males and females (Figure 1B). However, the incidence of fertile matings, defined as females with vaginal plugs and at least one fertilized ovum, were below control levels at all times and reached the lowest value in the 3rd week; the incidence of fertile matings in test groups was 80% less than in controls. Fertile females in the test group had a reduced number of total ova, both fertile and infertile, in the 3rd week. However, considering only fertilized ova per mating, this reduction below control levels was unequivocal in the 3rd week and persisted until the 5th week. In the 3rd week, the number of fertilized ova in test and control group were 6.6 and 10.0 per female, respectively. Thus, there was a 34% reduction in the number of fertilized ova in the test groups. This effect is less than the 80% difference between test and control groups in the incidence of mated females with at least 1 fertilized ova; the greater effects would have been missed if the vaginal plugs had not been checked.

Individual males: Data for 33 test and 20 control individual males in 1 replicate were analyzed. The fertility of the males did not seem to be directly related to the total number of matings; the overall percentage of fertile matings in the test and controls was 66.7% and 95.3%, respectively. Some treated males had periods of fertile matings followed by periods of no mating or only infertile matings, with subsequent return to fertile mating. Thus, it was possible to determine the periods of infertility induced by niridazole (Figure 2); these periods may be underestimates, as consecutive periods of failure to mate were excluded. In most males, the period of infertility was continuous. In the treated group, 2 out 33 were consistently fertile, and 6 showed no consistent periods of infertility. Of the 24 males with infertile periods, 22 were infertile between day 21 and 25, thus confirming observations in individual females (vide supra) that these effects were maximal on the 25th day following the treatment. In all of these 24 males, infertility commenced between days 17 and 19; mean and S.D. were $17.4 \pm 4.9$. The mean and S.D. of the duration of the infertile period in these males, including the 2 males which remained fertile, was $11.1 \pm 7.87$ days; median 10 days. Infertile matings in controls were few and scattered with no consecutive periods of infertility.

The only manifest effects of treatment of male mice was reduction in their fertilization rates, based on scoring of non-penetrated ova in females with vaginal plugs. This was evident from calculations based on individual ova, individual females with vaginal plugs, or individual males. Fertility began to decline on the 12th day following drug treatment, decreased progressively till the 25th day, and then gradually recovered until it was normal by the 39th day; 22 out of 24 males were infertile between days 21 and 25.

The spermatogenic cycle in the mouse, from spermatogonia to sperms in ejaculate, takes 42 days and is comprised of: spermatogonial mitosis, 6 days; spermatocytes, 14 days; spermatids, 9 days; testicular sperms, 5.5 days; epididymal sperms, 7.5 days. Meiosis occurs at the end of 3rd week[6]. Thus, niridazole primarily affects spermatids and meiotic division of spermatocytes, resulting in reduced fertility. These results are consistent with the findings in the dominant lethal assay in mice, where reduced incidence of pregnancies and the number of total implants were observed in females mated with males which had been treated 3 to 4 weeks previously with niridazole[3]. Infertility induced by niridazole is in marked contrast with the biphasic infertility, both pre-meiotic and post-meiotic, induced by mutagenic alkylating agents, such as TEPA. Infertility induced by TEPA, as evidenced by pre-implantation losses, is due to the effects on both pre- and post-meiotic stages of spermatogenesis. The mechanism involved, however, is different for those two stages. With post-meiotic drug-exposed sperms, fertilization is normal but subsequent development of the zygote is retarded and malformed embryos fail to implant. Contrastingly, with pre-meiotic effects infertility is characterized by failure of fertilization possibly due to aspermia[5,7,8].

Data reported here indicate that sterility induced by niridazole is due to inhibition of fertilization and not due to the induction of dominant lethal mutagenic effects. Reduction in total number of implants in the dominant lethal assay cannot then be necessarily equated with pre-implantation losses and mutagenic effects[3,5].

*Zusammenfassung.* Nach einmaliger i.p.-Injektion von 700 mg/kg Niridazol wurden männliche Mäuse wiederholt mit unbehandelten Weibchen wöchentlich über 2 Monate hinweg gepaart und die Ova gesammelt. Es konnte eine starke Herabsetzung der Befruchtungsraten 3–4 Wochen nach Paarung festgestellt werden, was die Annahme einer Wirkung auf die meiotische Teilung der Spermatozyten und Spermatiden nahelegt. Nach der 7. Woche trat eine Normalisierung der Fruchtbarkeitsrate ein.

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[6] A. J. Bateman, Heredity *12*, 213 (1958).
[7] S. S. Epstein, W. Bass, F. Arnold, Y. Bishop, S. R. Joshi and I. D. Adler, Toxic. app. Pharmac. *19*, 134 (1971).
[8] S. S. Epstein, E. Arnold, K. Steinberg, D. Mackintosh and H. Shafner, Toxic. appl. Pharmac. *17*, 23 (1970).
[9] This work was supported by NIH Grants No. C-6516 and No. FR 05526 and by the Environmental Protection Agency Contract No. CPA-70-17.
[10] Reprint requests to Dr. S. S. Epstein, Director Environmental Health Programs, Case Western Reserve University Medical School, Cleveland, Ohio 44106, USA.

## β-Sympatholytics as Non-Specific Inhibitors of Serum Cholin-Esterase

The therapeutic use of β-sympatholytics is not limited to their specific antiadrenergic effects. Also their non-specific actions have aroused clinical interest. It is therefore desirable to gather more information on the non-specific effects of this group of drugs. It was the aim of this work to investigate the inhibiting effects and the type of inhibition of 11-β-sympatholytics on the serum cholinesterase as a model of an isolated enzyme, and the correlation between the inhibitory potency of the drugs and their hydrophobic properties.

*Materials and methods.* The octanol-buffer partition coefficients ($P$) of the β-sympatholytics were determined

as previously described[1] (*n*-octanol, Merck, Darmstadt; phosphate buffer, 0.16 *M*, pH 7.0). The colorimetric measurements[2] of the kinetics of the serum cholinesterase were carried out with a purified enzyme preparation (Serum-Cholinesterase, Behringwerke, Marburg/Lahn)[3] at pH 7.2, and 25 °C. The β-sympatholytics[3] (racemates) investigated are shown in the Table.

*Results.* The partition coefficients (*P*) of the drugs are shown in the Table. They increased with increasing number of the methyl substituents of the drugs, both in the position of the amino group and of the aromatic ring. The inhibitory constants ($K_i$) of the β-sympatholytics on the serum cholinesterase, calculated from the double reciprocal plots[4,5], are also shown in the Table: there is a gradual decrease of the inhibitory concentrations of the drugs, from Kö 1439 to Kö 1292, except for Kö 1124 which showed the same mean $K_i$ value as Kö 707, in


[1] D. HELLENBRECHT, B. LEMMER, G. WIETHOLD and H. GROBECKER, Naunyn-Schmiedeberg's Arch. Pharmac. *277*, 211 (1973).

[2] G. L. ELLMAN, K. D. COURTNEY, V. ANDRES JR. and R. M. FEATHERSTONE, Biochem. Pharmac. *7*, 88 (1961).

[3] We thank Behringwerke AG, Marburg, for gifts of serum cholinesterase, and C. H. Boehringer Sohn for providing the β-sympatholytics.

[4] H. LINEWEAVER and D. BURK, J. Am. chem. Soc. *56*, 658 (1934).

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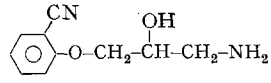
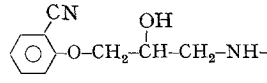
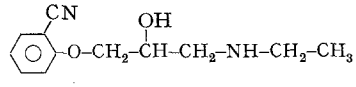
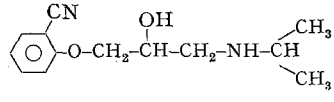
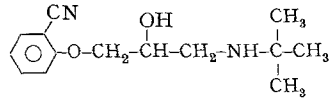
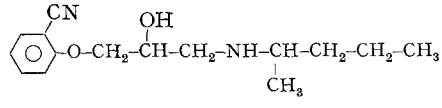
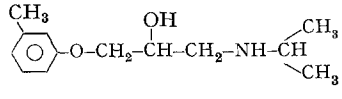
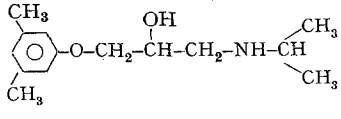
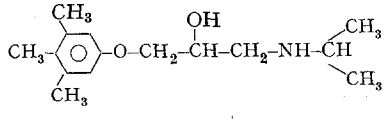
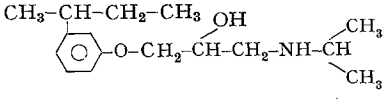
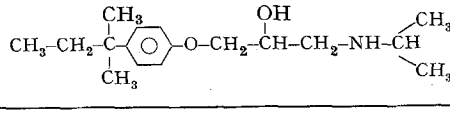
Chemical structure of the β-sympatholytics investigated; octanol-buffer partition coefficients ($\bar{x} \pm$ SEM from *n* determinations), and inhibitor constants ($K_i$)on purified human serum cholinesterase

| Compound | | Partition coefficient *P* | *n* | Inhibitor constants $K_i$ (*M*) |
|---|---|---|---|---|
| Kö 1439 |  | 0.0087 ± 0.0041 | 4 | $2.1\times10^{-3}$, $2.6\times10^{-3}$ |
| Kö 1561 |  | 0.0127 ± 0.0024 | 4 | $4.0\times10^{-4}$. $3.0\times10^{-4}$ |
| Kö 1560 |  | 0.0114 ± 0.0006 | 4 | $5.6\times10^{-4}$, $3.0\times10^{-4}$ |
| Kö 1313 |  | 0.0892 ± 0.0062 | 5 | $1.4\times10^{-4}$, $1.0\times10^{-4}$ |
| Kö 1366 |  | 0.139 ± 0.012 | 4 | $9.5\times10^{-5}$, $7.0\times10^{-5}$ |
| Kö 1500 |  | 1.483 ± 0.116 | 4 | $5.3\times10^{-5}$, $2.5\times10^{-5}$ |
| Kö 592 |  | 0.571 ± 0.038 | 4 | $7.5\times10^{-5}$, $5.5\times10^{-5}$ |
| Kö 707 |  | 1.789 ± 0.104 | 4 | $4.8\times10^{-5}$, $4.4\times10^{-5}$ |
| Kö 1030 |  | 3.671 ± 0.284 | 4 | $6.3\times10^{-5}$, $2.5\times10^{-5}$ |
| Kö 1124 |  | 15.465 ± 1.048 | 4 | $4.5\times10^{-5}$, $4.6\times10^{-5}$ |
| Kö 1292 |  | 43.240 ± 1.316 | 3 | $1.4\times10^{-5}$, $7.5\times10^{-6}$ |

The $K_i$ values refer to 2 inhibitory concentrations.

spite of a 10 times higher partition coefficient. Graphical analysis of the kinetic data showed a mixed type of inhibition for all the compounds investigated.

For statistical analysis of the data, the linear regression line was calculated by the method of least squares, from the logarithmic values of the partition coefficients (log $P$) of the drugs and from their respective inhibitory potency (log $1/K_i$) on the serum cholinesterase. The regression equation was $\log 1/K_i = 0.450 \log P + 4.200$ $n = 11$; $r = 0.922$.

Linearity of the regression function was tested by the F-distribution.

*Discussion.* Our investigations have shown a statistically significant correlation coefficient ($p < 0.001$) between the 2 parameters (log $P$ and log $1/K_i$) investigated. This suggests that hydrophobicity of the $\beta$-sympatholytics plays a significant part in the inhibitory potency of the drugs on the serum cholinesterase. The low slope of the regression line indicates, however, that the mechanism of action must be more complex, as was pointed out by HANSCH and DUNN[6] for a variety of biological systems. The unexpected low inhibitory potency of Kö 1124 can probably be explained by a steric effect of the m-isobutyl substituent of this compound. This effect, together with the experimental difficulties in estimating the partition coefficients of extremely polar compounds such as Kö 1439, may be responsible for a statistically significant deviation of the regression line from linearity ($F = 6.03$; $p < 0.01$). Omitting these 2 compounds (Kö 1124 and Kö 1439) from calculation the following regression line was obtained:

$$\log \frac{1}{K_i} = 0.414 \log P + 4.300;\ n = 9;\ r = 0.984.$$

As the test quotient for this regression function ($F = 0.66$) did not exceed the significance limit ($p > 0.05$), it can be assumed that this regression function is linear.

The relationship between the hydrophobic properties of the $\beta$-sympatholytics investigated and the inhibitory potency on the serum cholinesterase might be interpreted as a fairly non-specific binding of drug to the enzyme protein, because the mixed type of inhibition suggests that not only the free enzyme but also the drug enzyme complex is affected by the drugs. Stereospecifity, which is typical for the specific antiadrenergic activity of this group of drugs seems not to be involved. In our experiments the $K_i$ value of (−)–Kö 1313 was essentially the same as that of (+)–Kö 1313.

*Zusammenfassung.* Die hemmende Wirkung von $\beta$-Sympatholytica auf die Serum-Cholinesterase wird als unspezifische Wirkung angesehen und kann mit ihren hydrophoben Eigenschaften korreliert werden.

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## Changes in Brain Acetylcholinesterase Activity of Young Rats after Chronic Treatment with Tremorine

Oxotremorine or tremorine-induced tremors may offer some information concerning possible mechanisms involved in tremor diseases in general and in Parkinsonism in particular. Acute administration of tremorine[1,2] or its active metabolite, oxotremorine[3,4] causes an increase of brain acetylcholine and a causal relation between increased acetylcholine and the associated produced tremor by these agents has been proposed[2]. The present study was designed to investigate biochemical changes in rats treated chronically with tremorine, beginning at 60 days of age.

*Methods.* Sprague-Dawley male rats were purchased from Simonsen Laboratories, California and delivered to our laboratory at 60 days of age. 4 animals received daily for 7 days, the vehicle 0.9% NaCl i.p.; 4 animals received daily for 7 days either a) tremorine, 7.5 mg/kg body weight, or b) tremorine, 22.5 mg/kg body weight, or c) tremorine 30 mg/kg body weight. Body weights were recorded throughout the experimental period. 4 days following the 7-day injection period all animals were sacrificed by decapitation, the brains were rapidly removed, blotted free of moisture and weighed. The following CNS areas were rapidly dissected and used for biochemical determinations: cerebral cortex (gray matter only), caudate, hypothalamus and cerebellum.

Acetylcholinesterase (AChE) was determined colorimetrically by means of a Beckman DU spectrophotometer, using the rate of hydrolysis of the substrates acetylthiocholine (AcTCh), according to the method of ELLMAN et al.[5]. The determination of the enzyme activity was carried out 37 °C. Homogenate consisted of 1 mg tissue per ml of 0.07 $M$ phosphate buffer, pH 8.0, prepared with 0.07 $Na_2HPO_4$ and 0.07 $KH_2PO_4$. The final reaction mixture for determining AChE activity consisted of 2.9 ml pH 8.0 buffer, 0.1 ml homogenate, 100 µl (0.001 $M$) dithiobisnitrobenzoic acid (DTNB), and 20 µl acetylthiocholine iodide (0.075 $M$). Enzyme activity was expressed as µmoles of substrate hydrolyzed per min per g of wet tissue. To determine whether the means of parameters measured in controls and tremorine-treated rats differed significantly, the $t$-test for nonpaired data was applied[6].

*Results.* The 7-day treatment schedule was chosen in an attempt to produce spontaneously occuring tremors after chronic administration of tremorine. However, because

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[6] R. A. FISCHER, in *Statistical Methods for Research Workers* (Hafner, New York 1950).