Br. J. Pharmac. (1977), **59**, 539–549

CHARACTERIZATION OF (±)-METHADONE UPTAKE BY RAT LUNG

C.H. CHI & B.N. DIXIT

Department of Pharmacology, School of Pharmacy, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, U.S.A.

1 By use of a sensitive and specific fluorescence assay procedure it was shown that after subcutaneous administration to rats, (±)-methadone was concentrated in the lung. Lung to serum ratios ranging from 25 to 60 were obtained indicating that the rat lung tissue was capable of extracting (±)-methadone against a concentration gradient.

2 This phenomenon was investigated *in vitro* with rat lung slices incubated in Krebs-Ringer phosphate buffer (pH 7.4). The uptake was expressed in terms of tissue to medium concentration ratios (T/M ratio).

3 The principal observations were: (i) Studies on the time-course of the uptake showed that the T/M ratios of (±)-methadone increased rapidly during the first 60 min of incubation and then more slowly, with a plateau occurring at 180 min; (ii) The T/M ratio of (±)-methadone progressively increased from 9.5 to 17 as the pH of the incubation medium was varied from 6.2 to 7.8; (iii) When the concentration of (±)-methadone in the incubation medium was varied from 0.005 to 0.5 mM, the T/M ratio decreased rapidly suggesting self-saturation of the transport process. Beyond the medium concentration of 0.5 mM, the T/M ratio declined very slowly.

4 These results suggested that at low concentrations, (±)-methadone was transported predominantly by a self-saturable process while at higher concentrations it was transported by a process of simple diffusion.

5 At low concentrations (0.01 mM) the uptake of (+)-methadone was higher than that of (−)-isomer indicating stereo-specificity of the uptake process. The uptake of (±)-methadone at low concentration (0.01 mM) was significantly inhibited by low temperature, lack of O_2, lack of glucose, lack of Na^+ in the incubation medium, and by exposure of the tissue to high temperature (≈100°C). The uptake was also inhibited by relatively high concentration of iodoacetate (1.0 mM) and of naloxone (1.0 mM).

6 Kinetic analysis of data showed that the diffusion constant for (±)-methadone was 5.0 (h^{-1}) and the V_{max} of the active transport process was 6.5 μmol g^{-1} h^{-1}.

Introduction

During the past decade, (±)-methadone has been extensively used in the maintenance and rehabilitation programmes for chronic heroin users (Dole & Nyswander, 1968). In spite of the work that has been done on the metabolic disposition of methadone (Beckett, Mitchard & Shihab, 1971; Pohland, Boaz & Sullivan, 1971; Misra, Mulé, Bloch & Vadlamani, 1973) there are still gaps in our knowledge about its absorption, distribution and metabolism.

By use of a specific fluorescence assay method, it was recently shown that after subcutaneous administration of (±)-methadone, high concentrations of the drug were found in the lung tissue of the rat (Chi & Dixit, 1973). This paper describes studies that were conducted to elucidate and characterize the mechanisms involved in this preferential distribution of (±)-methadone in the rat lung.

Methods

Animals

All experiments were performed with lung tissue slices obtained from male albino rats of Wistar strain (Hilltop Lab Animals, Scottdale, Pa.), weighing between 150 and 200 grams. They were housed in group cages (approximately 20 rats per cage) in animal rooms maintained at 23 ± 1°C with a 12 h 'light-dark' cycle. Water and food were provided *ad libitum*. At least three days were allowed for acclimatisation.

Fluorescence assay for (±)-methadone

It was shown by McGonigle (1971) that when (±)-methadone reacts with paraformaldehyde in concentrated H_2SO_4 by heating at 100°C, a fluorophore

is formed. Based on this observation, a fluorescence assay was developed. It will be briefly described here.

Rats were killed by decapitation, the blood and other tissues were removed and were frozen and stored at −20°C until assayed. Just before assay, tissues were weighed and homogenized in distilled water (1:3). A 2 ml aliquot of the homogenate or 1 ml of serum (or plasma) diluted to 2 ml with distilled water was used for the assay. One ml of $ZnSO_4$ solution (5%, $ZnSO_4.7H_2O$) was added to each sample and the contents were thoroughly mixed before the addition of 1 ml $Ba(OH)_2$ solution (4.5%, $Ba(OH)_2.8H_2O$). Aliquots (2 ml) of the supernatant were transferred into 15 ml glass stoppered centrifuge tubes, and 0.4 ml borate buffer, pH 9.6, was added to give the final pH of 9.2. Six ml of 25% isobutanol in ethylene dichloride was added and the tubes were shaken for a minimum of 8 min to facilitate the extraction of methadone into the organic phase. Three ml or any other suitable aliquot of the organic phase was evaporated to dryness at 50–55°C by using a jet of water-washed air; 0.1 ml of 0.1% paraformaldehyde in concentrated H_2SO_4 was added to each sample and the contents were heated in a boiling water bath for 15 minutes. Distilled water (7.1 ml) was added to adjust the final normality of the solution to 0.5; 30 min later the fluorescence was read on an Aminco-Bowman Spectrophotofluorometer with a photomultiplier opening No. 5, an excitation wavelength of 275 nm and an emission wavelength of 450 nm (uncorrected). Tissue blanks, using tissues of untreated animals, and a set of standards were run simultaneously with each set of unknowns. With this method 50 ng of (±)-methadone/sample could be accurately determined.

The specificity of the extraction procedure was established by determining distribution ratio profiles (Brodie & Udenfriend, 1945) of authentic (±)-methadone and methadone extracted from the homogenates of tissues obtained from rats that were treated with (±)-methadone. It was found that the extraction procedure is specific for (±)-methadone, the two principal metabolites of (±)-methadone (metabolite I and metabolite II) do not form a fluorophore when reacted with the paraformaldehyde reagent.

Tissue levels of (±)-methadone

(±)-Methadone, 20 mg/kg, was administered subcutaneously to four groups of rats. They were killed 30, 60, 120 and 240 min after the injections. Serum, brain, liver and lung tissue samples were obtained and were analyzed for (±)-methadone.

Tissue uptake determination

(i) *Incubation medium and drug solutions:* Krebs-Ringer phosphate buffer was used as the incubation medium and was prepared as described by Umbreit, Burris & Stauffer (1971) with the modification that only 1/5 as much phosphate buffer was used to buffer the solution. The pH of this solution remained unchanged (7.4) for as long as 3 h of incubation at 37°C. The solution contained 2 mg/ml of glucose. Drugs were dissolved in Krebs-Ringer phosphate buffer or 0.9% w/v NaCl solution. The pH of all drug solutions was 7.4. Appropriate volumes of these solutions were added to the incubation medium.

(ii) *Incubation procedure:* Rats were killed by decapitation. The lungs were immediately removed and were placed in oxygenated ice-cold Krebs-Ringer phosphate buffer. Small slices (18–20 mg) were cut with a pair of iris scissors. They were pre-incubated for 15 min in Krebs-Ringer phosphate buffer at 37°C. At the end of the pre-incubation period they were transferred to another set of flasks, each containing 15 ml of Krebs-Ringer phosphate buffer. The incubating medium contained appropriate concentrations of (±)-methadone.

All incubations were carried out in a Dubnoff Metabolic Shaker under O_2 (gas flow, approximately 1000 ml/minute). Unless otherwise specified, the incubations were carried out at 37 ± 1°C. Depending on the experiment, the period of incubation varied from 2 to 180 minutes. Whenever effects of other drugs, chemicals or metabolic inhibitors were studied, the lung slices were pre-incubated with the respective drug or chemical for 15 min and (±)-methadone was added to start the reaction. In some experiments O_2 was replaced with N_2. (±)-Methadone was added after the tissue was exposed to N_2 for 15 min, and the incubation was continued under N_2.

After incubation, the tissue was removed from the medium, rinsed at least twice in fresh ice-cold Krebs-Ringer phosphate buffer and was gently blotted on a piece of Whatman filter paper (No. 3) to remove excess medium. The tissue was homogenized in 5.0 ml glass distilled water using a Polytron ultrasonic homogenizer (Type P.T. 20, OD Kinematica, GMBH). A suitable aliquot of the aqueous homogenate was used for the determination of (±)-methadone.

Extracellular space determination

Lung slices were incubated for various intervals and the incubating medium contained 0.01 mM of (±)-methadone and 0.5 μCi/ml of [^{14}C]-inulin. After incubation, each piece of tissue was removed from the medium, streaked on a piece of glass to remove excess medium (Lorenzo & Spector, 1973) and was weighed. The tissue samples were then dissolved in 1 ml of Soluene-100 in glass scintillation vials; 15 ml of toluene counting fluid was added to each vial and samples were counted for 10 min in a Packard Tri-Carb liquid scintillation spectrometer (Model 3310). To determine the [^{14}C]-inulin concentration in the medium, a 0.1 ml aliquot of the corresponding incubation medium was placed in similar vials and 15 ml of

Triton counting fluid was added. [^{14}C]-Inulin concentration was determined as above.

Total tissue water determination

Pieces of lung tissue were spread on watch glasses and weighed. They were dried to a constant weight at 100°C in an oven. The difference between the wet-weight and the dry-weight gave the total tissue water.

Expression of results and statistical analysis

The tissue concentration of (±)-methadone was expressed as μmol/g (wet weight). Tissue to medium concentration ratios (T/M ratios) were obtained by dividing the tissue concentration (μmol/g) by the medium concentration of (±)-methadone (μmol/ml). The uptake was expressed in terms of T/M ratios. The estimation of methadone in different tissues was done in duplicate. Group means, standard deviations and standard errors as well as Student's *t* scores were computed on an Olivetti-Programma 101 desk computer.

Chemicals

Sources of chemicals were as follows: all chemical reagents (A. R. or spectranalyzed grade) were from Fisher Scientific Co., Pittsburgh, PA 15238; (±)-methadone hydrochloride was from Mallinckrodt Chemical Co., Jersey City, New Jersey, 07303; the (+)- and (−)-isomers were kindly supplied by Eli Lilly Laboratories, Indianapolis, Indiana.

Results

In vivo *studies*

Figure 1 shows the time-concentration curves for the lung, liver, brain and serum after 20 mg/kg (s.c.) of (±)-methadone was administered to rats. It can be seen that the level of (±)-methadone in lung tissue is several-fold higher than that of the serum. For example, at 30 min the level in the lung was 54.2 ± 6.7 μg/g (mean ± s.e. mean) and the serum level was only 1.33 ± 0.12 μg/ml (mean ± s.e. mean) resulting in a lung/serum ratio of 41. The lung/serum concentration ratios (mean ± s.e. mean, $n=4$) at 30, 60, 120 and 240 min after 20 mg/kg of (±)-methadone were 40.5 ± 2.8, 43.0 ± 3.7, 54.8 ± 4.5 and 26.0 ± 3.7, respectively.

In vivo *studies*

The high lung/serum ratios of (±)-methadone obtained during *in vivo* experiments indicated that the rat lung tissue is capable of concentrating (±)-methadone against a concentration gradient. This phenomenon

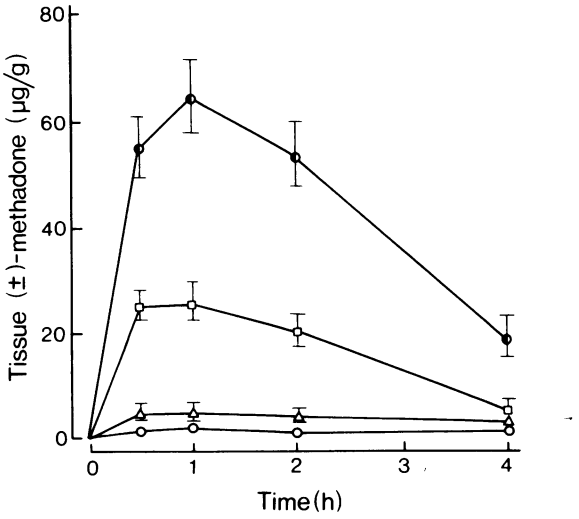

Figure 1 Concentrations of (±)-methadone in serum (○), liver (□), brain (△) and lung (●) in rat. (±)-Methadone, 20 mg/kg, injected subcutaneously at zero time. The individual points represent mean values obtained from at least 4 animals. Vertical bars indicate s.e. mean.

was investigated by conducting *in vitro* experiments with rat lung slices. The uptake of (±)-methadone by the rat lung slices was expressed as concentration ratio between the tissue concentration of (±)-methadone (T, μmol/g, wet weight) and the concentration of (±)-methadone in the incubation medium (M, μmol/ml). The final concentration of (±)-methadone in the medium was not determined in all experiments since the concentration of (±)-methadone was considered unaltered at the end of the incubation period as the volume of the incubation medium (5 ml) was much greater than that of the tissue employed (18–22 μl). Thus, in calculating the T/M ratio, the initial concentration of (±)-methadone in the incubation medium (M) was used.

Time-course of uptake

A 15 min pre-incubation of the lung slices had no significant effect on the uptake of (±)-methadone. The 60 min T/M ratio for pre-incubated tissue was 19.55 ± 2.04 (mean ± s.e. mean, $n=3$); for control tissue it was 18.02 ± 1.52 (mean ± s.e. mean, $n=4$).

It can be seen from Figure 2 that the T/M ratio increased rapidly up to 60 min of incubation and appears to reach a plateau in 180 min at which time the T/M ratio for (±)-methadone attained a mean value of 24 ± 2.67. [^{14}C]-Inulin T/M ratios were determined at 5, 15, 30 and 60 min of incubation. The [^{14}C]-inulin space was 30 ± 3.3% ($n=3$) for a 30 min

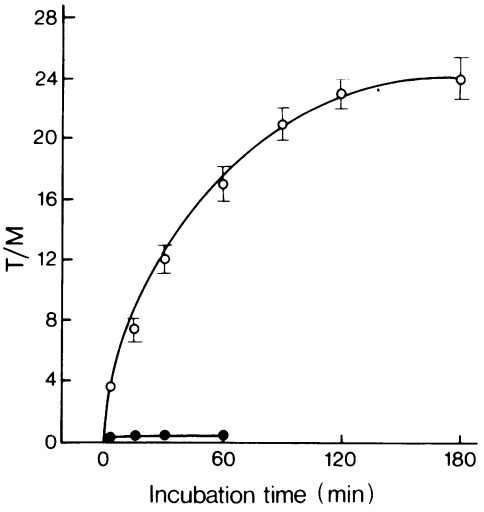

Figure 2 Time course of the *in vitro* uptake of (±)-methadone (○) and [^{14}C]-inulin (●) by rat lung slices. T/M = lung tissue/medium (±)-methadone concentration ratio. Incubations under O_2 (gas flow, 1000 ml/min) at 37°C in Krebs-Ringer phosphate buffer, pH 7.4. (±)-Methadone concentration in the medium, 0.01 mM. [^{14}C]-inulin concentration in the medium, 0.1 μCi/ml. The individual points represent mean values obtained from at least 3 duplicate determinations. Vertical bars indicate s.e. mean.

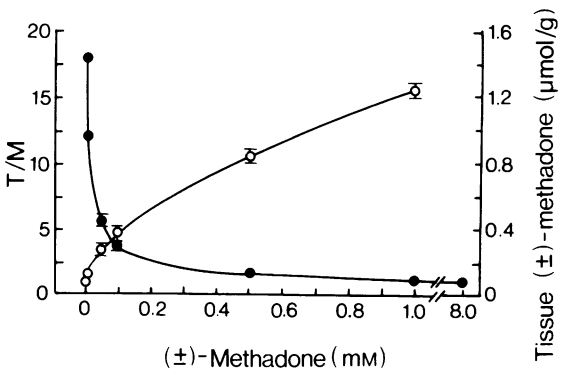

Figure 3 *In vitro* uptake of (±)-methadone at various concentrations. The net uptake (○) and T/M ratios (●) are shown. T/M = lung tissue/medium (±)-methadone concentration ratio. Incubation under O_2 (gas flow, 1000 ml/min) at 37°C for 30 min in Krebs-Ringer phosphate buffer, pH 7.4. The individual points represent mean values obtained from at least 4 duplicate determinations. Vertical bars indicate s.e. mean.

incubation period. Total tissue water content was 81.1 ± 0.1% (n = 10).

Effect of pH of the incubation medium

When incubations were carried out at pH 6.2, 6.6, 7.0, 7.4 and 7.8 for 30 min, and at M = 0.01 mM, the resultant T/M ratios (mean ± s.e. mean, n = 3) were 9.3 ± 1.7, 9.5 ± 0.7, 11.3 ± 0.5, 13.2 ± 1.3 and 16.1 ± 1.5, respectively. In the present study all experiments were performed at pH 7.4 which is within the normal physiological limits of the pH of the extracellular fluid.

Effect of concentration on the uptake

As the concentration of the (±)-methadone in the incubation medium was increased from 0.005 to 32 mM, the T/M ratio at first decreased very rapidly and then approached near unity at higher concentrations (Figure 3). The T/M ratios for the medium concentration beyond 8.0 mM are not shown in the figure.

At the (±)-methadone concentration of 32 mM in the incubation medium, the observed T/M ratio was not significantly different from the one obtained when osmolarity was maintained by decreasing Na^+ and Cl^- in the incubation medium (0.74 ± 0.1 and 0.72 ± 0.01, respectively). This indicates that the presence of 32 mM (±)-methadone in the medium does not affect the uptake characteristics of lung tissue due to possible changes in osmolarity.

The uptake of (+)- and (−)-isomers of methadone

With M = 0.01 mM, the T/M ratios (mean ± s.e. mean, n = 5) at 1, 3, 10, 30 and 60 min for (+)-methadone were 2.5 ± 0.5, 6.2 ± 0.6, 10.0 ± 1.5, 14.8 ± 0.9 and 19.9 ± 1.6, respectively. However, under similar conditions the T/M ratios for (−)-methadone were 1.7 ± 0.8, 3.4 ± 1.0, 6.3 ± 1.1, 8.1 ± 1.1 and 13.6 ± 0.7 indicating that the uptake of (+)-isomer was significantly higher than its (−)-isomer.

Effect of temperature

When incubations were carried out for 30, 60, 90 and 180 min at 0–4°C with M = 0.01 mM, the T/M ratios (mean ± s.e. mean, n = 3) were 6.3 ± 0.6, 7.8 ± 1.3, 7.6 ± 1.9 and 7.8 ± 1.0, while at 37 ± 0.5°C the corresponding T/M ratios were 21.1 ± 1.7, 19.6 ± 2.0, 21.5 ± 2.0 and 23.8 ± 2.7. These data indicated that the uptake of (±)-methadone was temperature-dependent.

Effect of anoxia

When incubation was carried out under N_2 instead of O_2, the uptake was inhibited by 73%

(T/M = 12.5 ± 0.2 under O_2 and 3.4 ± 0.3 under N_2). Results obtained with 30 min incubation indicated that the degree of inhibition decreased as the concentration of (±)-methadone in the incubation medium was progressively increased. At and beyond the concentration of 0.1 mM no significant inhibition of uptake was seen. Increase in the duration of incubation beyond 30 min did not have any significant influence on the inhibition of uptake.

Effect of lack of glucose

Omission of glucose from the incubation medium reduced the T/M ratio from 12.5 ± 0.2 to 3.1 ± 0.6 resulting in a 70% inhibition of the uptake. The degree of inhibition in uptake was not dependent on the period of incubation.

Effect of exposure of tissue to higher temperature

When the lung tissue was boiled for 3 min at 100°C in a water bath before incubation the uptake was inhibited by 85%.

Effect of iodoacetate

At a concentration of 0.01 mM of (±)-methadone in the incubation medium, a 15 min exposure to iodoacetate (0.01 mM) did not have significant effect on the uptake of (±)-methadone, but at 1.0 mM iodoacetate, (±)-methadone uptake was inhibited by 23%.

Effect of lack of Na^+

When Na^+ in the incubating medium was partially (50%) replaced by Li^+ as LiCl, the uptake was inhibited by 36%. A complete replacement of Na^+ by Li^+ resulted in 76% inhibition of the uptake indicating that the (±)-methadone uptake was Na^+-dependent and this dependency was related to the degree of Na^+ deficiency.

Effect of morphine and naloxone

Morphine in concentrations up to 1.0 mM had no significant effect on the (±)-methadone uptake. Similarly, 0.01 mM naloxone was without significant effect; however, at 1.0 mM naloxone, (±)-methadone uptake was inhibited by about 45%. In both cases, (±)-methadone concentration in the incubation medium was 0.01 mM.

Discussion

It was demonstrated *in vivo* that, after its subcutaneous administration, (±)-methadone was present in very high concentration in lung tissue of the rat. Lung to serum ratios ranged between 25 and 65, indicating that the rat lung is able to concentrate (±)-methadone against an apparent concentration gradient. The purpose of the *in vitro* experiments was to elucidate and characterize the mechanisms involved in this preferential *in vivo* distribution of (±)-methadone. *In vitro* incubation of lung slices seemed to be the most suitable procedure since it permitted detailed examination of the uptake characteristics and the factors that affect the uptake process. From the results obtained it can be safely concluded that (±)-methadone is taken up by the lung tissue by at least two processes: (i) At low concentrations in the incubation medium, (±)-methadone is taken up by a transport process that has the characteristics of an 'active transport' process. It was shown that (±)-methadone is taken up against an apparent concentration gradient; the transport process being inhibited by low temperature, lack of glucose, lack of Na^+ in the incubation medium and the presence of N_2 or the lack of O_2; (ii) At high concentrations of (±)-methadone, the major process of transport looks almost identical to that of 'simple diffusion' with the transport due to active process becoming nearly constant.

Studies on the time-course of the uptake showed that the T/M ratio of (±)-methadone increased more

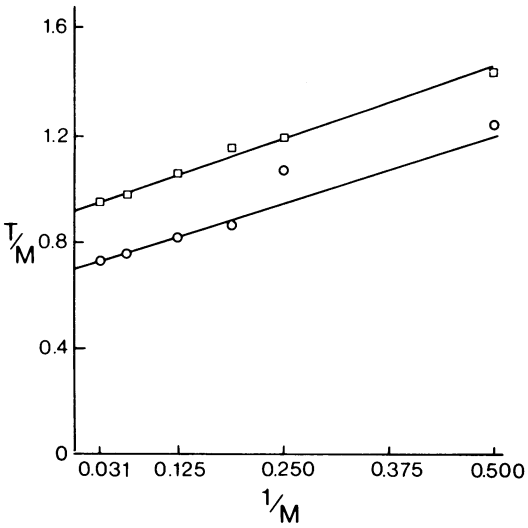

Figure 4 Relationship between T/M and 1/M. T/M = lung tissue/medium (±)-methadone concentration ratio. Incubations for 15 min (○) and 30 min (□) at 37°C under O_2 (gas flow, 1000 ml/min) in Krebs-Ringer phosphate buffer, pH 7.4. (±)-Methadone concentration in the medium, 0.01 mM. Linear fit by T/M = b(1/M) + a; a = 0.71, b = 0.93 for 15 min incubation, and a = 0.91, b = 1.20 for 30 min incubation.

rapidly during the first 60 min of incubation and then more slowly, with a plateau occurring at 180 minutes. Since this is the first time that lung slices have been used to study the uptake of a drug, it is not possible to compare the results obtained in the present investigation with other studies. Most other studies have been conducted on isolated lungs of rabbits or rats that were perfused either with artificial media with or without added constituents of blood or with whole blood (Alabaster & Bakhle, 1970; Rosenbloom & Bass, 1970; Junod, 1972a, b; Iwasawa, Gillis & Aghajanian, 1973; Orton, Anderson, Pickett, Eling & Fouts, 1973). The development of oedema has been the most common difficulty encountered in perfusion experiments using artificial media. According to Nicolaysen (1971), Lunde (1967) and Orton *et al.* (1973) whole blood is the most suitable perfusing medium. In the present studies the weight of lung slices increased by about 13% at the end of 60 min of incubation indicating that oedema formation was not a significant factor. When the lung is perfused, the perfusate is forced through the capillaries because of the 'pressure gradient' which causes the fluid to accumulate in the extracellular interstitial space resulting in oedema formation. Such a 'pressure gradient' is not a factor when lung slices are incubated *in vitro* and might explain the lack of significant oedema formation observed in the present experiments. The observation, that neither a 15 min pre-incubation at 37°C, nor keeping the lung tissue at 0°C for as long as 3 h in the Krebs-Ringer phosphate buffer adversely affected the capacity of the lung tissue to concentrate (±)-methadone, can be interpreted as an indication that under these experimental conditions the lung slices remain viable for a considerable period of time.

It was shown that the T/M ratio of (±)-methadone increased from 9.5 to 17 as the pH of the incubation medium was varied from 6.2 to 7.8. Experiments with pH above 7.8 were not conducted since Ca^{2+} and Mg^{2+} begin to precipitate as phosphates. The observed effect of pH change on the (±)-methadone uptake can be interpreted in different ways. The pKa of (±)-methadone is 8.3–9.0(Misra, 1972). As the pH of the incubation medium was increased from 6.2 to 7.8, the proportion of unionized form of (±)-methadone increased from less than 0.2% to 33% (Table 1). Since unionized compounds generally diffuse easily across cell membranes (Davson & Danielli, 1952; Schanker, 1962) more (±)-methadone may have partitioned into the lung tissue. Inherent in this explanation is the assumption that the intracellular pH changes vary little during the period of incubation. Even if this were true, it cannot explain the magnitude of the uptake as indicated by T/M ratios of the order of 10–15. One could also suggest that (±)-methadone is transported only in the unionized form by an active transport process. This might explain the increase in the uptake as the pH of the incubation medium is increased. In view of the fact that the active transport process works against a concentration gradient, the latter explanation may not be entirely acceptable unless one shows that the concentration of the unionized (±)-methadone in the incabation medium is much lower than the apparent *Km* of the active transport process. A critical evaluation of the apparent *Km* of the active transport of (±)-methadone has not been done in the present investigation, however, the data shown in Figure 5 indicate that the *Km* would be of the order of 0.05 to 0.4 mM. This value is 5 to 40 times higher than the concentration of (±)-methadone used (0.01 mM) when studying the effect of pH on the uptake. Therefore, the difference between the concentration of the unionized (±)-methadone and the apparent *Km* will even be greater. It can also be speculated that the change in pH of the incubation medium affects the tissue metabolism and consequent energy production in a manner that adversely affects the active transport process. No experimental data are available to support such a mechanism.

When investigating the uptake of morphine by choroid plexus, Takemori & Stenwick (1966) observed that as the pH of the incubation medium was varied from 6.5 to 7.5, the T/M ratio of morphine increased from 2.5 to 9.0 and beyond the pH of 7.5 there was no further increase in the T/M ratio. It was observed by Hug (1967) that varying the pH of the medium over the range of 6.8–8.0 produced no consistant effect on the uptake (T/M ratio) of dihydromorphine by choroid plexus. These investigators were unable to draw any definite conclusions as to why the uptake of morphine was facilitated with increasing pH but that of dihydromorphine was unaffected. It must be mentioned that no systematic investigation has been done to evaluate the effect of extracellular pH change on the active transport processes.

When the concentration of (±)-methadone in the incubation medium was varied from 0.005 to 32 mM the T/M ratio decreased rapidly (Figure 3) suggesting self-saturation of the transport system which is one of the most important criteria of an active transport process. Beyond a certain medium concentration of

Table 1 Effect of pH on the ionization of (±)-methadone

pH of incubation medium	*(±)-Methadone (ionized) / (±)-Methadone (unionized)*	
	pKa=8.3	*pKa=9.0*
6.2	125	630
6.6	50	250
7.0	20	100
7.4	8	40
7.8	3	16

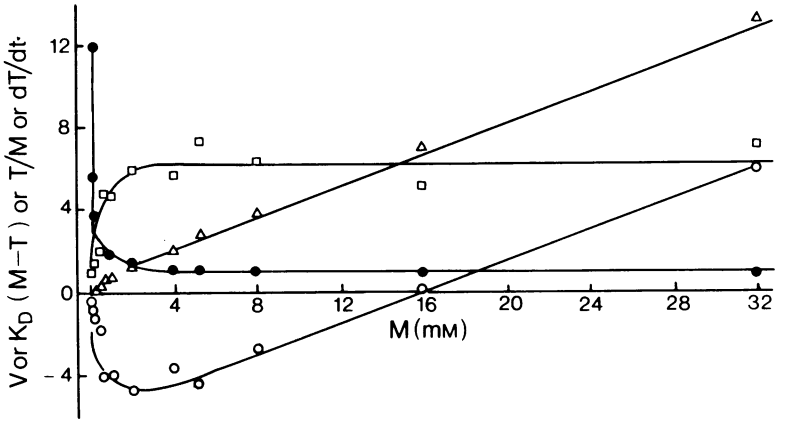

Figure 5 Plots of V, μmol g^{-1} h^{-1} (□); K_D (M−T), μmol g^{-1} h^{-1} (○); T/M, lung tissue/medium (±)-methadone concentration ratio; or dT/dt, μmol g^{-1} h^{-1} (△) vs M (mM). Incubation period 30 minutes. All values except M are calculated. See text for details.

(±)-methadone (approximately 0.2 mM), the T/M ratio declined very slowly. If one looks at the relationship between the tissue concentration and the medium concentration, it is easy to realize that at higher concentrations (>0.5 mM), (±)-methadone is being transported by a process of simple diffusion. These results suggest that at low concentrations (±)-methadone is transported predominantly by a self-saturable process while at higher concentrations it is transported by a process of simple diffusion.

The observation that the uptake of (+)-methadone was higher than that of the (−)-isomer indicates that the uptake process shows significant stereospecificity thus fulfilling one additional criterion of an active transport process. Although Orton *et al.* (1973) investigated the uptake of (±)-methadone by isolated perfused lung of rabbit, data on stereospecificity were not reported. However, Hug (1967) demonstrated that the *in vitro* rate of uptake of dextrorphan by the rabbit choroid plexus was greater than that of levorphan. He suggested that the transport mechanism was the same for either compound, but the relative affinity of dextrorphan and levorphan were different. Stereospecificity has also been demonstrated for the adrenergic neuronal uptake ($Uptake_1$) of noradrenaline (Iversen, 1967) and for several amino acids by different tissues (Schultz & Curran, 1970). However, it was not possible to demonstrate stereospecificity for the uptake of noradrenaline (NA) by perfused rabbit lung (Iwasawa *et al.*, 1973). The present data are not complete enough to draw any conclusions about the differential kinetic characteristics of the uptake of the two stereoisomers of methadone.

Significant inhibition of (±)-methadone uptake at low temperatures (0–4°C) indicates the temperature-dependent nature of this uptake process. Other investigators (Junod, 1972b; Orton *et al.*, 1973) have also shown that lower temperatures adversely affect the uptake of NA and 5-hydroxytryptamine (5-HT) by perfused lungs of rabbits and rats with inhibition ranging from 54 to 85%. In the present study it was observed that the inhibitory effect of low temperatures is greater when the substrate concentrations are lower. This, of course, would be expected if one considers that these substances are transported by an active process followed by simple diffusion.

Elimination of O_2 from the gaseous phase significantly inhibited the uptake of (±)-methadone suggesting that the 'energy' for the transport mechanism was supplied largely by aerobic or oxidative metabolism. This was particularly true at low concentrations because it was observed that the degree of inhibition decreased as the concentration of (±)-methadone in the incubation medium was increased. This would be expected, if one takes into consideration that (±)-methadone is transported by two processes, the O_2-dependent active transport being mainly responsible at low concentrations and simple diffusion becoming increasingly important as the concentration of (±)-methadone is increased.

Omission of glucose from the incubation medium produced a significant inhibition of (±)-methadone uptake, the degree of inhibition being nearly constant throughout the entire incubation period. These data would suggest that the energy for the transport process is mainly derived from the oxidative metabolism of glucose. A small amount of endogenous glucose is likely to be present and is probably utilized by the tissue to provide some energy. The inhibitory effect is in sharp contrast to the findings of Junod (1972b) and of Iwasawa *et al.* (1973), who

found that the lack of glucose did not have a significant effect on the uptake of 5-HT and NA by the isolated perfused lung of the rat. The results, therefore, would indicate that the site and the mechanism of (±)-methadone uptake may be different from those involved in the uptake of 5-HT and NA by the perfused lung. The results of Strum & Junod (1972) and that of Iwasawa *et al.* (1973) have shown that the uptake of NA and 5-HT occurs mainly in the capillary endothelium. At present, it is difficult to speculate on the site of the uptake of (±)-methadone in the lung tissue.

The need for adequate oxygenation and glucose availability indicated that the (±)-methadone uptake is an energy-dependent active transport process. This process is expected to perform optimally only under normal physiological conditions. When the lung slices were exposed for a very short period of time (3 min) to high temperature (≈100°C), the (±)-methadone uptake was drastically reduced. Such exposure to high temperature would disrupt all the 'energy' yielding processes and also the integrity of the cell membrane and the uptake observed is most likely to be due to simple diffusion. This observation provides indirect evidence to support the contention that (±)-methadone is taken up by the lung tissue by an active transport process.

The (±)-methadone uptake by the lung tissue was adversely affected when extracellular Na^+ concentration was reduced by replacement with Li^+ indicating that the transport process is dependent on extracellular Na^+. Junod (1972b) has recently shown that the uptake of 5-HT by the isolated perfused lung of rat is also adversely affected by a deficiency of Na^+ in the perfusion medium. These effects are similar to those reported for the transport of 5-HT and NA in other cellular systems (Bogdanski, Tissari & Brodie, 1968; Tissari, Schonhofer, Bogdanski & Brodie, 1969; Schultz & Curran, 1972). It has been suggested that the membranal transport of these substances is mediated by a 'carrier' that is dependent on the availability of Na^+ for the transport of the solute (Schultz & Curran, 1972). The affinity of the 'carrier' for the solute is increased when Na^+ is present in adequate concentrations, but is reduced in its absence. The movement of solute molecules, therefore, depends primarily on the availability of Na^+ in the extracellular fluid. Other ions such as K^+ and to a lesser extent Ca^{2+} and Mg^{2+} may also be involved in changing the affinity of the carrier for the solute. From these lines of evidence it is tempting to speculate that the (±)-methadone uptake by the lung tissue may involve a similar Na^+ dependent/sensitive carrier transport mechanism. Whether a Na^+–K^+ dependent adenosine-triphosphatase (ATPase) is involved in such a transport system must be determined by further experimentation. It is, however, important to note that the presence of such a Na^+–K^+ dependent ATPase has been demonstrated in the lung tissue (Mustafa, Ibrahim, Le & Cross, 1969).

It has been shown that the main pathway of metabolism of (±)-methadone involves N-demethylation followed by cyclization (Beckett *et al.*, 1971; Pohland *et al.*, 1971; Sullivan, Smits, Due, Booher & McMahan, 1972). These reactions occur mainly in the liver. The two main metabolites that have been identified are: 2-ethylidene-1,5-dimethyl-3,3-diphenyl pyrrolidine and 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (Beckett *et al.*, 1971; Pohland *et al.*, 1971). These metabolites are also found in the lung tissue after (±)-methadone is given to rats (Sullivan *et al.*, 1972). However, it is not known whether these metabolites are formed in the lung tissue or are transported there after being formed first in the liver. In the present investigation experiments were not conducted to determine quantitatively the extent to which (±)-methadone was metabolized by the lung tissue slices. However, preliminary results obtained with thin-layer chromatographic analysis of incubation medium and tissue homogenate indicate that under the conditions of incubation, (±)-methadone was not metabolized to a measurable degree. Recently Orton *et al.* (1973) have demonstrated that in the rabbit isolated blood-perfused lung preparation, (±)-methadone was metabolized to the extent of 15%. It is, therefore, quite unlikely that the lung slices used in the present study would metabolize much higher proportion of (±)-methadone present in the incubation medium. The fluorescence assay used in the present investigation is specific for (±)-methadone and the two major metabolites mentioned above do not interfer in this assay. Therefore, the T/M ratios calculated are not likely to be very significantly different from the corrected T/M ratios in which allowance for metabolism is made. Actual experiments will have to be conducted to verify this.

One of the more conventional ways of investigating the 'energy' dependency of an active transport process has been to study the effect of a number of chemicals which produce 'metabolic inhibition'. These agents, by a variety of mechanisms, disrupt the cellular metabolic reactions and the attendant 'energy' production and, therefore, adversely and non-specifically affect the active transport processes. Iodoacetate is such a metabolic inhibitor that produces its effect through alkylation of –SH groups of enzymes, particularly those that are involved in the glycolytic pathway (Webb, 1963). Dixon (1937) has shown that triose phosphate dehydrogenase, alpha-glycerophosphate dehydrogenase, and lactate dehydrogenase are inhibited by iodoacetate at concentrations of 10 mM or higher. Other investigators have shown that the inhibition of iodoacetate is relatively slow, not so much because of its rate of penetration, but more so because of the slow rate of alkylation of the enzymes. Fuhrman & Field (1943) have shown that in rat brain

slices and cell-free extracts, 0.01 mM iodoacetate produced about 50% inhibition of glycolysis and only 12% inhibition of 'respiration'. This effect was seen at the end of a 3 h incubation period with a latency of about 90 minutes. Only at very high concentrations (5 to 50 mM) was a significant inhibition (>65%) of 'respiration' seen. These results provide a rational explanation as to why in the present study only 20% inhibition of (±)-methadone uptake was seen in 30 min at 1.0 mM iodoacetate concentration. A variable degree of inhibition of the active transport of different compounds (morphine, dihydromorphine, 5-HT, NA, etc.) by iodoacetate has been observed by other investigators (Takemori & Stenwick, 1966; Hug, 1967; Rubin, Owens & Rall, 1968; Iwasawa *et al.*, 1973). One should, therefore, be careful in interpreting results obtained by using iodoacetate as a tool to evaluate the energy requirements or dependency of a transport process.

A mention was made earlier that the inhibitory effect of Na^+ lack on (±)-methadone uptake may be related to the involvement of Na^+-K^+ dependent ATPase in the transport of (±)-methadone. If this were true, then the inhibitory effect of iodoacetate may also be due to its interaction with the ATPase, since Shamoo & Brodsky (1970) have indicated that Na^+-K^+ dependent ATPase is a –SH containing enzyme.

From the foregoing discussion it can be concluded that at low medium concentrations (≤0.5 mM) active transport plays a predominant role in the transport of (±)-methadone. It is also the only demonstrable component at low concentrations because simple diffusion is expected to transport relatively insignificant amounts of (±)-methadone into the lung tissue cells. However, as the (±)-methadone concentration is increased, the active transport becomes saturated and the (±)-methadone uptake becomes concentration-dependent. In other words, at high medium concentrations (M > 1 mM) (±)-methadone is transported by the process of simple diffusion as indicated by the T/M ratio that approaches unity.

It should be noted that at low medium concentrations, when the intracellular concentration of (±)-methadone becomes several times that of the medium concentration it is quite possible that (±)-methadone will diffuse out of the cells into the medium. Thus it becomes difficult to obtain correct estimates of the rate of active transport at any given medium concentration below which the active transport process is not saturated. One way to overcome this difficulty is to analyse the transport of (±)-methadone by a method known as 'system identification' (Sage, 1972). This kind of approach has enjoyed wide attention in the field of engineering. The following is a summary of the analysis of the data, obtained in this investigation by this approach.

It is assumed that the transport of (±)-methadone occurs by two processes: (a) simple diffusion and (b) a saturable active transport process. These two processes can be represented by the equation

$$\frac{dT}{dt} = V + K_D(M - T) \qquad (1)$$

(Akedo & Christensen, 1962) where M is the (±)-methadone concentration in the incubation medium, T is the (±)-methadone concentration in the tissue, and t is the duration of incubation in hours. $K_D(M-T)$ and V are the velocities of the diffusion and of the active transport porcesses respectively. $K_D(M-T)$ is an expression of Fick's law of simple diffusion where K_D is the diffusion constant (h^{-1}). K_D is characteristic of the substance being transported and is considered to be independent of t, M and T. V is assumed to be independent of time of incubation (t) as well as of the tissue concentration of (±)-methadone (T). Integration of equation (1) yields:

$$\frac{T}{M} = \frac{V}{K_D}(1 - e^{-K_D t})\frac{1}{M} + (1 - e^{-K_D t}) \qquad (2)$$

In this relationship, K_D and V are the two unknown parameters that can be estimated from experimental data. At very high medium concentration of (±)-methadone, V by definition, becomes independent of M and nearly constant. Thus a T/M vs 1/M plot should result in a straight line. From this plot one can calculate K_D and V (Figure 4). If such a plot is not a straight line but a curve then it could indicate that V is dependent on time over the range of concentration used. After obtaining the value for K_D one can then calculate V at low medium concentrations. Under these circumstances V will vary with M. Analyses of the present data by this procedure were done and the results are summarized below:

(i) K_D was found to be 5.0 h^{-1} when incubations were carried out for 15 and 30 minutes.

(ii) At high medium concentrations of (±)-methadone (1.0–32.0 mM) V became nearly constant (V_{max}) and equalled 6.5 μmol g^{-1} h^{-1} (Figure 5). The V_{max} was identical when incubation period was 15 or 30 minutes.

(iii) At all incubation periods and at (±)-methadone concentrations greater than 1.0 mM, V was found to be independent of M. Thus, for 15, 30 and 60 min incubation periods and for medium concentrations (M) from 1.0 to 32.0 mM, calculated values for V (μmol g^{-1} h^{-1}, mean ± s.e. mean) were 5.05 ± 0.4, 5.92 ± 0.4 and 6.60 ± 0.5, respectively. At M ≤ 0.05 mM, V was dependent on M, since at these concentrations the 'active transport' process was not saturated. Extensive experimental work at low medium concentrations (M ≤ 0.5 mM), was carried out only for the incubation period of 30 min and, therefore, no definite relationship between the

time of incubation (t) and V can be suggested or established.

(iv) At low M, active transport will result in a much higher concentration of (±)-methadone in the tissue (T) than in the medium (M). This was clearly indicated by the fact that at low concentration $T/M \ggg 1$. Hence, the diffusion process will actually transport (±)-methadone out of the tissue and into the medium. This is indicated by the negative sign of diffusion process $[K_D(M - T)]$ at low concentrations of (±)-methadone (Figure 5).

(v) However, at high medium concentrations the active transport process becomes saturated and now (±)-methadone transport becomes concentration-dependent as described by the T/M value which approaches unity. Under these conditions then, simple diffusion process becomes the main transport mechanism. The velocity due to diffusion $[K_D(M - T)]$ became positive (Figure 5), as soon as M became greater than T. Note that the *net* velocity of uptake (dT/dt) by the tissue is positive at all times.

It must be mentioned that the values shown in Figure 5 are calculated values of $[K_D(M - T)]$ and of dT/dt. Therefore, this instantaneous velocity of net transport (dT/dt) at 30 min is not the same as the average net velocity of transport namely $\Delta(T/M)/\Delta t$ obtained at the end of the 30 min incubation period.

This study was supported in part by USPHS Training Grant GM-1217. Authors are grateful to Dr Donald N.H. Chi for his advice in the preparation of this manuscript.

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(Received July 29, 1975. Revised August 2, 1976.)