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THE EFFECT OF DI*ISO*PROPYL FLUOROPHOSPHONATE ON THE SENSITIVITY OF RATS TO CENTRALLY ACTING DRUGS

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The effect of a previous injection of an anticholinesterase, dyflos, on the sensitivity of rats to two centrally acting drugs, pentobarbitone and leptazol, has been measured. The sensitivity was determined at 12 and 35 days after birth and in full-grown animals. Though the dose of dyflos was of the order of two-thirds of the LD50, it did not affect the sensitivity of the young animals to sodium pentobarbitone and leptazol administered 20 hours later. The full-grown animals which had received dyflos in arachis oil were more sensitive to sodium pentobarbitone than litter mates which had received an injection of arachis oil only; this difference was significant at the 0.1% level. The dyflos treatment did not significantly affect the sensitivity to leptazol.

A previous paper (Elkes, Eayrs, and Todrick, 1955) described an attempt to modify the development of central nervous function in the rat by the injection of an anticholinesterase, dyflos (di*iso*propyl fluorophosphonate, DFP), regularly from the third day after birth. The results were negative. As an alternative approach to the study of the significance of cholinesterase in the central nervous system, the effect of a previous injection of DFP on the sensitivity of growing and full-grown rats to two centrally acting drugs, sodium pentobarbitone and leptazol, has been measured.

The results suggest that DFP affects the response of the full-grown animal to sodium pentobarbitone. They also indicate that the sensitivity of the rat to sodium pentobarbitone and leptazol varies with age.

MATERIALS AND METHODS

The object of the experiment was to compare the ED50 of rats previously treated with DFP with that of untreated animals. The measurement of an ED50 presents no unusual difficulties, but, since the population variance is high, it is desirable to use litter mates of the same sex at the different dose levels. This can be arranged if the number of doses employed is four. However, the comparison of the ED50 for two sets of experimental conditions rendered such a procedure impracticable. The principle adopted was therefore to balance the DFP and control groups at a given dose in respect of litter mates of the same sex, but to pay less regard to the distribution of litters and sexes between the different doses.

Another limitation in the design of the experiment arose from the use of young animals. It had previously been found that the effects of DFP were disabling for some hours (Elkes *et al.*, 1955); in the competitive conditions of a large litter, the treated animals tended to be eliminated by the mother. A modification of the standard split litter technique was therefore adopted.

Design of Experiment

Young Rats.—The experiment involved 42 litters born over a period of 18 days, and a total of 342 rats; they were of the Department of Pharmacology, Birmingham, strain. Each litter was split as close to a 3 : 3 : 4 ratio as possible, sexes being balanced during the operation; four-tenths of each of three litters were joined to form a control group, the others to form two experimental groups; they were then returned to the three mothers at random. Litters born within 48 hr. were split and grouped as they were born, and the mean age of the group was taken as the age of all the rats in the group. The larger numbers in the control groups tended to make the animals lighter than those receiving DFP; but the effect of the drug was expected to counteract this tendency at 12 days. By the thirty-fifth day the animals were feeding themselves and the two groups were expected to weigh approximately the same. In fact, the mean weight of the rats receiving DFP was 20.0 g. at 12 days compared with 18.7 g. for the controls. By 35 days the mean weights were 77.8 g. and 77.7 g. respectively.

The distribution of animals in the experiment by litter and sex may be summarized as follows: 76% of all the rats receiving DFP were paired with litter mates of the same sex which received an injection containing no DFP; a further 12% were paired with

litter mates of the opposite sex. In the test of the sensitivity of rats to leptazol the overall ratio males/females was 40/60; in the series used to test sodium pentobarbitone it was 50/50.

Owing to deaths, the population tested at 35 days was not identical with that tested at 12 days; the majority of the animals, however, were used on both occasions.

Full-grown Animals.—A fresh group of animals was used; owing to the difficulty of obtaining females, this was numerically unbalanced in respect of sex. The females (37 animals) comprised of the mothers of the litters used in the first part of the work; nothing was known of their parentage though some of them were almost certainly litter mates. The males (258 animals) were available in litters which were grouped equally into those receiving DFP and those which did not. The effect on the results of the lack of balance of the sexes was negligible, since the females were distributed equally between the treated and untreated groups.

Experimental Technique

Newborn rats are more sensitive to DFP than full-grown animals (Freedman and Himwich, 1948), but, at the same time, the depression of the brain cholinesterase activity by a single dose of the drug appears to be less (Elkes *et al.*, 1955). Rats aged between four and six weeks, on the other hand, appear to be less sensitive to DFP than full-grown animals (Elkes *et al.*, 1955). Since the aim was to give, at each age, the maximum dose of DFP compatible with survival, the following schedule was adopted.

DFP 2.2 mg./kg. in arachis oil (Boots) was injected subcutaneously on the fourth, seventh, and eleventh days after birth; the determination of the ED50 of the centrally acting drug was made on the twelfth day, 20 hr. after the last DFP injection. No further injections were given until the thirty-fourth day when 3.5 mg./kg. of DFP was injected subcutaneously; the determination of the ED50 followed 20 hr. later.

The fully grown rats at first received the same injection as the 35-day-old animals, but it was observed that the proportion of deaths was high. The dose of DFP was therefore reduced to 3.0 mg./kg. halfway through the experiment.

Animals in control groups received equivalent amounts of arachis oil. Animals were weighed prior to injection; injections were carried out using a micrometer syringe ("Agla" type).

Between the fourth and eleventh days, there were 15 deaths among the 198 animals receiving DFP, compared with 3 deaths among the 143 receiving arachis oil only. There were 12 deaths in the first 20 hr. among 165 full-grown animals injected with DFP. No deaths were recorded among 35-day-old and full-grown rats receiving arachis oil only.

Leptazol (Cardiazol, Knoll) solutions were made up freshly each day in physiological saline from the crystalline solid. On the basis of preliminary tests, dose levels of 40, 55, 70, 90, and 100 mg./kg. were selected. Sodium pentobarbitone (Veterinary Nembutal, Abbott) solutions were made up similarly; suitable doses were found to be 18, 25, 34, 45, 54, and 65 mg./kg.

Estimation of ED50 and Calculation of Results

The criterion of effectiveness of leptazol was the observation of at least one major tonic-clonic convulsion; minimal tremors or jerking movements, and pawing at the nose, which were observed in some animals, were disregarded.

The criterion of effectiveness of sodium pentobarbitone was anaesthesia, as determined by the disappearance of the response of the animal to (a) turning it over on its back, (b) pinching the tail and ear with forceps. It was found that these two tests gave parallel results. The corneal reflex was also observed. This, however, only disappeared in deep anaesthesia, and a fair proportion of the animals which failed to respond failed also to recover consciousness.

The results have been analysed statistically by the probit method as described by Finney (1952). Since "g" was too large to neglect, the more accurate form of the analysis was used. The significance of the differences between the values of the ED50 for the animals receiving DFP and for those receiving arachis oil were calculated by substituting the logarithmically transformed results in the equation

$$t = \frac{d}{\sqrt{s_1^2 + s_2^2}}$$

RESULTS

The effect of DFP on the sensitivity of rats of different ages to leptazol is shown in Table I; the results for the parallel series receiving sodium pentobarbitone are given in Table II.

TABLE I

EFFECT OF PRETREATMENT WITH DFP ON SENSITIVITY OF RATS TO LEPTAZOL

DFP injections were given as described in text.

Note on results with 35-day-old animals. The method of statistical analysis employed gives, besides the maximum likelihood estimate and fiducial limits of the ED50, a figure for the slope of the probit line "b" and its standard error. With a given number of animals, high accuracy in one estimate is attained at the expense of less accuracy in the other. The present experiments have aimed at giving accurate estimates of ED50 rather than of "b"; ten out of the twelve values obtained for "b" have shown standard errors lying between 22% and 27%. However, the standard errors of "b" in the experiments with leptazol on the 35-day-old animals were not lower, as might have been expected from the wider fiducial limits given below, but were 38% for the experimental and 35% for the control group. It must be concluded that these two sets of results possess a lower degree of accuracy than the remaining ten; no cause can be assigned to this observation.

Age (Days)	Animals Receiving DFP in Arachis Oil		Animals Receiving Arachis Oil Alone		Probability that DFP has *not* Affected Sensitivity to Leptazol
	No. of Animals	ED50 (mg./kg.) (Maximum Likelihood and 95% Fiducial Limits)	No. of Animals	ED50 (mg./kg.) (Maximum Likelihood and 95% Fiducial Limits)	
12	56	68·2 (63·0–73·7)	56	68·5 (64·3–73·0)	>0·9
35	40	75·6 (62·4–91·5)	40	66·2 (53·9–81·3)	0·3
Full-grown	53	50·4 (45·5–55·9)	54	45·2 (40·9–50·0)	0·1

TABLE II

EFFECT OF PRETREATMENT WITH DFP ON SENSITIVITY OF RATS TO SODIUM PENTOBARBITONE

DFP injections were given as described in text.

Age (Days)	Animals Receiving DFP in Arachis Oil		Animals Receiving Arachis Oil Alone		Probability that DFP has *not* Affected Sensitivity to Pentobarbitone
	No. of Animals	ED50 (mg./kg.) (Maximum Likelihood and 95% Fiducial Limits)	No. of Animals	ED50 (mg./kg.) (Maximum Likelihood and 95% Fiducial Limits)	
12	50	28·2 (24·5–32·2)	42	26·6 (23·6–29·9)	0·5
35	46	49·9 (46·5–53·6)	47	51·5 (48·3–54·8)	0·5
Full-grown	94	39·3 (35·4–43·5)	94	59·8 (55·0–65·1)	<0·001

The results do not suggest that DFP has in any way affected the sensitivity of young rats to leptazol; the fully grown animals are also not significantly affected, though, since the difference approaches the level of significance ($P=0.1$), no positive conclusion should be drawn regarding the ineffectiveness of the treatment with DFP.

The sensitivity of young rats to sodium pentobarbitone similarly shows no signs of being altered by pretreatment with DFP; the full-grown rats on the other hand are clearly more sensitive to sodium pentobarbitone 20 hr. after the DFP injection, the ED50 falling to 39 mg./kg. from the control value of 60 mg./kg.

This is significant at less than the 0.1% level.

The sensitivity of the rats to the two drugs appears to alter with age. In view of the change in the balance of the sexes between 35 days and maturity, it is necessary, before drawing conclusions, to analyse according to sex the results obtained at 12 and 35 days; leptazol seems to be equally effective against males and females at 12 days, and an indication of greater sensitivity in the male at 35 days may not be real when account is taken of the abnormal variability of this set of figures. With sodium pentobarbitone, the results do not suggest any difference between the sexes at either age.

It therefore seems permissible to compare the ED50 values at 12 and 35 days with those for the fully grown animals. The sensitivity to leptazol increases between the twelfth day after birth and full growth, the ED50 falling from 68 to 45 mg./kg. The sensitivity of the rat to sodium pentobarbitone decreases with age, the ED50 rising from 26 mg./kg. at 12 days to 52 mg./kg. at 35 days, and finally to 60 mg./kg. at full growth, that is the greater part of the change has occurred by the 35th day.

DISCUSSION

Feldberg (1950) has reviewed the evidence that acetylcholine acts as a central synaptic transmitter. While admitting the difficulty of obtaining direct evidence, such as is available for the peripheral nervous system from experiments on the perfused superior cervical ganglion of the cat (Brown, 1937), he concludes that many of the experimental results can only be satisfactorily accounted for on the assumption that acetylcholine is concerned in central synaptic transmission. Acetylcholine applied locally or injected through the vertebral or carotid artery has both stimulating and depressant central effects; anticholinesterases have similar actions, but, by comparison with acetylcholine, depression is even more pronounced and more easily obtained than stimulation (Feldberg, 1950).

The observations of Feldberg (1950) regarding the depressant effects of acetylcholine are supported by those of Richter and Crossland (1949) on the acetylcholine content of the rat brain; these workers found that this was significantly raised during anaesthesia and sleep and significantly lowered during emotional excitement, after electrical excitation, and during convulsions; they concluded that the level appeared to vary inversely with the degree of activity of the brain.

More recently, Sherwood and Feldberg (1954) have shown that the effects of intraventricular injections of eserine and DFP in the cat take place in three stages. The first is characterized by the appearance of itching or irritation, the second by changes in gait, stance and posture, increased tone of limb muscles and tremor, and the third by an alteration of awareness including the development of stupor resembling catatonia.

These results all point to the importance of the depressive component of the action of acetylcholine on the central nervous system. It appears to us that the positive result which we have observed, namely the increase in sensitivity of the mature rat to a central depressant drug caused by a heavy dose of DFP, is not inconsistent with the results quoted above. It is perhaps of interest that the only noticeable symptom observed 20 hr. after the injection of DFP was a marked " retardation " characterized by the animal remaining motionless in a crouching position, but even this was not observed in many of the animals.

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