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Characterization of the Transport System for Benzomorphans in Leukocytes

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SUMMARY

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The biological transport of pentazocine in leukocytes displayed characteristics of an active, carrier-mediated process. Within 5 min the uptake reached cellular equilibrium levels several fold higher than those in the medium. The transport process followed simple saturation kinetics, with an apparent K_m of 40 μM and V_{max} of 100 nmoles/g of cells in 5 sec. The uptake was sodium-independent. At decreased temperatures and in the presence of metabolic inhibitors, the transport of pentazocine was subject to noncompetitive inhibition. For temperatures between 0° and 37° the V_{max} for uptake had a Q_{10} of 1.88. Poisons of glycolysis were more effective in blocking drug uptake than were inhibitors of aerobic energy production, and oxygen deprivation did not affect the transport process. The rate of transport of pentazocine into leukocytes decreased linearly with the fall in cellular ATP content. Uptake of pentazocine in cells depleted of ATP amounted to one-fifth of the uptake in control cells and was equal to that in cells previously treated by heating or freezing. Efflux of the drug from leukocytes was rapid and sensitive to temperature. Exodux appeared to be the result of two first-order processes with greatly different rate constants. Only the higher of these rate constants was affected by temperature, and displayed a Q_{10} of 1.86. Both uptake and exodus exhibited the phenomenon of countertransport, showing transacceleration. Uptake of pentazocine and inhibition of this process at various pH values indicated that the drug was transported when it bore no net charge. Benzomorphan analogues and amines of wide structural variety, excluding quaternary amines or those with acidic character, competitively inhibited the uptake of pentazocine. Additional structural requirements in the uptake process have been elucidated. On the basis of its characteristics, the transport system for benzomorphans in leukocytes represents a novel process for the cellular uptake of amines.

INTRODUCTION

Benzomorphans, compounds exhibiting strong analgesic properties, were developed in the search for pain-relieving drugs with little or no addiction liability (1, 2). Among the benzomorphans pentazocine particularly has found wide clinical application as a potent, markedly fast analgesic (3, 4). This drug rapidly enters the brain of rodents after intraperitoneal (5), subcutaneous (6), or intravenous (7) administration. It was re-

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cently shown that pentazocine is accumulated in rat leukocytes by a process possessing many characteristics of an active transport system (8). Furthermore it was reported that the uptake into leukocytes of pentazocine, as well as of amphetamine, codeine, methadone, and naloxone, occurred through the intervention of a single, carrier-mediated process (9, 10). Pentazocine has also been found to be a potent inhibitor of the uptake of both 5-hydroxytryptamine and metaraminol into blood platelets (11).

The present study provides a detailed characterization of the process by which the uptake and exodus of pentazocine occurred in morphologically typical, nucleated mammalian cells, the rat leukocytes.

MATERIALS AND METHODS

Materials. Tritiated pentazocine (generally labeled) was a generous gift of the Sterling-Winthrop Research Institute, Rensselaer, N. Y. Its specific activity was determined by gas-liquid chromatography (12). Tryptamine, *N*-methyltryptamine, choline chloride, tyramine hydrochloride, ouabain, Tris, ascorbic acid, and iodoacetamide were purchased from Sigma Chemical Company. Phenethylamine was purchased from Aldrich Chemical Company. The central nervous system drugs used in this study were kindly provided by Drs. H. H. Swain and J. H. Woods of the Department of Pharmacology, The University of Michigan.

Reagents and enzymes used for the analysis of metabolites were obtained from Boehringer/Mannheim and Sigma, respectively. Protosol, a tissue solubilizer, was a product of New England Nuclear Corporation. Plasmagel, a modified gelatin solution used for the separation of leukocytes, was obtained from HTI Corporation, Buffalo, N. Y.

Isolation of blood cells. Leukocytes were isolated from the blood of 300-g male Sprague-Dawley rats as described previously (8, 9, 13). Briefly, erythrocytes were separated from the other blood cells by sedimentation in the presence of Plasmagel. The further separation of platelets and leukocytes was carried out by differential centrifugation. The separated leukocytes were obtained within 2 hr after the collection of blood, with an average yield of 59%. The purity and viability of the isolated cells were investigated in detail and ascertained (13). The leukocytes fulfilled the viability criteria of cellular content of K^+, Na^+, and ATP, trypan blue exclusion, and uptake of O_2 immediately after their isolation as well as during incubation at 37° for up to 3 hr (13). Leukocytes were counted either microscopically, using a Spencer Bright-Line hemacytometer, or electronically, in a Cytograf model 6301 instrument (Bio/Physics Systems, Inc., Mahopac, N. Y.). Approximately 10^9 cells corresponded to 1 g, wet weight, and 50 mg of protein.

Protein. Protein was determined according to Lowry *et al.* (14).

Cellular ion content. Aliquots of cellular suspensions corresponding to 2–4 × 10^7 leukocytes were centrifuged in polyethylene tubes at 3000 × *g* for 2 min at 2°. The cellular pellet was washed twice with ice-cold 0.3 M sucrose by resuspension and recentrifugation. The washed pellet was digested with 50 µl of 70% HNO_3 at 80° for 1 hr. After the addition of 5 ml of LiCl reference solution (15 mM) the concentrations of potassium and sodium were determined in a flame photometer.

Determination of metabolites. All operations in these experiments were carried out at 2–4°. Cell suspensions containing 5–10 × 10^7 leukocytes (2.5–5 mg of protein) were centrifuged at 3000 × *g* for 2 min. After the supernatant solution had been discarded, the cellular pellet was disrupted in an all-glass Potter-Elvehjem homogenizer with 500 µl of 0.6 M $HClO_4$. After centrifugation at 1900 × *g* for 15 min, 250 µl of the supernatant fluid were pipetted off and neutralized to pH 7 with a measured volume of 5 M K_2CO_3. The mixture was chilled for 15 min in ice, to allow precipitation of $KClO_4$, the tubes were briefly centrifuged at 1900 × *g*, and the supernatant fluid was decanted and stored at −70° until analysis. Aliquots of these extracts, corresponding to 4–8 × 10^6 leukocytes, were used for the determination of metabolites.

Cellular concentrations of ATP, ADP, AMP, lactate, and glucose were estimated

using enzyme assays coupled to the fluorometric measurment of reduced nicotinamide adenine nucleotides (15). Final concentrations of the reagents in the particular assays were the following. ATP: Tris HCl buffer (pH 7.5), 100 mM; $MgCl_2$, 5 mM; $NADP^+$, 0.05 mM; glucose, 1 mM; BSA, 0.01%; glucose 6-phosphate dehydrogenase, 2 μg/ml; hexokinase, 4 μg/ml. Glucose: Tris HCl buffer (pH 7.5), 100 mM; $MgCl_2$, 5 mM; $NADP^+$, 0.1 mM; BSA, 0.01%; glucose 6-phosphate dehydrogenase, 2 μg/ml; hexokinase, 4 μg/ml. Lactate: hydrazine buffer (pH 9.5), 200 mM; NAD^+, 0.2 mM; BSA, 0.01%; lactate dehydrogenase (beef heart), 40 μg/ml. ADP and AMP: phosphate buffer (pH 7.0), 50 mM; $MgCl_2$, 2 mM; NADH, 0.006 mM; ATP, 0.02 mM; phosphoenolpyruvate, 0.02 mM; BSA, 0.01%; lactate dehydrogenase (skeletal muscle), 8 μg/ml; pyruvate kinase, 0.5 μg/ml; myokinase, 1 μg/ml. Appropriate standards were included in each set of analyses. Progress of the enzymatic reaction was monitored on a recorder to ascertain the rate and completion of the assay.

General experimental procedure. Throughout the experiments a phosphate buffer was used, containing the following millimolar concentrations: NaCl, 133; KCl, 5.3; $MgSO_4$, 1.3; Na_2HPO_4, 13.3; glucose, 10. The pH of this medium was adjusted to 7.4 with 1 N HCl. After isolation, 1-ml aliquots of a suspension of leukocytes in the buffer (5–10 × 10^6 cells, 0.25–0.5 mg of protein) were incubated at 37° for 1 hr. The pH remained constant throughout this period. Cellular uptake was initiated by the rapid addition of 1 ml of buffer medium containing pentazocine at a concentration twice that desired during the incubation. In addition, this solution contained 0.05–0.1 μCi of the radiolabeled drug. Because of the markedly rapid cellular uptake, the incubation was routinely terminated 5 sec after the addition of pentazocine by quickly separating the leukocytes from the incubation medium as described previously (10). Briefly, the cellular suspension was quickly filtered on a Millipore assembly through glass-fiber filters supported by a stainless-steel screen. The cells on the filter were washed with three 6-ml portions of ice-cold 0.9% NaCl. For the determination of radioactivity, the filters were placed on the bottoms of counting vials and the biological material was digested for 1 hr at 60° with 0.3 ml of Protosol. After the addition of 10 ml of toluene scintillation fluid, containing 5 g/liter of 2,5-diphenyloxazole and 0.3 g/liter of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene, the radioactivity was determined in a liquid scintillation spectrometer. Efficiency of counting was determined periodically by using radiolabeled toluene as an internal standard.

The procedure outlined above was partly modified in specific experiments. Although details of the conditions are included in legends for the table and figures, several major modifications are outlined in the following sections.

Effect of pH. To provide buffering capacity between pH 6 and 11, the standard incubation medium was modified to include both phosphate and pyrophosphate as buffers. While the cells, prior to incubation with pentazocine, were suspended in the standard buffer at pH 7.4, the drug was dissolved in a solution of the following composition: NaCl, 133 mM; KCl, 5.32 mM; $MgSO_4$, 1.33 mM; Na_2HPO_4, 6.6 mM; and $Na_4P_2O_6$, 20.0 mM. The pH of the drug solution was adjusted to a value such that mixture of this solution with an equal volume of standard medium at pH 7.4 resulted in a solution with the desired pH.

Efflux of pentazocine. Owing to the rapid exodus of the drug, a technique using quick dilution of the cells was adopted. To load the cells with pentazocine, 0.25-ml aliquots of cell suspension, concentrated 4-fold more than usual, were incubated with an equal volume of a solution of radioactive drug. In order to achieve high cellular concentration of the drug, the loading of leukocytes was carried out at elevated pH (see RESULTS). Exit of pentazocine was initiated by dilution with 4.5 ml of drug-free medium. Also estimated was the efflux at various temperatures. Cells were loaded with drug at 37° as described above. Dilution with medium at 37° or at a lower temperature initiated exodus. After dilution at temperatures below 37°, the final temperature was measured and

designated as the temperature at which exit occurred. In general, because of the markedly rapid cellular exodus of pentazocine, efflux of drug was more conveniently determined at low temperatures.

Countertransport. Countertransport phenomena were investigated during both uptake and efflux of pentazocine. In uptake experiments 0.5 ml of cellular suspension, containing twice the usual concentration of leukocytes, was incubated with the same volume of a solution containing unlabeled pentazocine. In controls the latter solution was drug-free. After equilibrium had been reached between medium and cellular concentrations of the drug, radiolabeled pentazocine was added, and the cellular uptake was determined as usual. The effect of external pentazocine on drug efflux was determined by diluting the cells with medium containing various concentrations of unlabeled drug instead of drug-free medium as described above.

RESULTS

Time course and saturability of uptake. The uptake of [^{3}H]pentazocine by leukocytes was markedly rapid. Within 5 sec the cellular drug content was about 60% of that at equilibrium, and the uptake (60 nmoles/g) was virtually complete 2 min after the addition

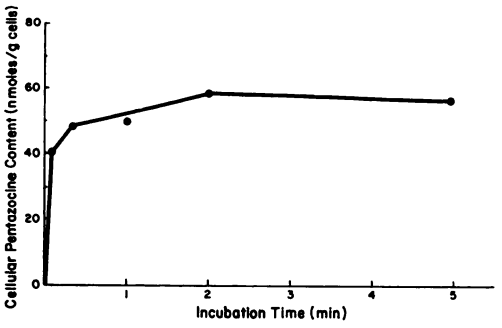

FIG. 1. *Time course of uptake of pentazocine into leukocytes*

Suspensions of cells were incubated at 37° in the standard medium containing 42 μM pentazocine. In addition the medium contained 0.1 μCi of the ^{3}H-labeled drug. At the times indicated, the cells were collected by rapid filtration as described under MATERIALS AND METHODS. The shortest incubation time was 5 sec. Plotted are results of a representative experiment.

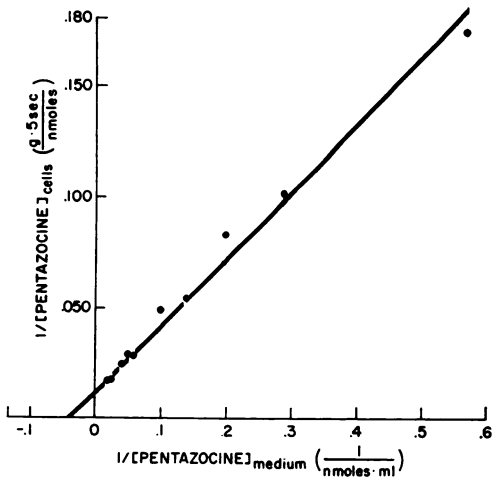

FIG. 2. *Lineweaver-Burk plot of uptake of pentazocine by leukocytes*

The conditions for incubation and collection of cells as well as quantitation of the drug are described under MATERIALS AND METHODS. Each point is the mean of 5–50 experiments, corresponding to 20–200 separate incubations. The apparent K_m and V_{max} values were determined to be 40 μM and 100 nmoles/g of cells·5 sec, respectively.

of pentazocine (42 μM) (Fig. 1). At low concentrations of pentazocine in the medium, the cellular accumulation of the drug was up to 12-fold. The identity of the measured radioactivity with pentazocine was established in initial experiments (8).

For drug concentrations in the medium ranging from 2 to 100 μM, the uptake of pentazocine by leukocytes conformed to simple Michaelis-Menten kinetics (Fig. 2). The apparent K_m and V_{max} for uptake were 40 μM and 100 nmoles/g of cells·5 sec^{-1}, respectively. At millimolar concentrations of the drug in the medium, the uptake deviated from that predicted by Michaelis-Menten kinetics, indicating the existence, under these conditions, of additional mechanisms for the entry of pentazocine into leukocytes.

Effect of temperature. Lowering the temperature of incubation decreased the V_{max} without changing the K_m of the uptake process (Fig. 3). The Arrhenius plot was linear in the range of temperatures studied. The Q_{10} for the V_{max} from three separate experiments was calculated to be 1.88.

Unlike the pronounced temperature effect

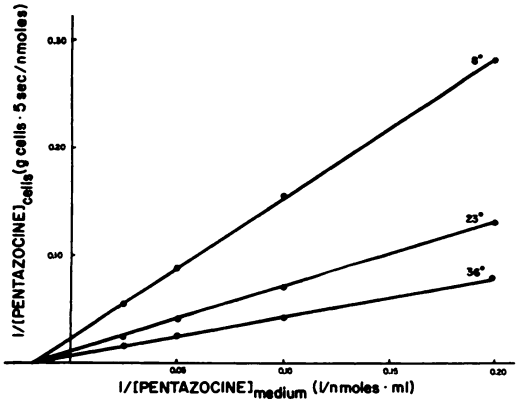

FIG. 3. *Effect of temperature on uptake of pentazocine*

Leukocytes, suspended in the standard medium, were incubated for 60 min at the indicated temperatures. Subsequently radioactive pentazocine was added and the cellular uptake at 5 sec was determined as described under MATERIALS AND METHODS. The temperature of the drug solution was the same as that of the cell suspension to which it was added. Shown are mean values of four separate experiments using different preparations of leukocytes.

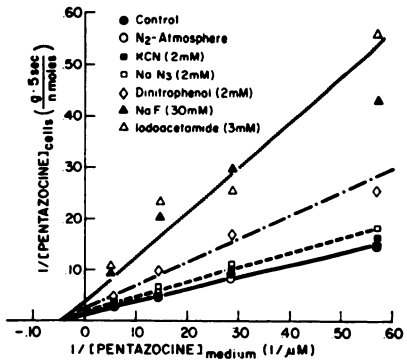

FIG. 4. *Effect of metabolic inhibitors on uptake of pentazocine*

Leukocytes, suspended in the standard medium, were incubated at 37° in the presence of 2 mM concentration each of either KCN, NaN_3, or dinitrophenol, 3 mM iodoacetamide, or 30 mM NaF. The osmolarity of the incubation medium was maintained constant by decreasing the concentration of NaCl equivalently. In separate experiments suspensions of cells were incubated for 60 min in a nitrogen atmosphere. After these treatments the cells were exposed to radioactive pentazocine for 5 sec. The separation of the cells and quantitation of the drug were performed as described under MATERIALS AND METHODS. Each point represents the mean of four experiments.

observed for viable cells, the uptake of pentazocine by heat-treated leukocytes was insensitive to changes in temperature. Also uninfluenced by temperatures in the range 0–37° was the binding of pentazocine to BSA.[2]

Effects of metabolic inhibitors and atmospheric composition. Prior incubation of leukocytes with metabolic poisons resulted in noncompetitive inhibition of pentazocine uptake. Most pronounced effects were obtained with inhibitors of glycolysis (Fig. 4). No significant difference in the cellular uptake of pentazocine was observed if the incubation was carried out in oxygen (18.34 ± 2.35 nmoles/g of cells·5 sec) or in a nitrogen atmosphere (17.90 ± 1.40 nmoles/g of cells·5 sec).

Correlation of drug uptake and energy reserves in leukocytes. The marked reduction of pentazocine uptake in the presence of glycolytic inhibitors mandated a closer investigation of the consequences of such treatment. In addition to elevated levels of glucose and markedly decreased cellular concentration of lactate,[2] leukocytes incubated with NaF were characterized by a strongly depleted content of ATP. The total adenine nucleotide content decreased by 75%, and the cellular energy charge, calculated as ATP + ½ ADP/ATP + ADP + AMP (16), was reduced by 40% relative to control cells. The rate of transport decreased as a linear function of the cellular ATP content (Fig. 5). The cellular concentrations of alkali metals were considerably less sensitive to changes in the levels of ATP. A pronounced decrease in the K^+ content and an increase in the Na^+ content occurred only after the cellular concentration of ATP fell to one-fourth the level found in control cells. Incubation of leukocytes with NaF did not result in either a decrease in the number of cells or an increase in their permeability to trypan blue.[2]

Sodium requirement of uptake process. Neither the exclusion of NaCl from the incubation medium nor prior incubation of leuko-

[2] M. J. Marks and F. Medzihradsky, unpublished observations.

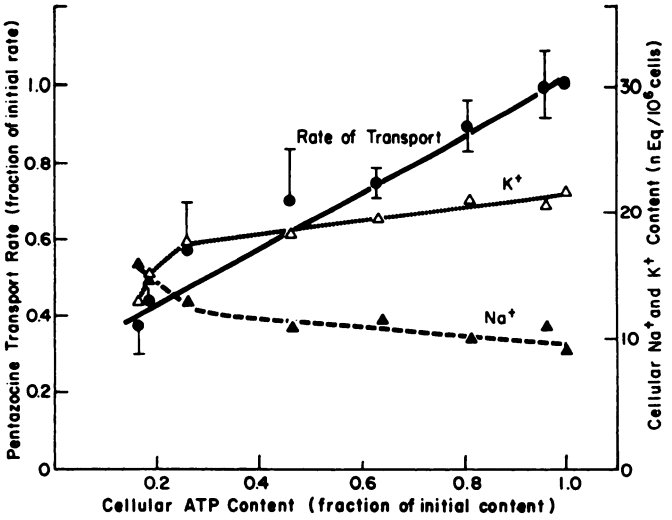

FIG. 5. *Correlations between rate of pentazocine transport, cellular concentration of K^+ and Na^+, and content of ATP in leukocytes*

Leukocytes were incubated for 60 min with various concentrations of NaF (from left to right, 0, 0.3, 1, 3, 12, 30, and 60 mM). Subsequently, using aliquots of the suspensions, the cellular contents of ATP, K^+, and Na^+ were determined as described under MATERIALS AND METHODS. In addition, the uptake of radiolabeled pentazocine by these cells was estimated at an external drug concentration of 20 μM. The cellular uptakes are expressed as a fraction of the uptake in control cells, incubated in the absence of NaF. Presented are mean values ± standard deviations of at least four experiments.

cytes with ouabain affected the cellular uptake of pentazocine.

Drug uptake by purposely disrupted cells. Cells treated by freezing and thawing or by heating exhibited greatly diminished capacities for pentazocine uptake. In cells exposed to either treatment the uptake, at a drug concentration of 10 μM in the medium, was reduced 80–90%.

Effect of pH on pentazocine uptake. The uptake was markedly sensitive to changes in hydrogen ion concentration. Maximal cellular uptake at various concentrations of the drug in the medium occurred at pH 10 (Fig. 6). Analysis of the data (Fig. 7) indicated a steady increase in the V_{max} with decreasing hydrogen ion concentration in the range from pH 7 to pH 10. The apparent K_m for pentazocine was unchanged between pH 8 and 10 but increased outside this range.

Possible cellular damage by the short exposure to nonphysiological pH levels was examined using several criteria. Neither the cell count nor the percentage of cells stained by trypan blue was altered after an 8-sec exposure to pH values ranging from 6 to 11. In addition, after the exposure to various pH values, leukocytes at pH 7.4 demonstrated unchanged uptake of pentazocine relative to control cells.

Exodus of pentazocine. The efflux of pentazocine from leukocytes was rapid and the rate of outflow increased with rising temperatures (Fig. 8). As a sample analysis (17) shows (Fig. 9), the exit of pentazocine was apparently the result of a process composed of two first-order components. The accelerated efflux at higher temperatures resulted from an increase in the rate constant for the more rapid component of the process, which displayed a Q_{10} of 1.86. The fraction of drug exiting with the latter rate constant increased from 0.55 at 8° to 0.90 at 36°. The slower component of the exodus was relatively independent of temperature.

Countertransport in uptake and exit of pentazocine. The rate at which pentazocine entered leukocytes increased in cells previously loaded with the drug. At a concentration of pentazocine of 143 ± 26 nmoles/g of cells the initial rate of uptake of labeled drug

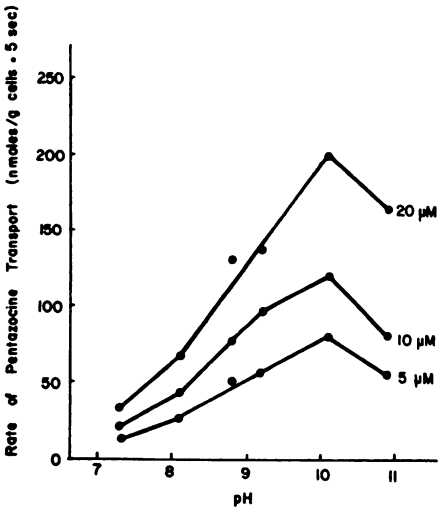

FIG. 6. *Effect of pH on uptake of pentazocine*
The uptake of pentazocine at several pH values was determined using the standard experimental procedure described under MATERIALS AND METHODS. The different pH values of the incubation medium were achieved by mixing equal volumes of a suspension of cells in the standard incubation medium (pH 7.4) and of drug solution of a different, appropriate pH. The following sets of numbers represent the initial pH of the drug solution and the final pH achieved after mixing with an equal volume of cell suspension: 7.4, 7.4; 8.4, 8.1; 9.9, 8.8; 10.5, 9.2; 11.0, 10.1; 11.4, 10.9. The concentrations of pentazocine in these experiments were 5, 10, and 20 μM, respectively. Each point corresponds to the mean of four determinations.

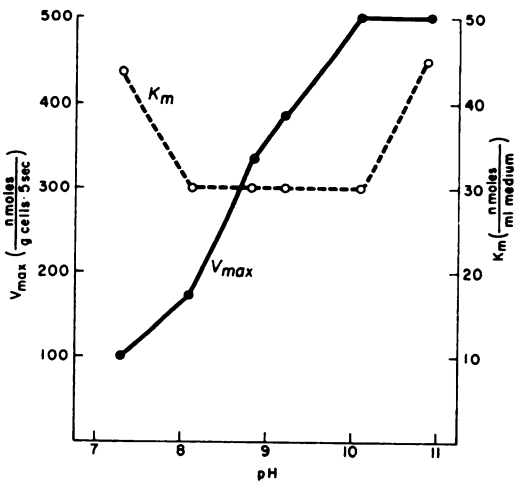

FIG. 7. *Kinetic constants for uptake of pentazocine at various pH values*
The values for K_m and V_{max}, obtained from Lineweaver-Burk plots of the data presented in Fig. 6, are plotted as a function of pH at which the uptakes were determined.

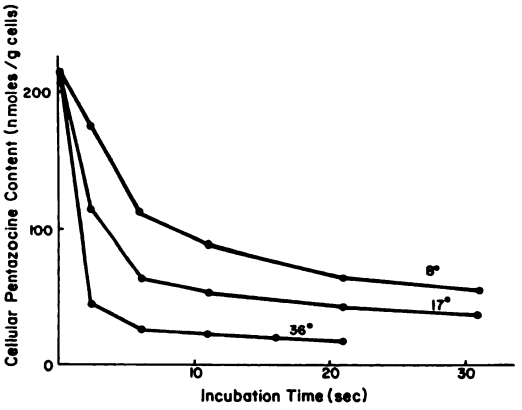

FIG. 8. *Efflux of pentazocine from leukocytes at various temperatures*
Leukocytes, suspended in the standard medium, the pH of which was adjusted to 9.8, were incubated with radioactive pentazocine for 8 sec at 36°. The external concentration of drug was 10 μM. The cells were either immediately filtered (cell content at zero time) or diluted 10-fold with incubation medium (pH 7.4) kept at various temperatures. Dilution with media at 0° and 12° gave final temperatures of 8° and 17°, respectively. In all instances the dilution resulted a final pH of 7.4, at which the efflux was monitored. The latter incubations were terminated at the times indicated. Plotted are results of a representative experiment.

was 81.29 ± 10.70 nmoles/g of cells·5 sec, as compared to the corresponding value of 64.83 ± 4.94 in cells not loaded with unlabeled drug ($p < 0.01$, t-test).

The exit of pentazocine from leukocytes previously loaded with the radiolabeled drug was accelerated when the external medium contained high concentrations of the benzomorphan (Fig. 10). An analysis of the data (17) indicated that under these conditions the first-order rate constants of exodus remained unchanged. The acceleration of efflux was apparently the result of a more rapidly established equilibrium, in the presence of pentazocine, in the external medium.

Effects of competitive inhibitors on pentazocine uptake. The uptake of pentazocine was

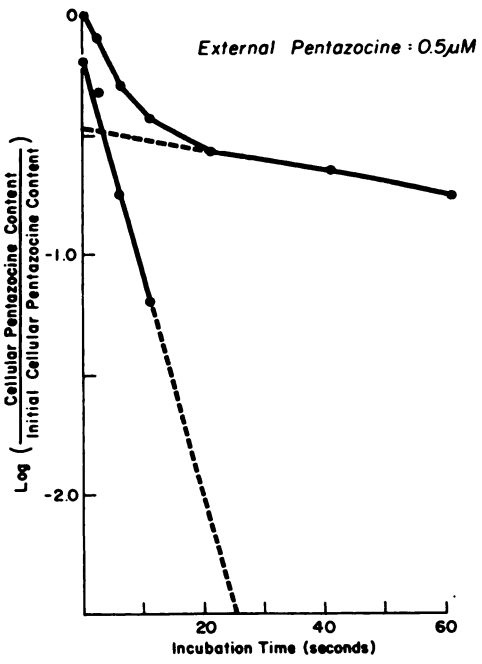

FIG. 9. *Analysis of efflux of pentazocine*

Analyzed according to Robertson (17) are the data from an experiment in which the efflux from leukocytes was determined at 17°. Previously the cells had been loaded with pentazocine by incubation with 5 μM drug for 8 sec at pH 10. Up to 60 sec after dilution the cell content at any time can be calculated as the sum of two first-order processes:

$$C_t = C_0[f_1 e^{-k_1 t} + (1 - f_1)e^{-k_2 t}]$$

where C_t and C_0 are the cellular concentrations of the compound at times t and zero, f_1 is the fraction of the efflux accounted for by the process with a rate constant k_1, and $(1 - f_1)$ is the remaining fraction of the efflux occurring with a rate constant k_2. As shown, this analysis indicates the intervention during the efflux of pentazocine of two processes with considerably different rate constants.

competitively inhibited by a number of compounds with structures similar to that of the benzomorphan. The competitive nature of the interactions at the transport site is illustrated by the data shown in Fig. 11. The K_i values obtained for various compounds are listed in Table 1. All benzomorphan analogues were potent inhibitors of pentazocine uptake. The latter process was also strongly inhibited by quinacrine, tricyclic antidepressants, and a number of narcotics

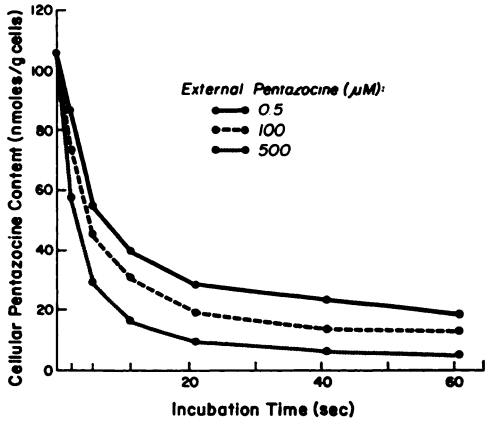

FIG. 10. *Effect of external pentazocine on efflux of drug from leukocytes*

The cells were incubated at pH 9.5 for 8 sec with radioactive pentazocine at a drug concentration of 5 μM. Subsequently the cells were rapidly diluted 10-fold with the standard incubation medium which was kept at 12° and contained different amounts of pentazocine to give a final concentration of the drug in the external medium of 0.5 μM, 100 μM, or 500 μM. The temperature and pH at which efflux occurred were 18° and 7.4, respectively. Each point is the mean of four determinations.

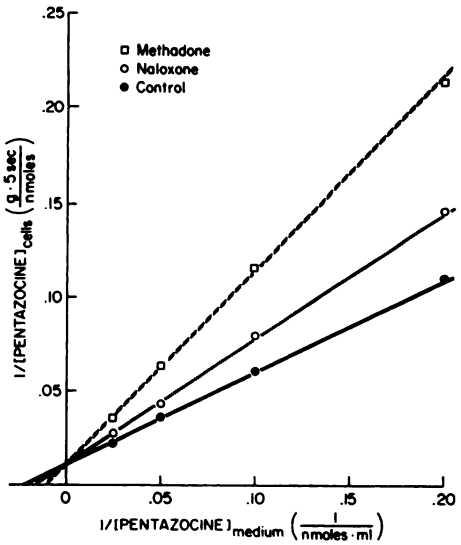

FIG. 11. *Competitive inhibition by methadone and naloxone of uptake of pentazocine*

The uptake of radiolabeled pentazocine (20 μM) by leukocytes was determined in the absence and presence of 30 μM methadone or 100 μM naloxone. Shown are results of a representative experiment. Each point is the mean of three separate incubations.

TABLE 1

Potency of various compounds as inhibitors of pentazocine uptake by leukocytes

The inhibition constants were determined at 37° and pH 7.4 using several methods. The K_i values were generated from log dose-response curves of the uptake of pentazocine in the presence of inhibitor. The rate of pentazocine transport was determined in control cells and in the presence of different concentrations of the inhibitor. By plotting the log of inhibitor concentration in the medium against the rate of pentazocine uptake, the point at which 50% inhibition of the latter process occurred was determined. In addition, the inhibition constants were estimated from Dixon plots of the data on the uptake and were also calculated from the apparent K_m values obtained in the presence of inhibitor (Lineweaver-Burk plots). Within the same experiment, the values for the inhibition constants obtained by the various methods were virtually identical.

Compounds according to classification	K_i	Compounds according to classification	K_i
	μM		μM
Benzomorphans		Phenethylamine analogues	
(±)-Pentazocine	40	Phenethylamine	130
(+)-Pentazocine	35	Amphetamine	100
(−)-Pentazocine	30	Tyramine	>1000
Etazocine	110	Dopamine	>1000
Cyclazocine	70	Norepinephrine	>1000
Phenazocine	40	Indolealkylamines	
Tricyclic antidepressants		Tryptamine	>1000
Amitryptiline	8	*N*-Methyltryptamine	600
Desipramine	8	*N*,*N*-Dimethyltryptamine	85
Imipramine	8	5-Hydroxytryptamine	>1000
Narcotic analgesics and antagonists		Miscellaneous	
Morphine	>1000	Choline	>1000
Codeine	220	Histamine	>1000
Thebaine	45	Cocaine	100
Naloxone	200	Phenobarbital	>1000
Nalorphine	350	Quinacrine	3
Levorphan	140	Tripelennamine	40
Levallorphan	40		
Meperidine	75		
Methadone	22		
Propoxyphene	75		

and narcotic antagonists. Morphine, however, was a decidedly weak inhibitor. In proceeding from primary to tertiary amines in the indolealkylamine series, the potency of inhibitors increased. Hydroxylation of phenethylamine strongly reduced its inhibitory properties. Similarly, inhibition of the uptake of pentazocine decreased with the additional presence of free hydroxyl groups in the series thebaine, codeine, and morphine.

DISCUSSION

The characteristics of the uptake of pentazocine by rat leukocytes are consistent with those expected for a carrier-mediated, active transport system (18–20): the uptake displayed saturation kinetics: at equilibrium the distribution of drug between cells and medium exceeded a ratio of 1; the temperature sensitivity of the uptake resulted in a Q_{10} of approximately 2; the uptake was markedly reduced in cells poisoned with metabolic inhibitors; the rate of uptake was closely correlated with cellular ATP content; chemical analogues competitively inhibited uptake; the uptake and exit of drug exhibited the phenomenon of countertransport; and the ability to transport the drug strongly decreased in cells disrupted by heating or freezing.

Both cellular uptake and exit of pentazocine occurred rapidly and were equally sensitive to changes in temperature. While the

V_{max} for pentazocine uptake exhibited a Q_{10} of 1.88, the K_m was unchanged between 1° and 37°. The rapid first-order component of efflux showed the same temperature dependence (Q_{10} = 1.86) as the V_{max} for uptake. Marked sensitivity to temperature is not limited to carrier-mediated processes but can also be the result of temperature-dependent binding (19, 20). However, the binding of pentazocine to heat-treated leukocytes as well as to BSA was unaffected by changes in temperature. The temperature independence of pentazocine binding, as well as the insensitivity of the K_m to changes in temperature, suggests that the activation energy for binding of this drug to protein or to the transport carrier is remarkably low.

The nature of the slower component of exodus is still uncertain. This component may be either a true exit process or an artifact arising from recapture of drug from the medium.

The effect of metabolic poisons on pentazocine transport was kinetically similar to that of temperature; i.e., inhibition of uptake was noncompetitive. The strong effect of inhibitors of anaerobic energy production is consistent with the dominant role of glycolysis in leukocytes suspended in artificial media (21–23). The rate of transport of pentazocine decreased both as a function of the duration of exposure to NaF and as a function of inhibitor concentration. Upon concurrent addition of drug and inhibitor[2] no change was observed in cellular uptake, indicating the lack of a direct effect of NaF on the carrier.

The importance of ATP in maintaining active transport processes has been demonstrated directly for erythrocyte ghosts (24, 25), squid axon (26–28), and *Escherichia coli* (29) by incorporation of the nucleotide into the previously depleted systems. Additional evidence for the role of ATP has been presented by the correlation of its concentration and transport in tissues with altered energy metabolism (30–34). Therefore the correlation between the rate of uptake of pentazocine and ATP levels in leukocytes presents strong evidence for the active nature of the transport process. The linear relationship of this correlation closely resembles that reported for glycine transport in brain slices (30, 31) and Ehrlich cells (33). At an extrapolated cellular ATP content of zero, the uptake of pentazocine was calculated to be identical with that obtained in leukocytes disrupted by heating or freezing. The residual fraction of uptake (less than 20% of control) under such conditions apparently represented a fraction of the drug which was nonspecifically bound to cellular protein, or which diffused into cells incapable of active transport.

The uptake of pentazocine was apparently independent of sodium concentration in the external medium and was insensitive to inhibition by ouabain. These results indicate that neither the electrochemical gradient of K^+ and/or Na^+ nor the participation of sodium as a cosubstrate was required for the accumulation of the drug by leukocytes.

The pH sensitivity of the uptake of pentazocine resulted from changes in both the K_m and V_{max}. The change in K_m outside the range of pH 8–10 indicated that only when it bore no net charge was pentazocine a substrate for the transport carrier (35). The 5-fold increase in V_{max} occurring between pH 7 and 10 cannot be explained as a simple ionization process (36). Various factors influencing the V_{max} of a transport system, e.g., ionization of the carrier, rate constants of translocation, and carrier distribution, might have been affected by the change in pH, resulting in a complex, composite change in V_{max}. The enhanced transport of pentazocine at high pH did not appear to result from an increase in diffusion or binding of the drug, for several reasons: inhibition of drug transport by NaF was unchanged throughout the investigated range of pH; uptake of pentazocine at high pH was strongly affected by competitive inhibitors; at high pH binding of the drug to disrupted cells accounted for an even smaller fraction of the uptake than at physiological pH; and at all pH values the cellular content of the drug was considerably higher than its external concentration.

The observation of countertransport is considered important evidence for the existence of carrier-mediated processes (18–20, 37). Both uptake and exit of pentazocine in

leukocytes exhibited transacceleration, indicating that translocation of the loaded carrier was more rapid than that of the empty one (37). Acceleration of exit by external drug occurred with an interesting alteration of the kinetic parameters of that process. While neither first-order rate constant of the process describing exodus changed, the fraction of drug exiting by the rapid component increased with rising external concentrations of pentazocine. Half-maximal acceleration of exit occurred at an external drug concentration of 50 μM, a concentration close to the K_m for uptake. Acceleration of uptake in loaded cells was less pronounced. Since no change in the rate constant for exit was seen for cellular drug concentrations as high as 200 nmoles/g of cells, the level to which the cells were loaded may have been sufficiently below the intracellular K_m for pentazocine to prevent significant acceleration of uptake.

At physiological pH the cellular transport of pentazocine was competitively inhibited by amines of wide structural variety. Interestingly, however, a number of endogenous compounds, including tyramine, dopamine, choline, histamine, tryptamine, and 5-hydroxytryptamine, were poor inhibitors. Certain structural changes considerably affected the inhibitory strength of a given compound. The addition of hydrophilic hydroxyl groups in progressing from levorphan to morphine, from phenethylamine to tyramine, and from levallorphan to nalorphine, greatly reduced the inhibitory potency displayed toward the uptake of pentazocine. On the other hand, inhibitory strength was increased by methylation of the hydroxyl groups, as observed in the series morphine, codeine, and thebaine. The phenolic hydroxyl group in the benzomorphan nucleus had less influence on inhibition, since cyclazocine and deoxycyclazocine displayed nearly identical K_i values. The increased inhibition by dimethyltryptamine relative to tryptamine was seemingly the result of the different extents of ionization of these compounds. With rising pH the inhibitory potency of both compounds increased as a function of the amount of uncharged amine present at a given pH. The latter observation provides additional support for the concept of an unprotonated amine as the substrate for the transport carrier. At high pH both primary and tertiary amines were strong inhibitors of the uptake of pentazocine. Apparently, a substrate for the benzomorphan carrier in leukocytes can be a primary, secondary, or tertiary amine, but it must be uncharged to be transported. The affinity of the transport system toward a compound is inversely related to the increase in the hydrophilic character of the latter. Although all the effective inhibitors of the uptake of pentazocine contained an aromatic ring spatially separated from the amino group, an absolute requirement for an aromatic nucleus has not been established. As investigated with optical isomers of pentazocine, no evidence for stereospecificity of the transport process was obtained.

On the basis of its characteristics, the transport process for benzomorphans in leukocytes is different from hitherto described processes for the uptake of biogenic amines by nervous tissue and platelets (38), as well as for the uptake of choline (39, 40) and of methylglyoxal bis(guanylhydrazone) (41, 42) by human leukocytes. Similarities in the accumulation of benzylamine in Ehrlich cells (43) and the transport of pentazocine by leukocytes described here are of interest, but further work is required to clarify the relationship among these systems. Uptake of various central nervous system drugs by preparations of nervous tissue has been reported, although the characterization of these processes is not unequivocal. In addition to pentazocine, several other central nervous system drugs, including codeine, naloxone, methadone, and amphetamine, were actively transported into leukocytes (9, 10). The results of the latter studies indicated that these compounds entered the cell by a single, common carrier system.

Parallels between the uptake of pentazocine by leukocytes *in vitro* and of various central nervous system drugs by brain *in vivo* exist. Results of Oldendorf *et al.* (44), obtained by a technique of carotid artery injection (45), show a good correlation between the specificity for cerebral drug uptake and the affinity of the leukocyte trans-

port system for a particular compound. Nevertheless, an evaluation of the biological role of the transport system for drugs existing in leukocytes will require the characterization of such processes in cellular preparations from nervous tissue.

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