Pharmacology. — *On the Possibility of Testing Analeptics with the Aid of Fish.* H. A. DIJKERMAN, T. NIJZINK and P. E. VERKADE.

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During the last few years extensive investigations have been carried out at our laboratory as to the reactions of the goldfish, *Carassius auratus L.* (?), to a number of (local) anesthetics; only a small part of this work has as yet been published [1]). Starting from work of ADAMS *c.s.* [2]), we were able to show that the goldfish is eminently suitable as test animal for comparing the activity of at least certain types of (local) anesthetics. In our respective publications we emphasized the fact — which should be stipulated here once more — that in this test we undoubtedly are concerned with an action of the substances in question on the central nervous system.

During these investigations the question soon occurred to us whether with goldfish, with fish in general, a stimulating effect of substances like cardiazole, benzedrine and the like on the central nervous system, thus an antagonism between said substances and the anesthetics, exists and can be readily ascertained; at the time, as far as we are aware, such an antagonism was only known to exist with man and with a few warm-blooded animals. If so, attempts might be made, while availing ourselves of the results of our above-mentioned investigations, to work out a method for comparing the activity of analeptics using the goldfish or possibly some other kind of fish as test animal. A reliable and simple test for analeptics was, and in our opinion still is, needed.

In the literature we soon found KOCH's paper [3]), then of recent date, on the reaction of the stickleback (*Gasterosteus aculeatus L.*) to the combinations of ethyl urethan — cardiazole and ethyl urethan — lobeline. The 2–3 cm long and about one-year-old test animals were anesthesized by a 30 minutes' stay in a 0.6 % solution of ethyl urethan and subsequently transferred to water or to a highly dilute solution of the analeptic to be examined; KOCH then ascertained the time elapsing until the beginnings of awaking, for which fluttering of the lateral fins served as criterion. The concentrations examined were 5, 25 and 50 mg per litre in the case of cardiazole and 2.5, 5, 10 and 25 mg per litre in the case of lobeline. All the experiments were carried out at 18° C and the test

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[1]) C. P. VAN DIJK, thesis Delft, 1946; P. H. WITJENS, thesis Delft, 1946; preliminary communication: P. E. VERKADE, C. P. VAN DIJK and P. H. WITJENS, Verslag Kon. Ned. Akad. v. Wetensch., Amsterdam, **55**, 5 (1946); P. H. WITJENS, C. P. VAN DIJK and P. E. VERKADE, Arch. intern. pharmacodynamie **74**, 178 (1947).

[2]) R. ADAMS, E. K. RIDEAL, W. B. BURNETT, R. L. JENKINS and E. E. DREGER, J. Am. Chem. Soc. **48**, 1758 (1926).

[3]) F. E. KOCH, Z. ges. exptl. Med. **108**, 695 (1941).

animals were previously kept at the same temperature for several days. The number of test animals was 100 in the control test and 50 in each of the tests with one of the analeptics. The numerical data thus obtained were elaborated statistically in the manner indicated by BURN [4]). Leaving aside the experiments with the lowest concentration of each of the two analeptics, the rather slight decreases observed in the average *duration of the anesthesia* if the fishes were introduced into a solution of an analeptic instead of into water are said to have been significant. KOCH thinks himself justified to the conclusion that his method is in principle suitable for ascertaining the "Weckwirkung" of central nervous system stimulants; his paper even contains a comparison of the activity of cardiazole, lobeline and an extract of *Lobelia inflata L.* on the basis of the rather unimpressive data obtained during the work in question.

During our investigations referred to above [1]) we found that the duration of the anesthesia of goldfish treated with a given solution of the anesthetics employed — 1-*n*-propoxy-2-amino-4-nitrobenzene and ethyl 4-aminobenzoate (anesthesine) — may fluctuate very considerably; for the latter anesthetic this had already been stated by ADAMS *c.s.* [2]). In our opinion one of the causes and perhaps even the main cause, of this phenomenon is the fact that the gill respiration, too, is affected by these anesthetics, such in degrees greatly varying from individual to individual; differences in the intensity of the gill respiration are bound to produce differences in the rate at which the anesthetic is removed from the organism.

It is obvious that the immediately preceding remarks give rise to serious objections to the test proposed by KOCH. This investigator also pointed out the pronounced scattering in the duration of the anesthesia of the sticklebacks used by him as test animals. This necessitated the use of large numbers of test animals, which, even though the test is simple, is to be considered a drawback. KOCH's paper does not contain information about the intensity of the gill respiration after a 30 minutes' stay of the fishes in the 0.6 % solution of ethyl urethan. It is, of course, conceivable that in this respect the reaction of the stickleback is quantitatively different from that of the goldfish, and also that the same applies to the effect of ethyl urethan as compared with that of the above-mentioned anesthetics employed by us. At any rate, for the test in question only such combinations of fish and anesthetic are suitable in which the gill respiration is not or only slightly affected by the anesthetic; indeed, differences in the intensity of the gill respiration are bound to produce differences in the rate of resorption of the analeptic by the fish.

On the other hand, it appears from the experience gained at our laboratory [1]) and by ADAMS *c.s.* [2]) that the *anesthesizing time, i.e.* the time elapsing between the moment the goldfish is introduced into a solution

[4]) J. H. BURN, Biologische Bewertungsmethoden (Berlin, Jul. Springer, 1937), p. 22.

of an anesthetic and the moment it ceases to react to the strongest permissible pressure on the caudal fin and the dorsal fin, can be reproduced quite well within reasonable limits. In our opinion it was, therefore, very attractive to investigate whether the anesthesizing time can be affected by the type of analeptics in question. This can be done in two ways:

1°. By comparing the anesthesizing time pertaining to a chosen solution of an anesthetic and determined in the usual manner, with those anesthesizing times occurring in solutions which contain, besides the anesthetic in the same concentration, the analeptic to be examined.

2°. By comparing the former anesthesizing time with those anesthesizing times occurring in the case of fishes which have previously been treated during a given time with solutions of the analeptic to be examined.

We started by investigating whether any useful results could be obtained with the aid of the technique mentioned *sub* 1, this being of course experimentally the simplest one. Meanwhile, from the very beginning we were by no means blind to the fact that this technique presents the indisputable and serious drawback of the simultaneous use of two drugs. As test animals we used goldfishes and as anesthetics 1-*n*-propoxy-2-amino-4-nitrobenzene or ethyl 4-aminobenzoate (anesthesine), because a good deal of experience had been gained with these combinations of fish

TABLE 1. Anesthesizing times in minutes.

No.	1-*n*.propoxy-2-amino-4-nitrobenzene 7 mg/l									
	no analeptic	cardiazole mg/l					benzedrine-HCl mg/l			
		20	100	500	1000	0	5	20	100	200
1	10.5	13	12	12	10	14	9.5	12	9	12.5
2	9	14.5	15.5	14	11	13	8.5	11.5	8.5	13.5
3	9.5	13	13.5	15	12	13.5	10	8.5	8	9
4	11	16.5	13.5	10	9.5	20.5	12	8	10	10
5	10	14	12	13.5	10.5	11.5	9	9	8	7.5
6	13.5	12.5	9	10.5	12.5	14.5	13.5	11	7.5	10
7	9.5	12	13	11	8	14.5	11.5	15.5	9	11
8	9	12.5	12	16	7.5	24	14.5	11	8.5	11
9	11.5	10	16	13	8	13	10.5	9	9	11
10	18.5	16.5	11	15	12.5	18	11	9	9	10
11	15	11.5	13	11.5	8	11.5	11	10	8	8
12	26.5	10	14	10	10	10	8	8.5	10.5	12
13	13	10	12	11.5	10	15	14	8	9	9.5
14	12.5	10.5	14.5	12.5	9.5	11	10.5	14	10	10
15	11	13	12	13.5	10.5	13.5	9.5	9.5	9	8
16	15	10.5	11.5	9	7	10.5	8	8.5	7.5	8
17	19	11	14	10	11	10	9	9.5	9	7.5
18	14	10	14	10	11.5	9.5	8	7.5	10.5	8
19	10.5	14	13	13	14	12	13.5	8.5	8.5	8.5
20	13.5	10	17.5	10	9.5	12	7.5	10.5	7	6.5
Aver.:	13.1	12.3	13.2	12.1	10.1	13.6	10.2	10.0	8.8	9.6

and anesthetic in the course of the work already carried out at our laboratory and in particular also because the substances mentioned are already active in very low concentrations; we consider this to be an advantage especially over the ethyl urethan used by KOCH and afterwards also by other workers. The analeptics used by us were cardiazole and benzedrine hydrochloride. The tests were carried out at 20.0° C. The technique for the determination of the anesthesizing time was exactly that described elsewhere [1]; for the determination of the average anesthesizing time holding for a given solution use was invariably made of twenty fishes, which proved to be quite sufficient.

TABLE 2. Anesthesizing times in minutes.

No.	anesthesine 50 mg/l			
	no analeptic	cardiazole mg/l		
		20	100	500
1	10.5	13	17	6
2	8	13	12	8.5
3	11.5	11	8.5	10
4	7.5	12	13	9.5
5	13	14.5	14.5	8
6	15.5	12	19	6.5
7	12.5	13	12	8
8	8	10.5	15	8
9	8	10.5	13	11
10	11	17	15.5	12.5
11	9.5	8	20.5	17
12	11.5	10	19	14
13	10	9.5	16	15
14	12.5	25.5	17	10.5
15	17.5	10	17.5	10
16	12.5	11	16	6.5
17	9	11.5	12	8
18	14.5	12.5	10	10.5
19	10	12.5	11	12.5
20	11.5	10	13.5	13.5
Aver.:	11.2	12.4	14.6	10.3

Some results of our experiments are given in the tables 1 and 2, which as a whole speak for themselves. Only column 7 of Table 1 requires some explanation. The fishes which had been used for the experiments with a solution containing, besides the anesthetic, 1000 mg of cardiazole per litre were used again 17 days later for experiments with a solution of the anesthetic alone; the anesthesizing times then found are mentioned in the respective column. The average anesthesizing time (13.6 minutes) agreed completely with that originally found (13.1 minutes; column 2 of Table 1). We thus arrive at the conclusion that even the relatively very high concentration of cardiazole of 1000 mg per litre does not produce any lasting

effect on the goldfish. If desired or necessary the goldfish may, therefore, be used repeatedly for experiments with cardiazole as here described, provided, of course, that sufficient rest periods are observed. According to our experience the same applies with respect to benzedrine hydrochloride.

It should be remarked here that the addition of cardiazole or benzedrine hydrochloride to the solutions of the anesthetics employed did not modify the pH to any appreciable extent. Moreover, as we soon hope to show in a separate paper, the results of experiments on goldfish with the above-mentioned anesthetics are surprisingly little affected by the pH of the solution.

The data collected by us, only part of which has been given above, lead to the conclusion *that in none of the cases studied was the analeptic found to have any effect on the anesthesizing time*; none of the differences found between the average anesthesizing times in the absence and presence respectively of an analeptic was significant. Further work in this direction, *e.g.* with other kinds of fish or other anesthetics, appeared to us to be useless: we are convinced that the technique in question cannot lead to a comparison of the activities of analeptics.

In uttering this conviction we bear of course in mind the drawback — already referred to — of the simultaneous use of two drugs. We came across difficulties of this nature during some experiments with the combination of cocaine hydrochloride and cardiazole. Whereas these substances separately, in a concentration of 1000 and 500 mg per litre respectively, are tolerated quite well by the goldfish — as far as the former substance is concerned, naturally apart from the anesthesia caused by it, which was complete in about 15 minutes [5]) — the combination of the two substances, while maintaining the said concentrations, was found to have a fatal effect within a very short time.

The technique mentioned above *sub* 2, *i.e.* the subsequent treatment of fishes with a solution of an analeptic and of an anesthetic, has meanwhile been applied by URBAIN and BEAUVALLET [6]). In view of the experiments so far carried out by us in the manner in question, we are strongly inclined to doubt the accuracy of the results obtained by the said investigators. We wish to collect more numerical data and then intend to devote a separate paper to the technique in question.

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[5]) Cf. J. RÉGNIER, R. DAVID and R. SITRI, Compt. Rend. Soc. Biol. **129**, 476 (1938). These investigators used for anesthesizing experiments on the stickleback a solution of only 100 mg of cocaine hydrochloride per litre. In the case of the goldfish this anesthetic must be used in much higher concentrations, *e.g.* 1000 mg per litre.

[6]) G. URBAIN and M. BEAUVALLET, Compt. Rend. Soc. Biol. **139**, 576 (1945); **140**, 44 (1946); M. BEAUVALLET and G. URBAIN, ibid. **140**, 41 (1946).