diazomethane is eliminated. The precision of the macromethod is better than that reported for the GLC assay (2), while that of the micromethod is equal to that of the GLC method. The micromethod permits monitoring of drug concentrations in small volumes of plasma, making pharmacokinetic studies in rats and children feasible.

As reported for the GLC method (2), this procedure can be used to determine oxaprozin concentrations in urine by extracting the free drug at pH 7 rather than at pH 2. This modification is necessary in both urine and rat plasma[11] to avoid the partial extraction of oxaprozin glucuronide.

[11] F. W. Janssen and S. K. Kirkman, unpublished data.

Due to the chromatographic separation of oxaprozin from its major metabolites, no other modification in the extraction procedure is necessary.

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Electronic Study of Receptor Binding of Analgesic Aryl Moiety II: Prodine Analogs

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Abstract □ Analogs of the prodine analgesics were prepared and tested for analgesic activity. A good correlation seems to exist between the energy level of the highest occupied molecular orbital and biological activity. The energy level of the highest occupied molecular orbital of the aryl moiety of these analogs may permit a charge transfer interaction between the aryl groups of the analgesic molecules and their receptors with the aryl groups acting as charge donors.

Keyphrases □ Prodine analogs—synthesis, testing for analgesic activity, correlation between activity and energy level of highest occupied molecular orbital of aryl moiety □ Analgesic activity—prodine analogs, correlation between potency and energy level of highest occupied molecular orbital of aryl moiety □ Charge transfer interactions—prodine analogs, correlation between analgesic activity and energy level of highest occupied molecular orbital of aryl moiety, complex formation between aryl moiety and receptor

The synthesis of 1-methyl-4-(3-thienyl)-4-propionoxypiperidine (I) and its 2-pyridyl analog (II) was reported previously (1). A similar type of replacement of the benzene ring by heterocyclic rings, such as pyridine, also was reported for propoxyphene, tetralins, and chromanes (2). Compounds I and II were designed to study the receptor binding of the analgesic aryl moiety. The ED_{50} data obtained for I and II at 3.9 and 16.0 mg/kg, respectively, using the mouse–hot plate method and subcutaneous administration pointed to the possible interaction of the aryl group by forces (1) other than van der Waals forces (3, 4) or hydrophobic bonding (5). No further attempt was made to identify these possible forces.

BACKGROUND

Organic compounds containing aromatic rings form charge transfer complexes with other compounds, and they may act as donors or acceptors of the charge (6). Some biochemicals were found to interact in the biological system through the formation of charge transfer complexes (7). Their role as charge donors or acceptors depends on their respective highest occupied molecular orbital or lowest empty molecular orbital energy levels. Correlations between electron densities and the energy levels of the highest occupied molecular orbital or lowest empty molecular orbital and biological activity were described for cholinesterase inhibitors (8, 9), several antimalarials (10), hallucinogens (11), benzothiadiazine antihypertensives (12), and other compounds (13).

Charge transfer complex formation between the aryl moiety of the analgesic molecules and the analgesic receptor is one possibility for explaining variations in analgesic potency. This possibility can be studied by the synthesis of analogs of I and II with aryl groups having different energy levels for their highest occupied molecular orbital or lowest empty molecular orbital. Study of the analgesic activity of these analogs, expressed as their ED_{50} values, together with that of I and II may give more understanding as to how these groups interact with the receptors.

This paper describes the synthesis and analgesic activity of some prodine analogs and correlates the activity with the energy state of the highest occupied molecular orbital or lowest empty molecular orbital of the aryl groups in these analogs.

EXPERIMENTAL[1]

Formation of Aryl Lithium—The aryl halide (0.1 mole) was dissolved in 100 ml of dry ether, placed in a three-necked 500-ml flask, and cooled with a dry ice–acetone bath. An equivalent amount of *n*-butyl lithium[2] (2 moles in 100 ml of hexane) was added dropwise, with stirring, to the aryl halide solution under nitrogen.

Formation of 1-Methyl-4-arylpiperidine-4-ols (IV–VIII)—To the prepared aryl lithium salts was added, with stirring and cooling at the temperature of a dry ice–acetone bath, a solution of 1-methyl-4-piperidone[2] (0.1 mole, 11.3 g) in 100 ml of dry ether over 20 min. The temperature of the reaction mixture then was allowed to rise to 0° and was maintained for 45 min. The reaction mixture then was poured over 100 g of ice–hydrochloric acid (1:1).

The ether layer was separated and washed twice (~50 ml) with dilute hydrochloric acid, and the washings were added to the aqueous acid layer. The mixture was made basic with 10% NaOH and extracted three times (~100 ml) with ether and once with 100 ml of chloroform. The organic washings were mixed and dried over anhydrous sodium sulfate. Removal of the organic solvents under vacuum gave the required alcohols.

Microanalyses were performed on the pure products after recrystallization from the appropriate solvent or on the quaternary salts. The quaternary salts were prepared by treatment of the alcoholic product with methyl iodide or benzyl bromide in methanol and recrystallization from an alcohol–ether mixture.

The IR spectra (chloroform solution) of IV–VIII showed absorption bands in the region of the alcoholic hydroxyl group at 3400–3150 cm^{-1} and at 3100–3000 and 1600–1500 cm^{-1} due to the aryl rings introduced in the piperidine ring.

Esterification of IV–VIII—A solution of IV–VIII (0.02 mole) in 50

[1] All melting points were recorded in an oil bath and are uncorrected. IR spectra were recorded on a Perkin-Elmer model 257 spectrophotometer. All products had IR spectra in agreement with the assigned structures. The aryl halides (Fluka, AG, Bucks, Switzerland) and toluene were distilled before use. Ether was dried over anhydrous sodium sulfate. Microanalyses were performed at Midwest Microlab, Indianapolis, Ind., or by the Department of Medicinal Chemistry, University of Kansas, Lawrence, Kans.

[2] Fluka, AG, Bucks, Switzerland.

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Table I—Data of IV–XIII

Compound	Salt	Formula	Melting Point	Recrystallization Solvent	Yield, %	Analysis, %	Calc.	Found
IV	Methyl iodide	$C_{12}H_{19}N_2O \cdot \frac{1}{2}H_2O$	245–247°	Methanol–ether	86	C	41.98	42.01
						H	5.83	5.62
						N	8.16	8.05
V	—	$C_{16}H_{19}NO$	185–187°	*n*-Hexane[a]	74	C	79.66	79.48
						H	7.88	7.94
VI	Benzyl bromide	$C_{23}H_{26}BrNO$	207–208°	Methanol–ether	88	C	66.99	67.11
						H	6.31	6.36
						N	3.39	3.28
VII	—	$C_{15}H_{18}N_2O$	195–196°	Benzene	85	C	74.37	74.54
						H	7.43	7.34
						N	11.57	11.33
VIII	—	$C_{10}H_{15}NO_2$	138–139°	*n*-Hexane[a]	90	C	66.29	66.53
						H	8.28	8.38
						N	7.73	7.79
IX	Benzyl bromide	$C_{17}H_{20}BrNO$	205–206°	Chloroform–ether	71	C	61.07	61.01
						H	5.98	6.15
						N	4.19	3.92
X	Benzyl bromide	$C_{21}H_{27}BrN_2O_2 \cdot H_2O$	175–176°	Methanol–ether	88	C	57.66	58.02
						H	6.63	6.50
XI	Benzyl bromide	$C_{26}H_{30}BrNO_2 \cdot \frac{1}{2}H_2O$	161–162°	Methanol–ether	77	C	65.40	65.01
						H	6.49	6.59
						N	2.93	3.19
XII	Benzyl bromide	$C_{26}H_{30}BrNO_2 \cdot \frac{1}{4}H_2O$	164–166°	Methanol–ether	82	C	66.03	66.08
						H	6.45	6.42
						N	2.98	3.13
XIII	Benzyl bromide	$C_{25}H_{29}BrN_2O_2 \cdot H_2O$	190–191°	Methanol–ether	72	C	61.60	61.56
						H	6.36	6.35
						N	5.74	5.66

[a] Skellysolve B.

ml of dry toluene was added dropwise, with stirring at room temperature, to a solution of propionyl chloride (0.06 mole, 5.5 g) in 20 ml of dry toluene over 20 min. The reaction mixture then was refluxed for 4 hr and allowed to stand overnight at room temperature. Removal of the solvent under vacuum gave a white solid, which was treated with 5% sodium bicarbonate solution and extracted three times (~50 ml) with ether and once with 50 ml of chloroform.

The combined extracts were dried over anhydrous sodium sulfate. The organic solvents were removed under vacuum, and the residue was treated with excess methyl iodide or benzyl bromide in methanol. The quaternary salts were recrystallized from methanol–ether or chloroform–ether mixtures. The physical constants and microanalyses are shown in Table I.

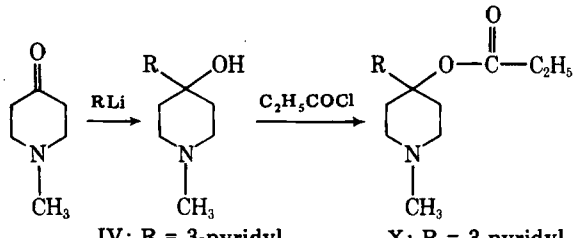
The IR spectrum of IX (chloroform solution) showed absorption bands at 3020, 2900–2800, 1620, and 1600 cm^{-1}. No absorption band was observed in the carbonyl region. The IR spectra of X–XIII (chloroform solutions) all showed strong absorption bands at 1740–1725 cm^{-1}, indicating an ester carbonyl group.

Biological Testing of X–XIII—Compounds X–XIII were tested for analgesic activity using the mouse–hot plate method and subcutaneous administration. The ED_{50} data are shown in Table II.

RESULTS AND DISCUSSION

Chemistry—Analogs of the prodine analgesics were synthesized according to Scheme I.

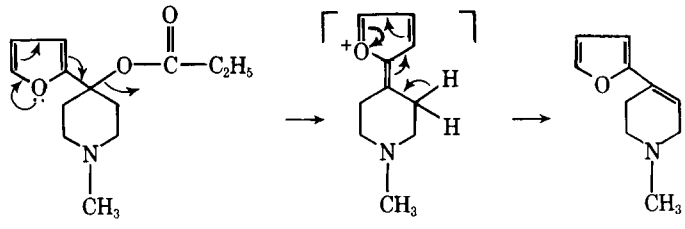
The lithium salts of the aromatic or heteroaromatic rings were prepared (14) by treatment of the corresponding bromo derivatives with an equivalent amount of *n*-butyl lithium. Addition of these lithium salts to



IV: R = 3-pyridyl
V: R = 1-naphthyl
VI: R = 2-naphthyl
VII: R = 3-quinolyl
VIII: R = 2-furyl

X: R = 3-pyridyl
XI: R = 1-naphthyl
XII: R = 2-naphthyl
XIII: R = 3-quinolyl

Scheme I



XIV IX

Scheme II

1-methyl-4-piperidone afforded the corresponding alcohols (IV–VIII) in high yields. Treatment of IV–VII with 3 equivalents of propionyl chloride afforded X–XIII. Similar treatment of VIII did not yield the expected ester; instead, olefin IX was the only product obtained from the reaction.

Evidence for the formation of IX was obtained from its IR spectrum, which did not show any absorption band in the carbonyl region and from microanalysis. Furthermore, treatment of 1-methyl-4-(2-thienyl)piperidino-4-ol with propionyl chloride under the same conditions afforded the same type of olefin (15), which was identified by IR and NMR spectroscopy and microanalysis. Changes in the reaction conditions (*i.e.*, ratio of VIII to propionyl chloride, temperature and time of the reaction, and workup of the crude product and solvent) did not change the situation. These results indicate that the heteroatom of the furan ring participates in the elimination of the propionate group after its formation.

If the mechanism of formation of IX was the facile *trans*-diaxial elimination of water from the alcohol during esterification, the same type of olefin would have been produced in all cases reported in this paper. However, the expected esters were obtained in high yields as the only products from the esterification of IV–VII as well as I and II (1). Since the olefin formation was limited to esterification of VIII and its 2-thienyl analog (15), a different mechanism may be involved in this type of elimination. The suggested mechanism involves the electron pair on the oxygen atom of the furan ring assisting in the elimination of the ester group after its formation to give XIV as an intermediate. Loss of a proton from the piperidine ring followed by a shift of the double bonds would produce IX (Scheme II).

Correlation of Pharmacological Data—Compounds IX–XII were tested for analgesic activity using the mouse–hot plate method and subcutaneous administration. The ED_{50} data are summarized in Table II together with those of I and II. The molecular orbital calculations (16) for the highest occupied molecular orbital and lowest empty molecular

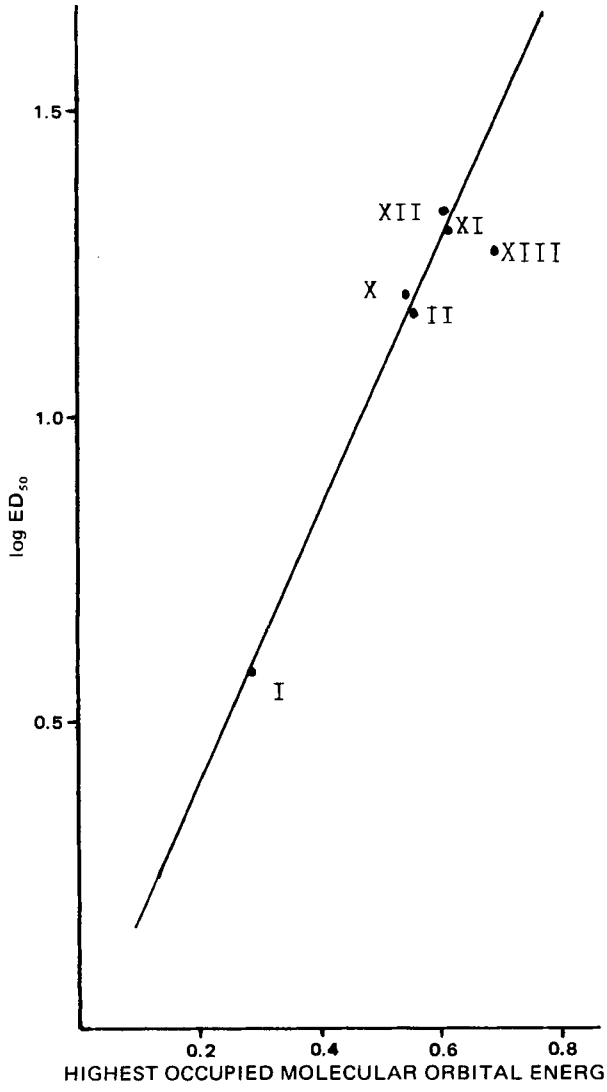


Figure 1—*Plot of log ED_{50} versus highest occupied molecular orbital energy.*

orbital energy levels of aromatic groups present in these molecules also are listed in Table II.

The choice of the aryl groups used in these derivatives was based on the energy level differences of the highest occupied molecular orbital and lowest empty molecular orbital and on the capability of these aryl groups to form charge transfer complexes *in vitro* (17) and *in vivo* (10). The molecular orbital treatment of these molecules was confined to the aromatic planar part, containing the π-electrons. These π-electrons are of primary concern in charge transfer complex formation (18). Other portions of the molecules that are not planar and are not conjugated with the major π-electron system were not included.

The electronic structure indexes considered here are the energy levels in units of the resonance integral (β) of the highest occupied molecular orbital and lowest empty molecular orbital. The energy of the highest occupied molecular orbital is a measure of electron donor ability, and that of the lowest empty molecular orbital is a measure of electron acceptor ability (7). These two energy levels are of primary importance in effecting charge transfer complex formation (7). They also are important in effecting charge transfer complex formation in the biological system (19). The smaller the energy level of the highest occupied molecular orbital, the lower is the energy required to remove a π-electron from the molecule and, thus, the greater are the electron donor properties (19–21). The closer to zero the energy level of the lowest empty molecular orbital, the greater is the electron affinity and, therefore, the greater are the electron acceptor properties (21, 22).

Compound I, the most active in the series, had an ED_{50} of 3.9 mg/kg. The energy levels of the highest occupied molecular orbital and lowest empty molecular orbital of the thienyl group were 0.2928932 and −1.00, respectively (16). These values indicate that the thiophene ring is most likely to form a charge transfer complex in the biological system and acts as a π-electron donor. However, it is the least likely π-electron acceptor because of the high energy level of the lowest empty molecular orbital relative to the other compounds.

Table II—ED_{50} Values and Highest Occupied Molecular Orbital and Lowest Empty Molecular Orbital Energy Data of I, II, and X–XIII

Compound	ED_{50}, mg/kg sc	Energy (π)	
		Highest Occupied Molecular Orbital	Lowest Empty Molecular Orbital
I	3.90	0.2928932	−1.00
II	16.00	0.5602315	−0.5602315
X	16.60	0.5602315	−0.5602315
XI	22.00	0.6180340	−0.6180340
XII	22.50	0.6180340	−0.6180340
XIII	20.00	0.7032833	−0.5270970

Compounds II and X contain a pyridine moiety at position 4 of the piperidine ring. The energy level of the highest occupied molecular orbital of pyridine is 0.5602315 (16), which is lower than that of thiophene. A charge transfer complex of II or X is expected to occur in the biological system in which the pyridine ring acts as a donor but requires a higher energy to transfer the π-electron than that required for the thiophene ring. Thus, a less firm charge transfer complex is expected in this case, which may have been reflected in the ED_{50} values of II and X at 16.0 and 16.6 mg/kg, respectively. The argument presented in comparing the biological activity of I with that of II and X also may hold for naphthalene- and quinoline-containing compounds. The energy levels of the highest occupied molecular orbital of XI–XIII were lower than those for I, II, and X. If a charge transfer complex is expected to occur between the biological system and XI–XIII, they would be weaker than those formed between the thiophene- and pyridine-containing compounds and the biological system. Also, they would require a higher energy for their formation. This difference in the energy requirement may have been the cause of the lower activity of XI–XIII as shown by their higher ED_{50} values.

To test the possibility of a charge transfer complex between the aryl groups of the analgesics and their receptors, the logarithm of the ED_{50} values given in Table II was plotted against the energy values of the corresponding highest occupied molecular orbital. Figure 1 shows a linear relationship between log ED_{50} and the energy of the highest occupied molecular orbital of these compounds. The line is the least-squares line (23) whose equation is $y = 2.2x - 0.04$. This linear relationship may indicate (13) the possibility of a charge transfer complex interaction between these compounds and their receptors, with the aryl groups acting as the charge donors. There was no simple linear relationship when the log ED_{50} values were plotted against the energy values of the lowest empty molecular orbital of these compounds. This finding may exclude the possibility that the aryl groups of these compounds function as electron acceptors.

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Correlation of Hydrophobicity with Protein Binding for Clorobiocin Analogs

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Received April 10, 1979, from the *Biochemistry Department, Pharmaceutical Division, May and Baker Ltd., Dagenham, Essex, RM10 7XS, England.* Accepted for publication February 1, 1980.

Abstract □ A new method of equilibrium dialysis was used to measure the binding of analogs of clorobiocin (18631 R.P.) to human serum albumin. Binding constants and numbers of binding sites on human serum albumin were calculated from the binding data and were used to calculate the percentage of compounds free in equilibrium with 4% albumin. Partition coefficients between *n*-octanol and phosphate buffer (0.05 *M*, pH 7.4) also were measured. A positive linear correlation ($r = 0.918$, $s = 0.240$, and $n = 10$) was obtained between log (bound/free compound) and log partition coefficient.

Keyphrases □ Hydrophobicity—clorobiocin analogs, correlation with protein binding □ Protein binding—clorobiocin analogs, correlation with hydrophobicity □ Clorobiocin analogs—correlation of hydrophobicity with protein binding

Clorobiocin (18631 R.P.) (1, 2) has been shown to be tightly bound to human serum albumin (3). In an effort to reduce this binding, various semisynthetic derivatives of the antibiotic were prepared, and the effect of these changes on the binding was monitored.

BACKGROUND

Binding of clorobiocin to human serum albumin was so high (99.97% with 10^{-5} *M* drug and 4% albumin) that the free drug concentration was in the range of 10^{-8}–10^{-9} *M*. To measure this drug level accurately by physicochemical means, radiolabeling of the drug would have been required. As an alternative, a method was developed whereby low concentrations of albumin were used so that the free drug concentration was sufficiently high for detection by UV spectrophotometry. From a plot of bound *versus* free drug, an iterative computer program gave binding constant values and the numbers of primary and secondary binding sites. These plots were used in a program that calculates the percentage of free drug in equilibrium with whole plasma.

The 4-hydroxycoumarin moiety probably contributes to the binding, since this group played a major part in the binding of the warfarin group of anticoagulants to human serum albumin (4). Various reports, listed by Jusko and Gretch (5), indicated that the hydrophobic nature was important for albumin binding. Accordingly, the partition coefficients of several clorobiocin derivatives were measured, and it was found that their hydrophobicity did play a major role in their binding to albumin.

EXPERIMENTAL

Equilibrium Dialysis—The technique was described initially by Coombs and Coulson (6). Two perspex blocks were screwed tightly together with a cellulose membrane[1] in between to form a complete unit with six compartments. Five units were used to hold 14 concentrations of compounds and two blanks (to allow for nonspecific UV absorbance).

For binding measurements, 2 ml of albumin[2] solution (0.25–2 mg/ml) was placed on one side of the membrane. The concentration of albumin appropriate for each compound was selected on the basis of the estimated partition using the Hansch aromatic substituent constants (7). The compounds[3] were dissolved in methanol at a concentration of 10 m*M* and diluted 1 in 50 in phosphate buffer (0.05 *M*, pH 7.4) to give the maximum concentration used. Further dilutions were made in the phosphate buffer. On the other side of the membrane was placed 2 ml of the compound solution (15–200 μM).

A plastic cap was fitted on each compartment to prevent evaporation. The units were shaken in a water bath for 8 hr at 37°, followed by 8 hr without shaking at room temperature. Aliquots from each side of the membrane were taken for the measurement of concentration by UV absorbance at a wavelength where there was no net change in absorbance on binding.

To examine the effect of methanol on the binding, methanolic and aqueous solutions of the sodium salts of clorobiocin and novobiocin were diluted in the buffer and tested; there was no significant difference in binding. The remaining compounds were dissolved initially in methanol since they were insoluble in water at higher concentrations.

Difference Spectra—The binding of each compound to human serum albumin was followed by UV spectroscopy as described previously (3) to find the wavelength for the measurement of the concentration of each compound, *i.e.*, to find the wavelength at which there was no net change in absorbance. Different albumin samples gave different results when binding clorobiocin derivatives according to the amount of dimer present. Therefore, albumin monomer was prepared using Sephadex G-100 as described previously (3).

Calculations—Binding constants and the number of binding sites were obtained from the binding data with an iterative computer program devised in these laboratories. The raw data were fitted to:

$$y = \frac{n_1 k_1 T}{1 + k_1 T} + \frac{n_2 k_2 T}{1 + k_2 T} \qquad \text{(Eq. 1)}$$

where y is the bound drug; T is the free drug; n_1 and n_2 are the numbers of primary and secondary binding sites, respectively; and k_1 and k_2 are the respective binding constants. The inclusion of a third set of binding sites did not improve the fit.

Arbitrary values for n_1, n_2, k_1, and k_2 were entered, and a standard deviation of the curve from the points was calculated. The value of n_1 is increased by 5%, k_1 and k_2 are kept constant, and the best value for n_2 is selected on the basis of a minimal standard deviation. If this standard deviation is lower than the previous one, then n_1 is increased further by

[1] Spectropor 2, M.S.E./Fisons, Crawley, Sussex, England.

[2] Miles.

[3] Clorobiocin was obtained from Dr. Jolles of Rhone Poulenc, novobiocin sodium was obtained from British Drug Houses, and the analogs were obtained from Dr. D. E. Wright, Dr. C. Smith, Dr. R. J. A. Walsh, and Mr. P. J. Warren of May and Baker Ltd. chemical research laboratories.

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