J. Pharm. Pharmac., 1974, **26**, 92–100 *Received September* 1, 1973

A new modification for studying 5-HT uptake by blood platelets: a re-evaluation of tricyclic antidepressants as uptake inhibitors*

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The effect of imipramine and other antidepressive drugs on 5-HT uptake by rabbit platelets was re-evaluated, since it had become obvious that, in previous studies, too high substrate concentrations and too long incubation times had been used. A Km of $1{\cdot}8 \times 10^{-7}$M was obtained for 5-HT uptake when 1 min incubation in plasma was used. Imipramine caused a half-maximal inhibition at 10^{-7}M or lower concentration. When the platelet-rich plasma was diluted with buffer solution, only about 2×10^{-8}M imipramine was required for 50% inhibition. This concentration is only 1/500 to 1/100 of concentrations described in most earlier data. In this study, other tricyclic drugs and phenothiazine derivatives were also much more effective than previously demonstrated. Their rank of potency order was, however, very similar to that earlier described. The correlation of uptake inhibition in platelets with 5-HT uptake inhibition in brain synaptosomes was found to be highly significant. It is concluded that the previous studies give valuable information on the relative potency of different drugs inhibiting 5-HT uptake. When the technique is modified as suggested by the present results, the level of uptake-inhibiting potency in an absolute sense is close to that described for other tissues, especially brain synaptosomes or slices. Therefore, platelets can more reliably be used as an easily obtainable model for nerve endings in uptake studies.

Blood platelets have been used extensively as easily obtainable models for nerve endings (for reviews see Paasonen, 1965, 1968, 1972; Paasonen, Ahtee & Solatunturi, 1971; Pletscher, 1968; Sneddon, 1973). These studies have dealt especially with the storage and release of 5-hydroxytryptamine (5-HT), but some work has also been done to evaluate inhibitors of amine uptake (cf. Sneddon, 1973).

It was recently demonstrated by Sneddon (1969) and by Lingjaerde (1970), as well as by our group (Tuomisto, 1972; Tuomisto, Walaszek & others, 1973), that the Km for 5-HT uptake by blood platelets is very low, and that by using substrate concentrations not exceeding the Km, the uptake lasts only for a few minutes. Thus about 90% of the substrate added was taken up in the first 10 min, when the initial concentration was 10^{-7}M (Tuomisto & others, 1973). These findings prompted a re-evaluation of some inhibitors of this uptake.

Marshall, Stirling & others (1960) originally showed that imipramine decreased 5-HT uptake. According to Stacey (1961), it was a competitive inhibitor, and it inhibited 50% of uptake at about 5×10^{-7}M. Lingjaerde (1970) found about 90%

* A preliminary report of some of these results was presented at the Fifth International Congress on Pharmacology, San Francisco, California, July 1972.

inhibition with 10^{-6}M imipramine. Most other workers have been able to confirm Stacey's results only qualitatively but not quantitatively. A 50% inhibition, if obtained at all, was achieved in most studies on human or rabbit platelets only with concentrations varying from 2×10^{-6} to 10^{-5}M (Yates, Todrick & Tait, 1964; Pletscher & Tranzer, 1967; Ahtee, Tuomisto & others, 1968; Todrick & Tait, 1969; Tuomisto, 1969). This is contrary to the high potency of imipramine and its congeners as 5-HT uptake inhibitors in brain slices (Ross & Renyi, 1969), or as a noradrenaline uptake inhibitor in rat heart (Iversen, 1965), aortic strips (Maxwell, Keenan & others, 1969) or brain slices (Hamberger, 1967). On the other hand, the amine uptake by brain slices or synaptosomes has also been reported as being inhibited in some conditions only with relatively high concentrations of imipramine or desipramine (Blackburn, French & Merrills, 1967; Ross & Renyi, 1967; Lidbrink, Jonsson & Fuxe, 1971).

In this study it was decided to utilize a concentration not exceeding the Km, and to test 5-HT uptake under conditions that allow for a claim that the measured rate of uptake represents the initial velocity. This is a necessary condition for meaningful kinetic studies, and these results were hence presumed to clarify the kinetics of the reaction and of its inhibition as well.

METHODS AND RESULTS

Rabbit platelet-rich plasma (PRP) was prepared as previously described (Tuomisto, 1968). Since the Km of 5-HT uptake is less than 10^{-6}M, and at these concentrations the availability of 5-HT present in plasma is limited to very few minutes, 1 min incubation time was used.

In the first experiments, imipramine and desipramine were added into the incubation tubes (15 ml polypropylene centrifuge tubes) in 0·2 ml saline. [^{14}C]-5-HT (The Radiochemical Centre, Amersham) was added to ice-cold PRP in 1/100 volume of dilute hydrochloric acid, to make a final concentration of 10^{-7}M. Two ml portions of this plasma were rapidly added to the incubation tubes which were in an ice-bath. The whole test-tube rack was then transferred for 1 min to a water bath at 37°, returned to the ice-water for about 5 min and centrifuged for 5 min in a refrigerated Sorvall centrifuge at 20 000 *g*. The pellet was washed with 2 ml saline, recentrifuged, solubilized in Soluene 100 (Packard Instrument Co.) and counted in a scintillation counter (Wallac Decem 314).

Both antidepressants exhibited a dose-response curve of a sigmoid type (Fig. 1). The maximal inhibition appeared to be about 80% of uptake. Imipramine was ten times more effective than desipramine, and the half-maximal inhibition was achieved at about 10^{-7} and 10^{-6}M concentrations, respectively.

The kinetics of the uptake process were studied in the following way. In addition to 10^{-7}M radioactive 5-HT, different concentrations of non-labelled cold carrier 5-HT were added to the tubes. The platelets were incubated as above with or without imipramine. When the uptake was plotted against 5-HT concentration, a two-phase curve was obtained (Fig. 2). There was a saturation-type curve between the concentrations 10^{-7} and 10^{-6}M. At higher concentrations, however, the correlation became linear. The linear portion was assumed as being due to diffusion or to other passive

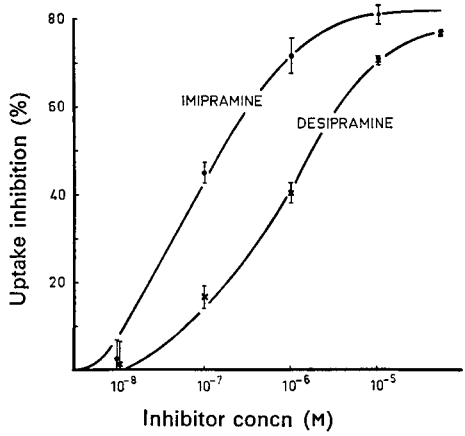

FIG. 1. Inhibition of 5-HT uptake in plasma by imipramine and desipramine during a 1 min incubation at 37°. 5-HT concentration 10^{-7}M. Mean ± s.e. of five to seven duplicate experiments.

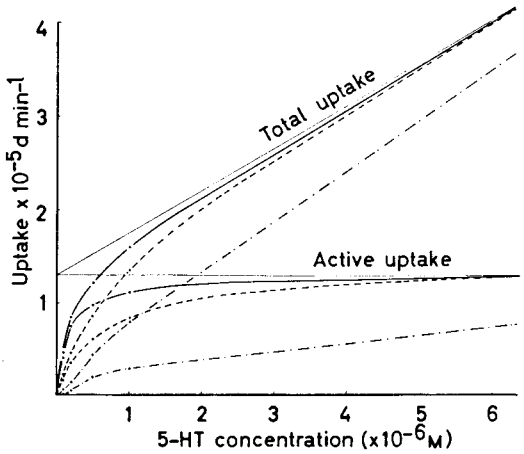

FIG. 2. 5-HT uptake as a function of 5-HT concentration (— control; - - - imipramine 10^{-7}M; —·—·— imipramine 10^{-5}M. The hyperbolic curve of the total uptake approached a straight line (thin line) and was linear to at least 10^{-4}M substrate concentration. This line was extrapolated to the ordinate and the linear part of the reaction was subtracted from the total uptake. The remaining part, assumed to be the active uptake, was used for kinetic analysis (Fig. 3). Mean of five duplicate experiments.

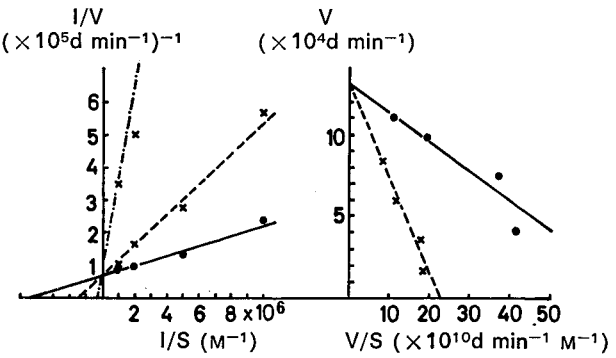

FIG. 3. A kinetic analysis according to Lineweaver & Burk (left) and Eadie & Hofstee (right) of the same results as shown in Fig. 2. The analysis reveals a competitive type of inhibition by imipramine.

non-saturable processes, and it was extrapolated to zero concentration. This line was utilized to calculate the linear process at different concentrations, as was done by Bogdanski, Tissari & Brodie (1970). The calculated non-saturable uptake was then subtracted from the total uptake. The resultant net uptake exhibited a typical saturation curve (Fig. 2), which was used for a kinetic analysis according to Lineweaver & Burk (1934) or Eadie and Hofstee (Eadie, 1952; Hofstee, 1952). A Km of about $1{\cdot}8 \times 10^{-7}$M was obtained by both methods. Imipramine 10^{-7}M raised the Km to $5{\cdot}9 \times 10^{-7}$M according to both plots (Fig. 3). According to the Lineweaver-Burk plot, the Km was about 5×10^{-6}M in the presence of 10^{-6}M imipramine. In the Eadie-Hofstee plot, which is more sensitive to distortions, this concentration no longer gave a linear correlation for various 5-HT concentrations.

In the second part of the study, the technique was adapted to allow a greater number of samples. Various inhibitors, as well as the radioactive 5-HT, each in 0·1 ml saline, were pipetted to 1·5 ml polypropylene tubes. One ml of plasma was rapidly added with an automatic pipette (Finnpipette) with a polypropylene tip. The test-tube rack was kept in an incubator at 37° for 1 min, and returned to the ice-bath. The tubes were rapidly transferred to an Eppendorff 3200 centrifuge and spun for 2 min at about 12 000 *g*. The pellet was washed with 1 ml saline, solubilized in Soluene and the radioactivity counted as above.

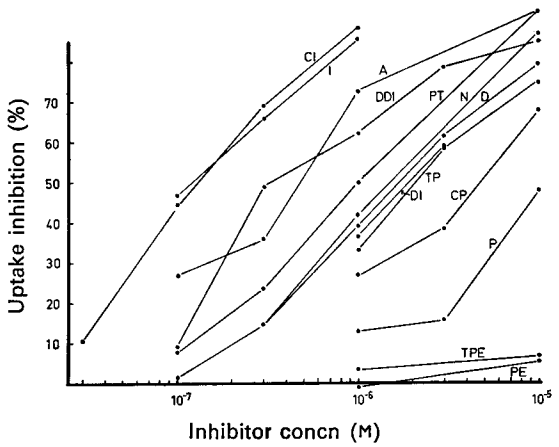

FIG. 4. Uptake inhibition in plasma by some tricyclic drugs and phenothiazine derivatives. Incubation time was 1 min, 37°, plasma volume 1 ml, the inhibitors and the radioactive 5-HT added in 0·1 ml saline each. Cl = chlorimipramine; I = imipramine; A = amitriptyline; DDI = didesmethylimipramine; PT = protriptyline; N = nortriptyline; D = doxepine; DI = desipramine; TP = triflupromazine; CP = chlorpromazine; P = promazine; TPE = trifluoperazine; PE = perfenazine. Mean of four to nine duplicate experiments.

A number of tricyclic antidepressants and phenothiazine derivatives was tested by using this modification. The results are given in Fig. 4. The most potent compounds, imipramine and chlorimipramine, caused a half-maximal inhibition at about 10^{-7}M concentration, followed in potency by amitriptyline and other antidepressants. Phenothiazine derivatives were clearly less potent uptake inhibitors. Those with a piperazine ring were at least 1000 times less potent than imipramine.

Even 1 min incubations in plasma do not strictly fulfil the requirements of the initial velocity measurements (Tuomisto & others, 1973). The difficulty of keeping the incubation time constant, however, prevented the use of incubations shorter

than 1 min. Therefore other means were sought to measure the uptake rate at the initial linear stage. The factor causing the decline in the reaction rate is the decrease of substrate concentration in the incubation medium due to the extremely rapid uptake (Tuomisto & others, 1973). To prevent this, the number of platelets per ml was reduced by diluting the platelet-rich plasma with Krebs-Henseleit bicarbonate buffer pH 7·4 (Krebs, 1950). 1·1 ml of the buffer, including the inhibitor and the radioactive amine, was pipetted into the 1·5 ml tubes. At the beginning of the incubation, 0·1 ml of PRP was added to the buffer, and the samples were incubated for 1 min. Then the tubes were rapidly transferred to the ice-bath, and then centrifuged for 2 min, and the pellets were treated as above.

The uptake in the buffer was more active than in plasma. The number of platelets in the former was only 1/10 of that in the latter. Yet the average uptake in the buffer was 1/3 of that in the plasma. The amount taken up in the Krebs-Henseleit buffer was 3 to 13% of 5-HT added, in plasma it was 7 to 26%. The variation was mainly due to the different number of platelets in the PRP derived from different rabbits. Thus there was a clear decrease in the 5-HT concentrations during the incubation in plasma, whereas in the buffer the change was usually less than 10%. The Krebs-Henseleit phosphate buffer (Krebs, 1950) was also tried instead of the bicarbonate buffer. In the phosphate buffer the initial rate was about 2/3 of that in the bicarbonate buffer, but on the other hand the uptake continued longer. After 8 min in the bicarbonate buffer, the amount of radioactivity again began to decrease, whereas in the phosphate buffer it increased for at least 15 min. This may be due to a more stable pH in the phosphate buffer. In both cases, however, the rate of uptake was much reduced after the first minute and 1 min incubation was used only for studies with inhibitors. In these experiments imipramine was about twice as effective in Krebs as it was in plasma.

To get an idea of the actual incubation time and its possible effects on the results, the rise and fall of the temperature in different tubes was determined. The results (Fig. 5) suggest that the slow warm-up in thicker tubes causes a lower uptake. To avoid the influence of the slow and possibly variable rise of temperature the following method was used: the 0·1 ml of PRP was added to 1·1 ml of prewarmed buffer containing the radioactive amine. After 1 min the sample was poured through a

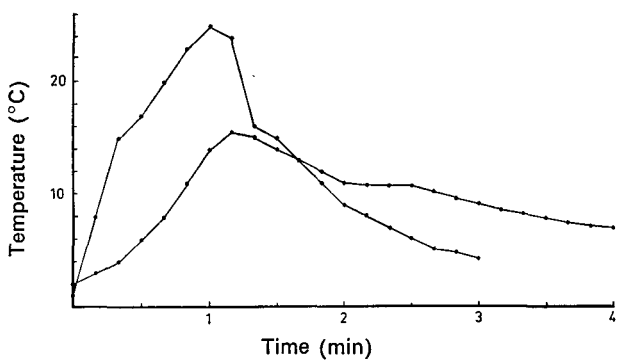

FIG. 5. Temperature change during 1 min incubation in two different tubes. The temperature was recorded every tenth second with an electric thermometer TE 3, Elektrolaboratoriet, Copenhagen. The tubes were transferred in a test-tube rack from ice-bath to a metabolic shaker shaking continuously 65 times a minute at 37°. After 1 min they were transferred to the ice-bath again. Lower curve: 15 ml thick-walled centrifuge tube with 2·2 ml distilled water. Higher curve: 1·5 ml polypropylene tube with 1·2 ml distilled water. A single representative experiment.

membrane filter. This would render the incubation time much more accurate and allow the use of even shorter incubation times. These experiments were not successful, however. The membrane filters (Schleicher & Schüll, Selectron BA 85, 0·45 μm pore size) broke down the platelets. Most of the radioactivity passed through the filter, and most of the endogenous 5-HT as assayed fluorimetrically (Weissbach, Waalkes & Udenfriend, 1958) was also found in the filtrate rather than on the filter.

Finally, the uptake-inhibiting potency of compounds presented in Fig. 4 was also tested on rat brain synaptosomes. The method used was essentially that of Snyder & Coyle (1969), and the modification has been described in detail elsewhere (Tuomisto, Tuomisto & Smissman, 1973). Crude synaptosomal preparations of rat brain stem were incubated for 5 min in Krebs-Henseleit bicarbonate buffer at pH 7·4 with 10^{-7}M ^{3}H-5-HT in the absence or presence of inhibitors. After the incubation the particles were separated by filtration, and their total radioactivity was counted. The potency of inhibitors was expressed as ID50 (concentration inhibiting 50% of uptake as estimated graphically by log probit paper). The results are given in Table 1. The

Table 1. ID50 *of tricyclic drugs and phenothiazine derivatives on* 5-HT *uptake by rat brain stem synaptosomes.* Incubation 5 min at 37° in Krebs-Henseleit bicarbonate buffer pH 7·4. Approximate ID50 on platelets is also given. A maximal response of 80%, i.e. an inhibition-resistant 20% of uptake was assumed in platelet estimations. For comparison the results of Todrick & Tait (1969) are shown on the right.

Inhibitor	Synaptosomes (concn M)	Platelets (concn M)	Platelets (T&T) (concn M)
Imipramine	$1{\cdot}1 \times 10^{-7}$	8×10^{-8} *	$2\text{–}3 \times 10^{-6}$
Chlorimipramine	2×10^{-8}	9×10^{-8}	3×10^{-7}
Amitriptyline	$1{\cdot}2 \times 10^{-7}$	3×10^{-7}	5×10^{-6}
Didesmethylimipramine	2×10^{-7}	3×10^{-7}	7×10^{-6}
Protriptyline	5×10^{-7}	6×10^{-7}	9×10^{-6}
Nortriptyline	4×10^{-7}	9×10^{-7}	1×10^{-5}
Doxepine	4×10^{-7}	1×10^{-6}	—
Desipramine	$1{\cdot}1 \times 10^{-6}$	1×10^{-6}	1×10^{-5}
Triflupromazine	8×10^{-7}	$1{\cdot}5 \times 10^{-6}$	—
Chlorpromazine	1×10^{-6}	3×10^{-6}	4×10^{-5}
Promazine	3×10^{-6}	7×10^{-6}	—
Perfenazine	5×10^{-6}	1×10^{-4}	—
Trifluoperazine	5×10^{-6}	$>10^{-4}$	—
Prochlorperazine	5×10^{-6}	not tested	—

* or lower depending on the technique.

potency in rat synaptosomes is in all cases close to that in rabbit platelets. The greatest deviation is the group of phenothiazine derivatives with a piperazine ring in the side-chain. They were very poor inhibitors of 5-HT uptake by platelets with an ID50 of 10^{-4}M or higher. Although these compounds were the group with the lowest potency also in synaptosomes, they had an ID50 of 5×10^{-6}M. If this group is excluded, since the ID50 is far outside of the therapeutic concentrations, the linear correlation coefficient for potency in platelets *vs* potency in synaptosomes is $r = 0{\cdot}972$ ($P < 0{\cdot}001$). Even if the piperazine compounds are included, there is a highly significant correlation between the potencies in these two tissues ($r = 0{\cdot}839$, $P < 0{\cdot}001$).

DISCUSSION

These data confirmed our previous results: that by using a low substrate concentration and brief enough incubation time, the kinetics of 5-HT uptake in platelets can be determined much in the same way as that of synaptosomal uptake (Bogdanski & others, 1970). An essential feature is that the linear part of the uptake is subtracted. Otherwise no Km can be determined and, especially in the Eadie-Hofstee plot, the linear function of V *vs* V/[S] is distorted to a hyperbolic curve. In addition, non-competitive inhibitors may appear competitive in the Lineweaver-Burk plot (Tuomisto & others, 1973).

In these conditions imipramine caused a half-maximal inhibition at about 10^{-7} to 5×10^{-8}M concentration in plasma. After diluting the platelets with buffer, only about 2×10^{-8}M imipramine was required for 50% inhibition of 5-HT uptake.

Until now, the widest study on the potency of tricyclic drugs and phenothiazine derivatives as inhibitors of 5-HT uptake by platelets is that of Todrick & Tait (1969). In this study the rank of the order of most drugs is the same as in the present study, with some minor exceptions. The most notable exception was that they found chlorimipramine to be about ten times more effective than imipramine, which was not so in this study. What is more remarkable, however, is that in their study, the level of potency of the whole group was different from that in the present study. They obtained a 50% inhibition with a concentration of about 2 to 3×10^{-6}M imipramine, 5×10^{-6}M amitriptyline or 4×10^{-5}M chlorpromazine. These concentrations are 20 to 150 times higher than the concentrations required in plasma in this study. The difference is still greater if compared with the experiments performed in buffer/plasma mixture. A comparison with other previous data on rabbit or human platelets gives the same or greater difference (Yates & others, 1964; Fuks, Lanman & Schanker, 1964; Pletscher & Tranzer, 1967; Ahtee & others, 1968). In all of the above studies, an initial substrate concentration exceeding the Km by a factor of 30 to 50 was used, and the incubation prolonged for 30 to 60 min or more. By necessity this means two things: firstly, since the inhibitors are competitive, the high substrate concentration renders the inhibition less effective; secondly, the role of diffusion increases (Fig. 2). Therefore only a moderate maximal inhibition was achieved in many of these studies, especially in rabbit platelets (Pletscher & Tranzer, 1967), unless concentrations were used that also caused release (Ahtee & others, 1968). This is contrary to the maximal inhibition of 80 to 90% in the present experiments. The imipramine-resistant 10 to 20% corresponds well with the linear type of uptake at the level of 10^{-7}M substrate concentration (compare Figs 1 and 2). This fraction is most probably diffusion aided by granular storage, which effectively keeps the cytoplasmic side of the cell membrane free of 5-HT (Solatunturi & Tuomisto, 1968). Stacey (1961) used a relatively short 15 min incubation and 2×10^{-6}M 5-HT concentration, which probably contributed to the fact that his results on imipramine are closer to the present results than are most other data. The accordance is still better with the recent results of Lingjaerde (1970). He used 10^{-6}M 5-HT and 5 min incubation time. The latter study is the only one where the technique also allowed for the estimation of the Km.

Of the three techniques used in this study, an incubation in the Krebs-Henseleit bicarbonate buffer obviously gives the most reliable kinetic data, which best represents the initial velocity. However, the 1 min incubation in plasma also seems to give

quite reliable results and can be used instead as a more practicable possibility. As shown in Fig. 5, the temperature never reached 37° even in the most favourable conditions. This points to the remarkable activity and speed of the uptake process, since even at subnormal temperature as much as 7 to 26% of the total radioactivity was taken up in 1 min. In our previous study (Tuomisto & others, 1973) around 40% was taken up in a minute when a thin-walled polypropylene tube was used for incubation. In the same study, a clear difference was noted in the apparent Km when the incubation was performed in thin-walled tubes as opposed to thick-walled ones. Therefore, heating of the buffer beforehand, a very short incubation, and a rapid separation of platelets would be the most reliable method for kinetic studies. Unfortunately the separation with the filtration procedure was not successful, and therefore conclusive results of the initial rate at 37° were not obtained.

The rank order of potency of the drugs tested as 5-HT uptake inhibitors, was very similar in platelets and in the nerve endings of the central nervous system (see Table 1). Taking technical differences into account (e.g. variable binding of the drugs to plasma protein in the platelet studies, obviously different temperature pattern in platelet and synaptosome studies), the correlation is to be considered excellent. On the other hand, there is no obvious correlation to either noradrenaline or dopamine uptake inhibition by antidepressive drugs as calculated on the basis of the data of Horn, Coyle & Snyder (1971). This suggests that molecular requirements of the 5-HT uptake mechanism in the platelets as well as in the brain are very close to one another or identical, but different from both those of noradrenaline and those of dopamine uptake mechanisms. This supports the conclusion of Sneddon (1973) that the platelet may only be applicable as a model for the study of serotonergic but not of other neurons.

The present results extend the previous information on the use of platelets to predict the effects of antidepressive drugs in order to screen some aspects of their relative effectiveness. Compared with synaptosome studies, the platelet technique is simple and the reproducibility much better. Therefore it appears that the use of platelets as neuronal models is worthwhile in uptake studies.

Acknowledgements

The author wishes to thank Mrs Anneli v. Behr and Miss Aira Klemelä for skilful technical assistance. This study was supported in part by grants from the National Research Council for Medical Sciences, Helsinki, Finland, and from the Yrjö Jahnsson Foundation, Helsinki, Finland. The following pharmaceutical firms generously supplied the drugs tested: Ciba-Geigy AG, chlorimipramine HCl and desipramine HCl; H. Lundbeck & Co., nortriptyline HCl, Lääke Oy, perfenazine, prochlorperazine maleate, chlorpromazine HCl; Oy Medikalis Ab, imipramine HCl; Merck, Sharp & Dohme, protriptyline HCl; Orion Oy, Doxepin HCl; Pharma Rhodia, trifluoperazine HCl; and Oy Star Ab, amitriptyline HCl, and promazine HCl. Didesmethylimipramine (Lakeside Labs.) was donated by Professor M. K. Paasonen, and trifluopromazine HCl (E. R. Squibb & Sons) by Dr. Liisa Ahtee.

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