrical stimulation occurred within 30 min, recovery of sensitivity to a repeated dose of **6** was exceptionally prolonged. Prior treatment with naloxone inhibited the depression of the twitch, and when given after a dose of **6**, naloxone immediately reversed the depression.

The responses of whole mice and electrically stimulated guinea pig ileum to the levorphanol analogue **6** clearly demonstrate its ability to interact with the opiate receptor. Recovery from the opiate effects presumably indicates the reversibility of binding of the nonphotolyzed photochemical label.

Binding of Photochemical Label to Biological Material upon Photolysis

Binding to bovine serum albumin. Earlier reports (4, 7, 8) using photochemical labeling described the necessary control experiments required to verify incorporation of the label into macromolecular species upon photolysis. In the present studies, the following minimum criteria for covalent binding of radioactive photochemical label to biological material were adopted. (a) There should be no incorporation of activity when substituents (label and biological material) are combined but not photolyzed (*dark* run). This control also determines the effectiveness of wash procedures to remove noncovalently bound material. (b) When the label and biological material are photolyzed separately and then combined immediately after photolysis, no incorporation of radioactivity should occur (*separate* run). This control assures that no photolysis products are subsequently tightly bound to the biological material. (c) When label and biological material are combined and photolyzed, there must be incorporation of radioactivity into biological material using purification procedures which have been shown by the first two criteria above to remove unbound label (*together* run).

TABLE 2

Bovine serum albumin labeling with [^3H]analogue 6

The concentration of **6** was 7.7 μM; the BSA concentration was 14 μM. See METHODS for description of runs. Values are the means ± standard errors of triplicate runs.

Run	Radioactivity
	cpm/mg BSA
Dark	9 ± 1
Separate	32 ± 3
Together, Tris	121 ± 16
Together, phosphate	130 ± 30

Bovine serum albumin was chosen as a test protein presumed to have no specific binding related to opiate activity. A summary of results is given in Table 2. It is clear that radioactivity is incorporated into bovine serum albumin upon photolysis, which demonstrates that "nonspecific" labeling can occur.

Binding to mouse brain total particulate fraction. Total particulate matter of whole mouse brain homogenate was obtained and photolyzed in the presence of analogue **6** with the variations listed in Table 3 and METHODS. The following observations were made. The results of the *dark* run and *separate* run with respect to the change in distribution of counts from the organic to the aqueous phase upon photolysis is consistent with unpublished results from this laboratory which demonstrated the high water solubility of the photolysis products. The *separate* run clearly demonstrates that photolysis products can bind tightly to the

TABLE 3

Distribution of radioactivity; photolysis of mouse brain total particulate and [³H]analogue 6

The concentration of **6** was 7.7 μM. One-half brain was used per run. For detailed description of runs, see METHODS. Runs 2–5 were photolyzed for 15 min. Runs 1–3 were in the absence of protector; runs 4 and 5 included levorphanol and dextrorphan, respectively, at a concentration of 3.8 mM. Values are the means ± standard errors of triplicate runs.

Run	Radioactivity incorporated			
	Water-soluble[a]	Organic-soluble[a]	Pellet[a]	
	% *dpm*	% *dpm*	% *dpm*	
1. *Dark*	11.9 ± 0.3	85.8 ± 0.4	2.2 ± 0.1	
2. *Separate*	48.1 ± 3.6	30.8 ± 4.3	21.1 ± 1.6	14.6
3. *Together*	19.4 ± 0.8	45.1 ± 1.1	35.7 ± 1.5	
4. *Together—levorphanol*	23.7 ± 1.1	49.8 ± 4.0	26.0 ± 2.4	9.7
5. *Together—dextrorphan*	25.6 ± 1.0	45.2 ± 2.0	29.2 ± 1.0	

[a] For detailed description, see METHODS.

pellet (approximately 21%) and are not effectively removed by the procedure as described.

The increase in radioactivity in the organic phase upon photolysis of **6** in the presence of total particulate (*together* vs. *separate*) may reflect covalent binding to $CHCl_3$–CH_3OH extractable biological components, but the data as presented give no indication of the change in efficiency of light absorption by **6** in the presence of a heterogeneous mixture of total particulate. The approximately 15% difference in the pellet observed between *separate* and *together* runs may be a *minimum* net incorporation because of the possibility that less of **6** is photolyzed in the *together* runs relative to the *separate* run.

There is no significant difference in the radioactivity observed in the organic phase in the three *together* runs. Of greater interest is the change in count distribution between the pellet and the water-soluble fractions for the *together* runs, depending on the presence or absence of dextrorphan or levorphanol as "protectors" of binding sites. There are significant differences (95% confidence) between *together* and *together—levorphanol* runs and between *together* and *together—dextrorphan* runs in both the water-soluble and pellet fractions. When levorphanol or dextrorphan is present, radioactivity increases in the water-soluble fraction and decreases in the pellet relative to the *together* run (which has no "protector").

TABLE 4

Incorporation of [³H]analogue 6 into guinea pig ileum longitudinal muscle strips upon photolysis

The concentration of **6** was 3.8 μM. See METHODS for description of runs. Values are means ± standard errors.

Run	Radioactivity incorporated[a]
	dpm/cm²
Dark	42 ± 10
Separate	91 ± 12
Together	266 ± 33
Together—levorphanol	241 ± 29
Together—dextrorphan	371 ± 37

[a] "cm²" refers to the measured area of the strip.

This is what would be expected if the "protector" is preventing covalent binding to macromolecular binding sites in the pellet upon photolysis. The effectiveness of the protection, however, is not complete.

Binding to guinea pig ileum longitudinal muscle strips. Guinea pig ileum longitudinal muscle strips with attached myenteric plexus have been shown to be sensitive to opiates in the manner described for the intact ileum (24). The longitudinal strips were chosen for two main reasons: (a) as opposed to mouse brain homogenate, the strips provide a simpler system which can be handled under conditions *in vitro* and (b) the longitudinal strips are suitable for radioautography.

Intact strips were photolyzed in the presence of analogue **6** with the variations listed in Table 4 and METHODS. Clearly, radioactivity is incorporated into the strips in the *together* runs relative to the *dark* and *separate* controls. Yet blocking of the incorporation is not observed when high concentrations of dextrorphan or levorphanol are present during the photolysis.

DISCUSSION

The aryl azido analogue of levorphanol, **6**, certainly fulfills in part the potential advantages of this approach for labeling the opiate receptor as described under INTRODUCTION. It does interact with the opiate receptor *in vivo* as evidenced by its pharmacological activity prior to photolysis. Irreversible incorporation of radioactivity into biological material occurs upon photolysis *in vitro*. However, evidence for increased specificity of binding by this method over more conventional approaches is not provided by the observations reported here.

Levorphanol fails to effectively block the incorporation of radioactivity into mouse brain total particulate or guinea pig ileum longitudinal muscle strips. Several explanations are possible. First of all, levorphanol may simply not compete well with **6** for binding sites. Analogue **6** is more potent pharmacologically and more lipid-soluble than levorphanol and hence may have a greater affinity for receptor sites. Second, levorphanol and analogue **6** may occupy *different* nonspecific binding sites. One also cannot eliminate the possibility from the present data that the specific sites are not saturated with the concentrations of levorphanol used. As an estimate of the number of molecules of **6** bound, 15% net incorporation of the analogue into the pellet of the mouse brain experiments (see Table 3) corresponds to 2.5×10^{16} molecules bound per brain. This value is greater than estimates of the number of receptor sites, which range from 7×10^{11} sites per brain (rat) (25) to 6×10^{14} sites per brain (mouse) (11).

A more likely explanation for the failure of levorphanol to block incorporation is that there is extensive nonspecific labeling. In fact, incorporation of radioactivity into BSA upon photolysis in the presence of analogue **6** clearly demonstrates that nonspecific labeling can occur. The wide range of reactions available from the reactive nitrene intermediate is an advantage when the affinity-labeling reagent is located within the receptor site, but may be a disadvantage otherwise, especially if loosely bound label does not react preferentially with solvent. Whether the energy of the nitrene in this system in sufficiently high for it to react with solvent has not been demonstrated. The number of nitrenes generated in an experiment is a function of many variables (such as concentration, time of irradiation, and wavelength), but within a specified experimental procedure the number should be constant. Consider the explanation of the results diagrammed in Fig. 1. If all the photochemically generated nitrene can react with nonspecific sites in preference to solvent, then the net incorporation will be the same whether or not the specific receptor site is blocked by levorphanol.

Levorphanol does, on the other hand, partially block the incorporation into the pellet in the mouse brain experiments (see Table 3). The apparent partial effectiveness of dextrorphan as a protector prompts the question whether it can occupy the receptor site although it does not elicit the opiate response.

It should be pointed out that Westheimer and co-workers (4e) recently observed in an enzyme system that a very high affinity for the site of interest may be required for the success of the photochemical labeling technique. It is absolutely crucial to demonstrate in a system containing the opiate receptor that protection of the receptor sites during photolysis can be achieved.

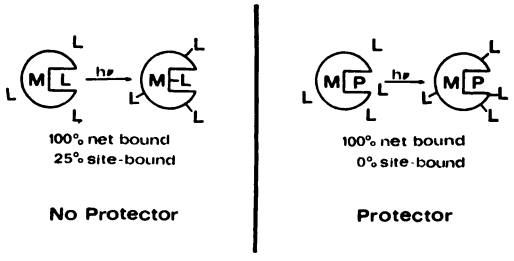


FIG. 1. *Possible mechanism of incorporation of a photochemical label (L) into a macromolecule (M) with and without the presence of a site protector (P)*

Experiments with this objective are in progress. The preparation of higher specific activity analogue **6** would permit the use of concentrations well below 1 μM. Radioautography using the longitudinal muscle strips of guinea pig ileum is being employed as an independent route to determine whether there is cellular localization of radioactivity.

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