A COMPARISON OF THE BEHAVIORAL EFFECTS OF VARIOUS LEVELS OF CHRONIC DISULFOTON POISONING

David W. Pearson, Ph.D.
George Clark, Ph.D.
Carl M. Moore

Approved by

J. Robert Dille, M.D.
Chief, Civil Aeromedical Institute

Released by

P. V. Siegel, M.D.
Federal Air Surgeon

October 1969

Department of Transportation
FEDERAL AVIATION ADMINISTRATION
Office of Aviation Medicine

A COMPARISON OF THE BEHAVIORAL EFFECTS OF VARIOUS LEVELS OF CHRONIC DISULFOTON POISONING

I. Introduction.

In jobs related to aerial application of toxic pesticides, there exists the danger of accidental exposure. Such exposure of general aviation pilots has been reported as a possible source of impairment of flying performance; in some instances exposure of pilots to pesticides may have contributed to fatal crashes.[27] Of particular concern are the organophosphates that are known to be brain AChE (acetylcholinesterase) inhibitors. It is generally believed that, if ACh (acetylcholine) is one of the most important transmitter substances in the central nervous system, it would seem almost assured that severe alterations in cholinesterase (which is responsible for the rapid destruction of ACh)[16] would markedly affect the function of ACh as a transmitter agent. Thus, it might be thought that significant changes in AChE levels would affect such processes as memory, learning, and/or similar mental performances.

Although there are several reviews and reports on anticholinesterases and their physiological and pharmacological significance,[25 30] few formal studies have been concerned with the behavioral consequences of cholinesterase inhibition. Of the animal studies on the effects of AChE inhibition, a large number deal with the *acute* stages of AChE reductions. Many of these report only the immediate toxic reactions of the organism such as fasciculation, tremor, diarrhea, etc., rather than the interjective effects upon an ongoing conditioned response[10] after administration of an AChE inhibitor.[11 12] Further, in all of the studies of *acute* poisoning, injection or intubation techniques were used. The *S*s (subjects) were given rather large doses of an AChE inhibitor in a relatively brief period of time; and with the exception of a study by Russell, Watson, and Frankenhaeuser,[26] all of the *chronic* studies (AChE reductions maintained over a period of time) used similar techniques.[2 9 23 24]

Most of the acute and chronic AChE inhibition studies noted above generally reported no differences in learning abilities between *S*s with reduced AChE activity and control *S*s, but they did find differences in rate of extinction of a conditioned response, i.e., *S*s with severely reduced AChE activity seem to take a longer period of time to extinguish a response than do control *S*s. Although injection techniques are commonly used in both chronic and acute studies, equivocal and, in many cases, contradictory results are reported. For example, Glow and Richardson[9] investigated the behavioral effects of AChE reductions on both selective and nonselective sites. They reported no differences in rate of extinction of poisoned versus control *S*s in selective-site investigation, i.e., reductions restricted to the brain, which was in contrast to the effects "of an acute, 'nonselective' reduction in AChE activity, i.e., reduction throughout the body" (p. 430). Natoff[17] states that one reason for these dissimilar results may be due to the fact that the administration of drugs to animals via different methods or routes can produce different results, even to the same strain of animals. Despite a growing consensus concerning the importance of using a noninjective technique, i.e., one more analogous to the natural feeding habits of animals, many investigators continue to ignore this problem.

As indicated earlier, only one study reported the effects of both chronic and acute AChE reduction upon performance. Glow, et al.[10] suggested that any differences noted between *S*s with reduced AChE activity and control *S*s may have been due entirely to "the malaise brought on by the treatment," i.e., the administration of "booster" injections to maintain a chronic AChE reduction level may have produced adverse physiological effects. Thus, the present study was designed to explore the extent to which chronic AChE inhibition at various levels of reduction might affect the performance of an

animal in a maze under apparently less traumatic conditions.

II. Method.

Subjects. Forty male Charles River rats (albino) approximately 30 days old were randomly assigned in equal numbers to a control condition or to one of three level-of-poison conditions immediately upon arrival at the laboratory. Each group was housed separately and fed a powdered form of a normal diet food. The experimental groups received either a 10-p.p.m., 25-p.p.m., or 50-p.p.m. addition of the AChE inhibitor Disulfoton (O, O-diethyl S–2–(ethylthio) ethyl phosphorodithioate), a semi-reversible organophosphorus AChE inhibitor, mixed in with their normal diet of ground-up pellet food. Both food and water for the next 3 months were available *ad libitum*. Since our animals received the poison, Disulfoton, in their food, the trauma of repeated intramuscular or intraperitoneal injections was obviated. Also, our experimental rats gained weight on the diet (until, by design, a weight loss was introduced to ensure a hunger drive) which indicates that long before training began the rats were well into the tolerant phase.[5] There were thus two distinct advantages to our procedure. First, since neither injections nor intubations were made, the rats could not associate removal from their home cages with pain which made them, perhaps, more amenable to handling. Secondly, the experiment was more comparable to the situation in man for we were not interested in acute effects, but rather with the long-term chronic effects such as might occur in agricultural pilots.

Other, similarly poisoned animals were used to determine the amount of weight-loss that could be used to assure motivation (food searching) without damaging the rats.

Apparatus. A closed-field intelligence test maze for rats[14] was used (see Figure 1). The

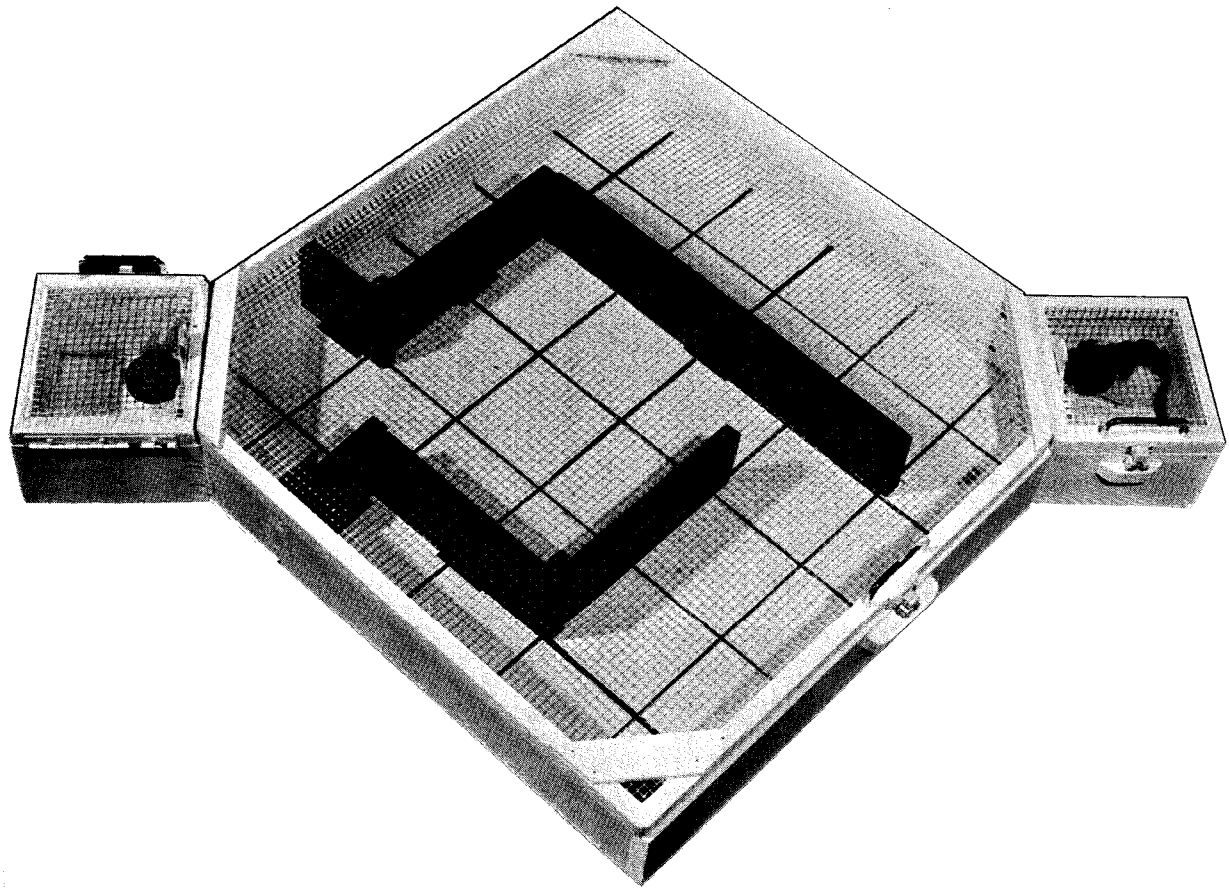

FIGURE 1. The closed-field maze arranged for presentation of Rabinovitch and Rosvold's "Problem 5."

maze had a start and goal box at opposite ends of a square configuration wherein removable barriers of varying lengths could be arranged in such a manner as to provide twelve different maze tasks, of which Problems 1, 3, 5, and 11 were used.[21] A wire-mesh screen top provided *E* (experimenter) with an unobstructed view of the entire maze from a wooden cubicle (42″ x 72″ x 42″) with a one-way vision glass insert. This arrangement also prevented possible *E* distraction for the *S*s. A chair, built-in counter in the cubicle, stop watch, and scales for weighing the animals completed the test equipment.

Procedure. Since there were too many animals to run in a single session, each group of 10 was subdivided randomly into three smaller groups of four, three, and three rats. The first animals tested consisted of four controls, four of the 10-p.p.m. group, four of the 25-p.p.m. group, and four of the 50-p.p.m. group. These were run in random order. In all cases the reward was the food to which they were accustomed. Thus, the control group received unpoisoned food, the 10-p.p.m. group received food containing 10-p.p.m. of poison, etc. Testing and training of the subgroup of four began after 13 weeks of exposure, and of the subgroups of three each after 14 or 15 weeks.

Prior to each familiarization and test session, all animals were weighed to ensure that relatively equal body-weight losses were maintained. This procedure was used to minimize possible *S* differences in drive as a function of body weight. All *S*s received five familiarization and five test trials on each of the four separate maze problems in a sequential manner (i.e., Rabinovitch and Rosvold's[21] problems 1, 3, 5, and 11, in that order) with familiarization and test trials given on alternate days, i.e., all *S*s in each of the four groups were given familiarization trials on problem No. 1 one day, and five test trials on the same problem the following day, at approximately the same time of day the familiarization trials were administered. This procedure was followed until four different maze problems had been presented. Alternating days for familiarization and test trials were employed as a means of reducing performance fatigue and/or food satiation effects. Prior checks indicated that most of the initial learning effects occurred during the first five familiarization trials. It should be noted that since prior experience may have cortical or subcortical ACh activity effects,[3] all animals received identical treatment during all phases of the experiment.

Scores for a trial consisted of the number of errors made and the length of time taken by a *S* in traveling from the start box to the goal box. Errors were counted as the number of wrong turns including retracing maneuvers. Upon entering the goal box the *S* was permitted to feed for 30 seconds as reward, after which he was replaced in the start box. All *S*s were maintained at 15 to 20 percent bodyweight loss during all phases of the experiment.

Performance measures consisted of the mean number of errors made and mean trial times for a five-trial session for each of the four problems presented. Immediately prior to sacrifice, all *S*s were weighed for a final time. Following sacrifice, whole-brain assays were made.*

III. Results.

AChE levels for the 10-p.p.m., 25-p.p.m., and 50-p.p.m. treated groups showed statistically significant reductions of 59 percent, 67 percent, and 74 percent, respectively, with the mean AChE level of controls representing zero reduction. There was, however, no statistically significant difference in the amount of reduction among the poisoned groups. All AChE levels were near or beyond the 60 percent critical reduction level considered necessary to severely affect ACh content.[1] Means and standard deviations of the number of errors and trial times for each group in each of the four test sessions are shown in Table 1.

An analysis of variance test of errors[29] indicated significant between-group differences as a function of poison; however, no differences were found to be present as a function of the four maze problems (probably attributable to the five practice trials each animal had in each maze prior to the second performance trials, and to the order of maze presentation, i.e., all animals began with the least difficult maze), nor were interaction effects present. (See Table 2.) Orthogonal comparisons revealed significant differences between the controls and the 10-p.p.m. *S*s ($F=102.95$, $p<.001$), the 25-p.p.m. *S*s ($F=$

* We wish to thank Mr. Leonard C. Ryan of CAMI's Pharmacology-Biochemistry Laboratory for performing these assays.

TABLE 1.—Number of errors and trial times in each test session for the control and Disulfoton-exposed groups.

Test session	Group	Mean number of errors	SD	Mean trial time (min.)	SD
I	Control	2.6	1.07	2.33	2.16
	10–p.p.m.	1.8	1.31	0.54	0.42
	25–p.p.m.	1.8	1.68	0.35	0.33
	50–p.p.m.	1.7	1.25	0.45	0.37
II	Control	3.1	1.28	1.59	1.13
	10–p.p.m.	2.2	1.03	0.24	0.14
	25–p.p.m.	1.6	0.84	0.36	0.31
	50–p.p.m.	1.6	0.84	0.20	0.12
III	Control	3.0	1.49	1.37	1.09
	10–p.p.m.	1.1	0.73	0.21	0.13
	25–p.p.m.	1.4	1.17	0.20	0.14
	50–p.p.m.	0.9	1.10	0.16	0.14
IV	Control	3.8	2.14	1.23	1.16
	10–p.p.m.	1.5	1.26	0.51	0.47
	25–p.p.m.	1.4	0.69	0.26	0.30
	50–p.p.m.	1.4	0.84	0.46	1.16

117.42, $p<.001$), and the 50-p.p.m. *S*s ($F=140.85$, $p<.001$). Additional orthogonal comparisons revealed no significant performance differences between the groups with reduced AChE. An analysis of variance test of the "trial times" data indicated significant group differences as a function of poison. Orthogonal comparisons revealed that the significant differences in mean trial times were between control animals and each of the 10-p.p.m. ($F=107.19$, $p<.001$), 25-p.p.m. ($F=126.35$, $p<.001$), and 50-p.p.m. ($F=120.44$, $p<.001$) groups. (See Table 3.) Similar orthogonal findings of error scores for the between-exposed-groups were obtained for the trial times. Again, no significant differences were found as a function of the four maze problems.

TABLE 2.—Summary analysis of variance of the effects of various levels of chronic Disulfoton exposure on number of incorrect responses.

Source	df	MS	F
Groups (G)	3	26	15.38*
Sessions (S)	3	2.33	1.59
G x S	9	1.22	.83
Error (G)	36	1.69	
Error (S plus G x S)	108	1.46	

*p <.001

TABLE 3.—Summary analysis of variance of effects of various levels of chronic Disulfoton exposure on trial times.

Source	df	MS	F
Groups (G)	3	9.62	*14.80
Sessions (S)	3	1.17	.99
G x S	9	.58	.49
Error (G)	36	.65	
Error (S plus G x S)	108	1.18	

*p <.001

IV. Discussion.

Organophosphates are inhibitors of cholinesterase, which is considered necessary in preventing excessive acetylcholine accumulations. It is generally thought that excessive accumulation of ACh leads to hyperactive and/or irregular synaptic transmissions at all cholinergic sites so affected. Thus, it is possible that the central processes of those routinely exposed to AChE may be detrimentally affected, resulting in measurable performance impairment. The results of this study indicate that rats fed relatively high amounts of Disulfoton were capable of performing a task with fewer errors and shorter trial times than their controls, although the AChE reduction in the most severely exposed group was more than 75 percent of normal. These re-

sults obtain, however, only in the event that physiological effects do not inhibit the behavior under investigation, i.e., if running time in a maze is the dependent variable, then the animal must be able to run. For example, inhibition effects occurred twice during familiarization tests (i.e., one 50-p.p.m. *S* had an epileptic type seizure and another 50-p.p.m. *S* had body tremors to the degree that walking became impossible). These latter effects also suggest that lethal dose levels were approached for the 50-p.p.m. group. It should be stressed that, except in these latter two instances, the 50-p.p.m. group (with brain AChE reduced to less than 75 percent of normal) was able to perform as well as the 10-p.p.m. group (with brain AChE reduced to 59 percent of normal) and significantly better than the controls. Differences in errors or trial times between the Disulfoton-exposed groups were not found and may have been due to the fact that the AChE reductions for the 10-p.p.m., 25-p.p.m., and 50-p.p.m. animals were near or below the 60 percent reduction level considered by Aprison[1] as necessary to critically affect ACh content.

In some respects the results reported here are different from those obtained by other investigators. Thus, Merrill[19] found no time-to-solution differences between albino mice that had been poisoned at various dose levels and controls (the poison presumably resulting in various AChE reductions) in a place learning task. Glow, et al.,[10] using female Wistar rats, reported significant differences in time to obtain 20 reinforcements between AChE-reduced groups and their controls, i.e., the AChE-reduced animals performed slower than the control animals. Glow, et al.[10] report, however, that their results were obtained, in part, as a result of the immediate effects of booster injections although "these slowings in the press rate became attenuated with each successive injection" (p. 156). Additionally, Merrill[19] reported that both the *highest* and *lowest* AChE-reduced groups made significantly more errors than the middle-range of AChE-reduced groups or the control animals. This is, in part, in contrast with the findings of this study which showed significantly fewer errors by the AChE-reduced groups than the controls.

Since other evidence indicates that ACh has manifold functions in addition to that of synaptic transmitter,[22] and that AChE can exist at both functional and reserve levels,[18] much more information is needed, in addition to behavioral data, to suggest any theory (e.g., an increased general activity drive) which might explain the manner in which the lowered AChE levels in these rats resulted in fewer errors and more rapid traversion of a maze task, or to extrapolate any of the findings reported above to human subjects. The fact that the performance of Disulfoton-treated animals was facilitated in this study may be contrasted with the physiological changes (e.g., seizure and incapacitation) induced by the chemical treatment in two animals from the most severely exposed group.

Hebb[13] in his discussion of "Emotion and Motivation; the Social Context" states that, in general, stimuli at lower levels of intensity promote waking up and, at progressively higher levels, increasing alertness and, finally, disorganization. Similarly, intoxication with ethanol can seriously impair performance, while lower levels may cause no decrement,[15] or even an improvement in behavior.[4] Coffee and other external or mental stimulants all may increase the level of excitation and of activity and, up to a certain level, may produce an apparent improvement in behavior, while higher levels may promote disorganized behavior and decreased performance. Our rats, which were exposed to critically high amounts of Disulfoton, showed not only no performance decrement, but a statistically significant increase in performance so long as the animals were not incapacitated by tremors or convulsions. The extent to which these results may be applied to man is not known although there are some indications in the literature that, in man, low blood AChE levels are not necessarily associated with decreased performance. This can be seen in the reports of Sumerford, Hayes, Johnston, Walker, and Spillane,[28] of Gershon and Shaw,[8] of Dille and Smith,[6] and of Smith, Stavinoha, and Ryan.[27] However, the possibility of either antagonism or synergism with a combination of insecticides is very real and this interaction should be investigated.

NOTE.—While the albino Charles River rats used in this experiment have very limited or no vision and thus the stimuli used by the animals in traversing the maze were not visual, we have replicated the experiment (one problem only) with Long Evans rats. These hooded rats have excellent vision. The results with the Long Evans rats were identical to those with the Charles River Strain.

Moreover, with chronic exposure to organophosphates and chronic lowering of AChE, the margin of safety is markedly lessened and a slight increase in exposure level may well bring overt symptoms which might mean disaster for an agricultural pilot.

REFERENCES

1. Aprison, M. H.: The relationship between cholinesterase activity and acetylcholine content in the cortex and caudate nucleus of rabbit brain. *Recent Advances in Biological Psychiatry*, 4:133, 1962.
2. Banks, A. and Russell, R. W.: Effects of chronic reductions in acetylcholinesterase activity on serial problem-solving behavior. *Journal of Comparative and Physiological Psychology*, 64:262–267, 1967.
3. Bennett, E. L., Diamond, M. C., Krech, D., and Rosenzweig, M. R.: Chemical and anatomical plasticity of brain. *Science*, 146:610–619, 1964.
4. Birch, H. G.: Personal communication, 1945.
5. Brodeur, T. and Dubois, K. P.: Studies on the mechanism of acquired tolerance by rats to 0,0-Diethyl S-2-(Ethylthio) ethyl phosphorodithioate (Di-Syston). *Archives International Pharmacodynamics*, 149:560–570, 1964.
6. Dille, J. R. and Smith, P. W.: Central nervous system effects of chronic exposure to organophosphate insecticides. *Aerospace Medicine*, 35:475–478, 1964.
7. Frawley, J. P., Fuyat, H. N., Hagan, E. C., Blake, J. R. and Fitzhugh, O. G.: Marked potentiation in mammaliam toxicity from simultaneous administration of two anticholinesterase compounds. *Journal of Pharmacology and Experimental Therapeutics*, 121:96–106, 1957.
8. Gershon, S. and Shaw, F. H.: Psychiatric sequelae of chronic exposure to organophosphorus insecticides. *Lancet*, 1:1371–1374, 1961.
9. Glow, P. H. and Richardson, A. J.: Chronic reduction of cholinesterase and the extinction of an operant response. *Psychopharmacologia*, 11:430–434, 1967.
10. Glow, P. H., Richardson, A., and Rose, S.: Effect of reduced cholinesterase activity on the maintenance of an operant response. *Journal of Comparative and Physiological Psychology*, 63:155–157, 1967.
11. Glow, P. A. and Rose, S.: Cholinesterase levels and operant extinction. *Journal of Comparative and Physiological Psychology*, 61:165–172, 1966.
12. Glow, P. H. and Rose, S.: Influence of secondary reinforcers on extinction in rats with reduced levels of cholinesterase activity. *Journal of Comparative and Physiological Psychology*, 61:292–294, 1966.
13. Hebb, D. O.: A textbook of psychology. Philadelphia: Saunders, 1966.
14. Hebb, E. O. and Williams, K.: A method of rating animal intelligence. *Journal of Genetic Psychology*, 34:59–65, 1946.
15. Higgins, E. A., Davis, Jr., A. W., Vaughan, J. A., Funkhouser, G. E., and Galerston, E. M.: The effects of alcohol at three simulated aircraft cabin conditions. *FAA Office of Aviation Medicine Report* No. AM 68-18, 1968.
16. Nachmansohn, D.: *Chemical and molecular basis of nerve activity.* New York: Academic Press, 1959.
17. Natoff, I. L.: Influence of the route of administration on the toxicity of some cholinesterase inhibitors. *Journal of Pharmacy and Pharmacology*, 19:612–616, 1967.
18. McIsaac, R. J. and Koelle, G. B.: Comparison of the effects of external, internal and total acetylcholinesterase upon ganglionic transmission. *Journal of Pharmacology and Experimental Therapeutics*, 126:9–20, 1959.
19. Merrill, K. H.: The effects of a cholinesterase inhibitor on place learning in mice. *Psychonomic Science*, 14:110–111, 1969.
20. O'Brien, R. D.: *Insecticides action and metabolism.* New York: Academic Press, 1967.
21. Rabinovitch, M. S. and Rosvold, H. E.: A closed-field intelligence test for rats. *Canadian Journal of Psychology*, 5:122–128, 1951.
22. Reeves, C.: Cholinergic synaptic transmission and its relationship to behavior. *Psychological Bulletin*, 65:321–335, 1966.
23. Richardson, A. J. and Glow, P. H.: Discrimination behavior in rats with reduced cholinesterase activity. *Journal of Comparative and Physiological Psychology*, 63:240–246, 1967.
24. Richardson, A. J. and Glow, P. H.: Post-criterion discrimination behavior in rats with reduced cholinesterase activity. *Psychopharmacologia*, 11:435–438, 1967.
25. Russell, R. W.: Neurophysiological and biochemical correlates of effects of drugs on behavior: the acetylcholine system. In H. Stienberg (Ed.), *Animal behavior and drug action.* Boston: Little, Brown, 144–162, 1964.
26. Russell, R. W., Watson, R. H. J., and Frankenhaeuser, M.: Effects of chronic reductions in brain cholinesterase activity on acquisition and extinction of a conditioned avoidance response. *Scandinavian Journal of Psychology*, 2:21–29, 1961.
27. Smith, P. W., Stavinoha, W. B., and Ryan, L. C.: Cholinesterase inhibition in relation to fitness to fly. *Aerospace Medicine*, 39:754–758, 1968.
28. Sumerford, W. T., Hayes, Jr., W. J., Johnston, T. M., Walker, K., and Spillane, T.: Cholinesterase response and symptomatology from exposure to organic phosphorus insecticides. *A. M. A. Archives of Industrial Hygiene*, 7:383–398, 1953.
29. Weiner, B. J.: Statistical principles in experimental design. New York: McGraw-Hill, 1962.
30. Zeller, E. A. and Foutz, J. A.: Enzymes as primary targets of drugs. *Annual Review of Pharmacology*, 3:9–32, 1963.