Chemical and Biochemical Analyses on Brain Tissue Preparations during the Epileptiform-Like Activity of Dieldrin and Other Cerebral Convulsants

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This investigation was undertaken to determine the etiological factors associated with cerebral convulsions. Because many conditions are capable of producing the same end result in animals—cerebral convulsions—it was believed that these could have some common feature. Convulsing animals had a mixture of betaine CoA esters released in their brains through damage of brain mitochondria. Similar results could not be obtained with normals. It was concluded that because these betaine CoA esters possess acetylcholine-like properties, their release from brain mitochondria may be an important step in elucidating the etiology of epileptiform-like convulsions.

CHLORINATED HYDROCARBONS as a group can produce in mammals, as a consequence of administration or ingestion, most or all of the following symptoms: nervousness and excitability, excessive blinking, cold skin (or fur), loss of appetite, muscular weakness, fine tremors due to muscular fibrillation, paralysis, and finally convulsions which could terminate in death (*26, 27*).

The most commonly used chlorinated hydrocarbons for insecticidal purposes are: dichlorodiphenyltrichloroethane (DDT), aldrin, dieldrin, chlordan, benzene hexachloride, lindane, and methoxychlor. All these substances are extremely insoluble in water but soluble in fat and organic solvents, and on incorporation into the animal body, they are usually deposited and stored in the fat (*10, 17*). Consequently, their metabolism and excretion could be either a slow or a rapid process, dependent in some manner on the rate of metabolism of the body fat stores (*17*). It is perhaps because of these properties that, depending on the amount of the agent incorporated within the fat stores of the body, toxic symptoms in mammals may immediately be apparent or be delayed for several days or months.

In this paper, chemical and biochemical studies on the epileptiform-like activity of dieldrin and other chemically unrelated cerebral convulsants are discussed. The LD_{50} of dieldrin in rats is 50 to 75 mg. per kg. (*18*), which probably makes it less toxic than aldrin, which has an LD_{50} of 40 to 50 mg. per kg. (*6*). Depending on the dosage administered, the symptoms of dieldrin poisoning may be delayed for several days. In vitro studies by McArdle, Hosein, and Denstedt (*21*) showed that the addition of 25 mg. of dieldrin (as a solution in olive oil) to rat brain cortex slices did not inhibit oxygen consumption by this tissue in the presence of various substrates. If, however, the brain slices were preincubated with the dieldrin for 30 minutes at 37.5° C., subsequent measurement indicated inhibition of the oxygen consumed by the tissue when it was incubated in the presence of various substrates. This inhibition was found to be due to the action of dieldrin on various dehydrogenases and on cytochrome b. Other studies by these authors showed that dieldrin could inhibit anaerobic glycolysis and the hydrolysis of acetylcholine (ACh) by brain acetylcholinesterase. Dieldrin also was found to increase the activity of the nonspecific serum esterases, confirming the earlier findings of Crevier, Ball, and Kay (*9*).

Effects of Dieldrin on the Rat

Other studies in our laboratory (*13*) have shown that the intraperitoneal injection of 200 mg. of dieldrin to normal adult rats produced violent convulsions which were fatal. These large quantities of dieldrin were necessary in order to produce convulsions within a reasonably short time.

About 25 minutes after intraperitoneal injection of the drug, the animal goes into convulsions which may persist from about 30 seconds to 1 minute. About 20 minutes after this convulsion, a second one of similar duration usually takes place. The third convulsion may appear about 15 minutes after the second, the duration being slightly more than 1 minute. The fourth convulsion occurs about 10 minutes after the third, and invariably the fifth is fatal. This latter convulsion occurs about 5 minutes before death and persists throughout this time interval. At the time of each convulsion, the animal salivates profusely. Gowdey *et al.* (*11*) were able to produce similar effects in cats (convulsions and salivation) and reported that application of dieldrin to the chorda tympani did not elicit salivation. From these results it appeared to us that the profuse salivation produced on dieldrin administration was due not to dieldrin itself, but probably to some substance released in the brain of the animal as a result of treatment with this agent.

Precipitation of Reineckate

One of the main purposes of our investigation was to determine whether some substance was released in brain as a consequence of the dieldrin administration which could be responsible for the salivation and the convulsions.

In experiments performed in our laboratory (*13, 15*), 10 groups of 20 hooded rats of either sex and weighing about 200 grams were injected intraperitoneally with 1 gram of dieldrin per kg., in order to produce convulsions in a relatively short time. During the last 5 minutes of life when the convulsions were continuous, the animals were decapitated and the excised brains were immediately homogenized in ice-cold water containing $10^{-5}M$ eserine. After centrifugation at 2000 r.p.m. (1500 g) for 15 minutes, the supernatant was removed and recentrifuged at 18,000 g for 20 minutes. The volume of this latter supernatant was then reduced to 5 ml. by vacuum evaporation at 40° C. The concentrated extract was

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deproteinized with an equal volume of cold methanol. Dilute ammonium hydroxide was then added to the protein-free methanol-water extract so as to bring the pH of the solution to 8.5 to 9.0, as suggested by Bregoff, Roberts, and Delwiche (*5*). Any precipitate formed at this time was removed by centrifugation. Then 3 ml. of 5% reineckate solution in methanol were added to the clear supernatant and the solution was left in the refrigerator for 3 hours. The precipitate formed was collected by centrifugation, washed with 1-propanol, and finally dissolved in a minimum quantity of an acetone-water (50–50) mixture.

In certain experiments, the supernatant from the alkaline reineckate precipitation was adjusted to pH 1.2 by the addition of 6*N* hydrochloric acid and the precipitate formed on leaving this acid solution in the cold was treated like the precipitate obtained in alkaline medium. In either case, the individual acid or alkaline reineckate precipitate dissolved in the acetone-water mixture was treated as described by Banister, Whittaker, and Wijesundera (*3*) with just sufficient saturated silver sulfate solution to remove the soluble reineckate. Any excess of silver was removed by careful addition of just sufficient 10% barium chloride and any excess barium was removed by addition of a saturated solution of sodium sulfate. The clear supernatant was then reduced to dryness by vacuum evaporation and the solid residue was extracted twice with absolute methanol. The volume of the pooled methanol extracts was reduced to 0.1 ml. under nitrogen gas at 40° C., this was applied as a band on a strip of Whatman No. 1 filter paper $1^1/_2$ inches wide and 20 inches long, and the chromatogram was developed in a butanol-water system (*3*). The developed chromatogram was air-dried and sprayed with a 1% solution of iodine in ethanol (*4*).

The reineckate precipitate formed in alkaline solution contained material which had R_f values of 0.10 to 0.14, 0.21, 0.30, and 0.50 to 0.70. Material contained in the acid-precipitated reineckate fraction had an R_f of 0.1. Aqueous brain extracts made from 20 normal animals in a similar manner contained no acid-reineckate precipitable material, while the alkaline reineckate precipitate contained only material which had R_f values of 0.10 to 0.14, 0.21, and 0.30.

From these results it appeared that as a consequence of the administration of dieldrin, certain substances could be separated from the brain extract of the convulsing animals which could not be found in normal brain extract. Further experiments confirmed the earlier findings of Banister *et al.* (*3*), who used ox spleen in their experiments, that the substances on our chromatograms with values of 0.10 to 0.14 and 0.21 were choline, ACh, and propionyl choline, respectively. We observed, as did these workers, that the band with R_f 0.50 to 0.70 contained substances which were physiologically active as tested on the frog rectus preparation. The material from the acid reineckate precipitation which had an R_f of 0.1 was devoid of physiological activity.

Bregoff *et al.* (*5*) have shown that this method of reineckate precipitation enables the separation of conjugated betaines from free betaines. Reineckate precipitation in alkaline medium enables the removal of the conjugated betaines from solution, while the free betaines are precipitated in acid media. The results from our experiments with extracts made from convulsing animals indicated that both free and conjugated betaines might have been liberated in the brain of these animals. Again, as the conjugated betaine fraction contained substances which were similar in physiologic action to ACh, it was believed that these substances could well be associated with the initiation and propagation of the convulsions, since topical application of ACh on the eserinized cat brain cortex elicits epileptiform activity (*22*).

Because the intraperitoneal injection of dieldrin caused the release of certain substances in brain which could not be found in aqueous extracts of normal animals prepared in a similar manner, it was of interest to investigate whether this effect was limited to dieldrin or whether other agents and conditions which are known to cause cerebral convulsions would produce similar results. Metrazol (50 mg. per kg.), camphor (1 gram per kg.), and ammonium chloride (250 mg. per kg.) were each administered intraperitoneally to groups of 20 rats at a time. A similar number of rats were made to convulse with electroshock, by the application of the necessary threshold voltage delivered through the leads of a physiological stimulator. During the convulsions, the animals were decapitated and aqueous brain extracts were made and analyzed by paper chromatography as described above. In every instance, results similar to those obtained with dieldrin were obtained. It appeared, therefore, that the release of these substances in brain may well be associated with convulsions of the epileptiform type.

Identification of Substances from Brain Extracts

The next phase of the investigation was to identify these substances which were found in the brain extracts derived from convulsing animals and shown to be present in both the alkaline- and acid-precipitable reineckate fractions. The material in the acid-precipitable reineckate fraction, possibly free betaines with an R_f of 0.1, was eluted from the pooled paper strips with a methanol-water (90–10) mixture and the eluate was analyzed using the procedure described by Linneweh (*19*) for the separation and identification of betaines by forming their gold salts. The gold derivatives were prepared by boiling the eluate with 100 mg. of gold chloride in 1% HCl. The salts formed on cooling were then separated on the basis of their solubility in 1% HCl, as described by Carter and others (*8*). The separated crystals were dried and were found to melt at about 152°, 182°, and 211° C., all with decomposition. On determining the melting points of the gold derivatives of authentic crystalline carnitine, γ-butyrobetaine, and crotonbetaine, similar values were obtained. When the crystals formed from the eluate of the chromatograms were mixed with those of authentic crystalline material, there was no depression of the melting points. From these results it was concluded that the material contained in the acid-precipitable fraction of the aqueous brain extract originally made from convulsing animals was comprised of a mixture of free betaines—namely, γ-butyrobetaine (gBB), crotonbetaine, and carnitine. This was further confirmed by determining the R_f values of authentic samples of these substances in the butanol-water system, which was found to be 0.1.

The eluate of the band with an R_f of 0.1 was found to be physiologically inactive. As we had identified the substances on this chromatogram, it was of interest to investigate the influence of these crystalline betaines on the frog rectus preparation and on the intact animal. These substances were all without effect on the frog rectus preparation, and intracranial injections of up to 1 mg. into unanesthetized rats produced no observable response. On the other hand, the intracranial injection of less than 100 μg. of the methyl, ethyl, or choline esters of these betaines caused violent and fatal convulsions in the unanesthetized rat. In addition, these betaine esters caused immediate contraction of the frog rectus preparation and this effect was abolished by atropine. From these results it appeared that betaine esters were capable of producing convulsions in animals and that their pharmacologic effects were similar to those of ACh (*13*). The similarity of the actions of betaine esters and those of ACh has been described by several authors (*7, 25*).

The presence of these betaines has not previously been reported in brain tissue, although their presence in muscle extracts has been known for a long time (*12*). Because the free betaines

have been found to be without physiologic activity but their esters were active (*7, 13, 25*), it seemed reasonable to surmise that these substances might be normally present in brain as esters, especially in view of the fact that we were unable to detect any free betaines in aqueous extracts made from normal animals. In such a case, it was also possible that the free betaines could have been liberated from their naturally occurring esters during the preparation of the extract and the subsequent manipulation required by the reineckate fractionation procedure. Accordingly, an aqueous extract was made from the brains of normal animals and divided into two parts; 1 mg. of the ethyl ester of gBB (gBBE) was added in a saline solution to one part of the extract. The entire extraction procedure for the isolation of free betaines was repeated with both samples. Acid reineckate–precipitable material (free gBB) was found in the fraction to which the gBBE was added but none in the companion control sample. These results indicated that the free betaines previously isolated from the aqueous brain extract made from convulsing animals could have been liberated from some easily hydrolyzable betaine ester precursor during the extraction process and accordingly, their presence in the extract could have been an artifact, as a consequence of hydrolysis.

As it has been reported that alkaline reineckate precipitation selectively removes conjugated betaines from solution (*5*), the next investigation was to determine the identity of the material found in this fraction with R_f 0.50 to 0.70 derived from the aqueous extract made from the brains of convulsing animals, since none of this material could be found in similar extracts made from normal animals.

The material in this band obtained from several chromatograms was eluted with methanol-water and tests were performed on the pooled eluate. A positive hydroxamic reaction was obtained when the eluate was treated with hydroxylamine and ferric chloride (*14*), indicating that some carboxylic acid derivative was present (*20*). Accordingly, a portion of the eluate was hydrolyzed by boiling with 0.1*N* HCl for 10 minutes. The solution was cooled and neutralized and its volume reduced to 0.5 ml. by vacuum evaporation. A portion of the concentrated hydrolyzate was applied as a spot on paper and developed in the butanol-water system as described above. Spraying the developed chromatogram with 1% iodine in ethanol revealed a spot with an R_f of 0.1. The material in this spot was eluted and gold derivatives were prepared as described above. The melting points of the crystals formed were determined and compared with those previously shown in this paper. Similar values were obtained. On the basis of results obtained in these and previous experiments, it was concluded that the same three betaines were also present in the alkaline reineckate fraction of the extract made from the brains of convulsing animals, and that these betaines were conjugated in some manner, probably through the carboxylic acid group, since a positive hydroxamic test was obtained and alkaline reineckate precipitation selectively removed conjugated betaines.

Since the eluate of the band derived from alkaline reineckate precipitation had an R_f of 0.50 to 0.70 and its acid-hydrolyzed product had an R_f of 0.1 in the same system, it appeared that the chemical group which was released by hydrolysis significantly altered the mobility of the betaines in this system. The R_f value of the methyl and ethyl derivatives of gBB was determined and found to be 0.21 in the butanol-water system (*13*). It, therefore, appeared that the group released by hydrolysis of the naturally occurring conjugated betaines may not have been of the hydrocarbon type. Aliquots of the acid hydrolyzate of the eluate were then applied as a spot on paper for two-dimensional chromatography using propanol-water (70–30) and phenol-water (3–1) systems, respectively. When the developed chromatogram was sprayed with 1% ninhydrin, a number of spots were observed, one of which was identified as that of β-alanine. Since this amino acid has an R_f of less than 0.1 in butanol-water, it was assumed that it could have been part of the structure of the conjugated betaine. The amino acid does form part of the structure of coenzyme A, a substance which forms thiol esters with carboxylic acids (*24*). Coenzyme A contains several substances, two of which are ribose and phosphoric acid as phosphate (*1*). Chemical analyses on the acid hydrolyzate of the concentrated eluate revealed the presence of both of these substances. Further identification of the other substances normally present in the coenzyme A structure was vitiated by chromogenic material. The CoA derivative of gBB has been synthesized enzymatically and found to have an ACh-like activity as tested on the frog rectus preparation (*16*). This material is precipitable by alkaline reineckate and is easily hydrolyzed in acid or alkaline media when heated. It has an R_f of 0.55 in the butanol-water system.

It was concluded that the aqueous extracts made from brains of animals which had received either convulsant drugs or electroshock contained a mixture of minute quantities of the CoA derivatives of gBB, crotonbetaine, and carnitine. Because of the severe losses involved during the entire manipulation, it is very difficult to say precisely the relative proportion of these esters in the extracts.

Origin of CoA Betaine Derivatives

Because CoA derivatives of betaines have been shown to be present in aqueous extracts made from the brains of convulsing animals and they could not be found in extracts prepared similarly from normal animals, the next phase of the investigation was to determine their origin in this tissue preparation. Further investigation (*15*) revealed that these substances are normally present in brain, but are released only when the tissue homogenate is treated with agents such as chloroform, ether, and ethanol. These substances were found to be present within the mitochondria. Similar results have been obtained with normal muscle mitochondria (*16*).

It appeared, therefore, that in our aqueous extracts made from the brains of convulsing animals, these betaine esters could have been released from the mitochondria, presumably through the action of the agents and conditions used to initiate convulsions in our experimental animals. If this were the case, we should expect to find more damaged mitochondria in brain preparations made from convulsing animals than in those made from normals. A number of enzyme systems are located exclusively within the mitochondria, and rupture of these bodies results in increased activity of these enzyme systems (*23*). This method of enzyme assay has been to determine the extent of mitochondrial damage under our experimental conditions, by measuring the succinoxidase activity of the isolated mitochondria. Brain mitochondria were isolated following the procedure described by Balazs and Lagnado (*2*). As there are many environmental factors which rapidly cause mitochondria to swell and eventually burst (*23*), we have measured the succinoxidase activity of mitochondria isolated from animals treated with convulsant agents and other conditions previously described, and from normal controls, for only 12 minutes in the presence of the substrate. In this way we were able to determine the succinoxidase activity of the isolated mitochondria before there was further rupture of the intact structures. The results shown in Table I are derived from experiments in which animals treated with cerebral convulsants and electroshock served as experimentals, and normal untreated animals as controls. The enzyme activity is reported in terms of microliters of oxygen per minute consumed by mitochondria contained in 1 gram of fresh brain. Table I shows that in all the mitochondrial preparations derived from the experimental animals, there was increased

succinoxidase activity as compared with the controls, especially within the first minutes of the incubation, which we assume is indicative of greater mitochondrial damage.

Conclusions

The results of these experiments seem to indicate that as a consequence of the administration of the cerebral convulsants used in this work or electroshock, to animals, the mitochondria in the brain is damaged. This, in turn, could lead to the detachment and release of the CoA derivatives of the betaines into a new environment. These betaine esters are structurally similar to ACh, having the same quaternary ammonium nitrogen and carbonyl groups. In addition, the bond distance between these two functional groups in the two molecules is not very different. Hence, it is not surprising to find that the betaine esters are pharmacologically active substances. It is therefore possible that these betaine esters could attach themselves to the same receptors in brain as does ACh itself. Miller *et al.* (*22*) have observed that the topical application of ACh to the eserinized cerebral cortex can elicit the electrical activity normally associated with convulsions of the epileptiform type. It would appear therefore that betaine esters released in brain as a consequence of the administration of cerebral convulsants could act in a manner similar to that observed on the topical application of ACh to the cerebral cortex.

Table I. Damage of Brain Mitochondria in Vivo by Cerebral Convulsants as Assayed by Succinoxidase Activity

(μl. of oxygen consumed by mitochondria in 1 gram of fresh brain per minute)

Time, Min.	Agent	Control	Expr.	Diff.	Agent	Control	Expr.	Diff.
0–3	NH_4Cl	5.6	9.6	4.0	Camphor	7.1	11.6	4.5
3–6	(4.5 g.)[a]	6.2	9.0	2.8	(4.0 g.)[a]	6.0	7.1	1.1
6–9		7.8	8.6	2.4		5.3	6.4	1.1
9–12		5.8	7.3	1.5		4.9	4.9	0
0–3	Dieldrin	7.5	14.3	6.8	Electroshock	13.2	20.0	6.8
3–6	(7.0 g.)[a]	7.8	9.2	1.4	(5.0 g.)[a]	15.0	16.9	1.9
6–9		7.9	9.3	1.4		11.2	11.6	0.4
9–12		7.9	10.2	2.3		13.0	13.0	0

[a] Weight of fresh brain from which mitochondria were isolated.

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INSECT RESISTANCE TO INSECTICIDES

Biochemical Factors in the Acquired Resistance of Houseflies to Organophosphate Insecticides

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THE RAPID DEVELOPMENT of resistance to certain organophosphates is proving to be a limiting factor in their use and a deterrent to the search for new insecticides of this type (*9, 10*). Houseflies have been most frequently used for investigations on the mechanism of this resistance. Although strains of houseflies have developed resistance to almost all the organophosphates, the resistance pattern varies with the strain (*11, 20, 22, 28, 31, 36, 48*, and others). The varying resistance pattern in different strains, along with genetical studies (*35, 37*), indicates that houseflies may have more than one mechanism of resistance to organophosphates. The resistance mechanism is even more complex, since selection with organophosphate insecticides can result in resistance to carbamates (*17, 18, 20, 29*) and *vice versa* (*32*). In addition, exposure to either phosphates or carbamates may result in high side resistance to chlorinated hydrocarbon insecticides (*27, 31, 32*), whereas the reverse situation does not hold (*27, 31*), although DDT-resistant flies developed organophosphate resistance faster than normal flies (*26*).

Many experimental approaches have been used in studying the mechanism of organophosphate resistance in houseflies. Attempts have been made to