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# THE BIOLOGICAL ACTION OF CHLOROPHENOLS

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The biological action of a series of chlorinated phenols has been investigated. With increasing chlorination there is an increase in toxicity in which the convulsant action of phenol is replaced by the signs characteristic of poisoning by dinitrophenol; the higher chlorinated phenols produce a contracture of the isolated rat phrenic nerve diaphragm and a stimulation of *in vitro* oxygen uptake of rat brain homogenate. These actions of the chlorinated phenols have been correlated with their dissociation constants; it is suggested that the higher chlorinated phenols interfere with oxidative phosphorylation, and that this property may be attributed to the chlorophenate ion. The convulsant action of the lower chlorinated phenols is probably associated with the undissociated molecule.

Chlorination of phenol produces a series of nineteen compounds comprising mono-, di-, tri-, and tetrachloro- isomers, and one pentachloroform. All are crystalline solids at room temperature with the exception of *ortho*chlorophenol which is a liquid. The lower compounds in the series are employed medically for their powerful antiseptic properties which, in many cases, are greater than those of phenol itself (von Oettingen, 1949). Tetra- and penta-chlorophenols are important wood preservatives, while the latter is also used as a herbicide, fungicide, insecticide, and molluscicide. These uses of the higher chlorophenols have been reviewed by Truhaut, Vitte and Boussemart (1952).

On account of the differences in test animals and injection techniques employed by authors, including the fact that only seven chlorophenols appear to have been studied, it is difficult to trace the way in which chlorination affects the toxicity of phenol (Bechold and Ehrlich, 1906; Binet, 1896; Deichmann, 1943; Kehoe, Deichmann-Gruebler, and Kitzmiller, 1939). The general effect of chlorination is to increase the toxicity while reducing the convulsant activity characteristic of phenol. Pentachlorophenol is non-convulsant and is characterized by its stimulant effect on oxidative processes, exhibited both *in vitro* and *in vivo*, which is very similar to the activity of dinitrophenols as shown by Weinbach (1954).

Employing eleven chlorophenols as well as phenol itself, a systematic study has been made of the quantitative changes in toxicity occurring with progressive chlorination of phenol. Median lethal doses have been taken as the basis for comparison and have been determined under strictly controlled conditions. An attempt has been made to correlate these quantitative changes in toxicity with changes in the qualitative nature of the effects, and also with the action on oxidative processes studied *in vitro*. In turn, these changes have been considered in conjunction with the dissociation constants of the phenols.

The effect of the progressive chlorination of phenol upon the isolated rat diaphragm has also been examined.

## METHODS AND MATERIALS

*Acute Toxicity Tests.*—All toxicity tests were carried out on male albino rats, weighing 125 to 175 g., which had been fasted for 24 hr. Experiments on rats which had received food prior to dosing showed a considerably lower toxicity.

Median lethal doses were determined by injecting the chlorophenols intraperitoneally in 10 ml. olive oil/kg. body weight. Using the method of moving averages (Thompson, 1947), successive doses were spaced by a factor of 2.0, four rats being injected at each dose level. Fiducial limits for $P=0.05$ were calculated by the method of Weil (1952). All injections were carried out at a room temperature of $23° \pm 1°$.

*Enzyme Experiments.*—The effects of chlorophenols on the oxygen uptake of rat brain homogenate were

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studied by means of the Warburg manometric method. The chlorophenols were added to the flasks as chlorophenates. Stock solutions, prepared by dissolving the chlorophenols in equivalent volumes of N-NaOH, were diluted in substrate-buffer; 0.5 ml. of these solutions was added to the side-arms of the flasks to give final flask concentrations ranging from $10^{-4}$ to $10^{-3}$ M. In control flasks, substrate-buffer alone was added. For absorbing carbon dioxide produced, 0.2 ml. of 20% potassium hydroxide and filter papers were added to the central wells.

For preparing the enzyme, the method adopted was essentially that of Tyler (1950). Using an all-glass hand homogenizer, a 10% homogenate of rat cerebrum was prepared in Krebs-Ringer phosphate (0.154 M, *p*H 7.4) containing $10^{-2}$ M-glucose (Umbreit, Burris, and Stauffer, 1949). The homogenizer was immersed in an ice-water mixture. After straining the homogenate through gauze, 1.0 ml. was added to the main compartment of each flask together with 0.5 ml. buffer. The flasks were set in the water bath at 38° within 20 min. of the rats being killed. The atmosphere in the flasks was air. After temperature equilibration for 15 min. followed by control readings for 30 min., the contents of the side-arms were tipped in and readings then taken 6 and 36 min. later for measuring the oxygen uptake. These 30 min. oxygen uptakes were compared with those of control flasks run concurrently.

*Rat Phrenic Nerve-diaphragm Preparation.*—Preparations were set up according to the method of Bülbring (1946), in Tyrode solution containing 0.2% glucose and aerated with 5% $CO_2$ in oxygen. Supramaximal, rectangular wave stimuli of 0.2 m.sec. duration were applied to the nerve at the rate of 7/min. The capacity of the bath was 60 ml. Sodium chlorophenates were added to the bath in doses of 1 to 200 $\mu$ moles. At the time of testing, 3:4:5- and 2:3:6-trichlorophenols were not available.

Relationships between the different variables were estimated by calculation of co-efficients of correlation (Snedecor, 1948).

## Results

*Median Lethal Doses.*—Median lethal doses are presented in Table I. With doses around these values, death usually occurred within 1 hr. of injection; the highest doses caused death within 5 to 15 min. No further deaths occurred after 3 hr.

*Convulsions.*—Injections of phenol, the monochlorophenols, 2:6-dichlorophenol, and 2:4:6-trichlorophenol were all followed by the same syndrome. Occasionally there was an initial brief bout of excited behaviour with rapid running, or vigorous nose-rubbing. Tremors appeared in 40 to 120 sec. and almost immediately became generalized; sometimes the head and forepaws were seen to be affected first. When poisoning was sufficiently great for the tremors to increase in severity, intermittent convulsions developed and the rat usually fell on its side with loss of righting reflexes. When recovery followed, the rats were sluggish and hypotonic for several hours. If poisoning was fatal, coma and dyspnoea set in. Rats injected with 2:3:6-trichlorophenol often convulsed violently when handled, but otherwise lay prostrate with hypotonia.

*Hypotonia.*—All compounds proved capable of producing hypotonia although this was difficult to detect at first after injections of the convulsant phenols. When the convulsions lessened, however, the hypotonia became obvious and persisted for several hours.

After injecting 2:4-dichlorophenol, 2:3:6-, 3:4:5-, and 2:4:5-trichlorophenols, tetrachlorophenol, or pentachlorophenol, hypotonia was observed in the hind-limbs in 2 to 3 min. This gradually progressed to involve the forelimbs and neck so that the rat was completely prostrate. The

TABLE I

ACUTE TOXICITIES OF CHLOROPHENOLS DETERMINED BY INTRAPERITONEAL INJECTION IN OLIVE OIL INTO MALE ALBINO RATS

The rats weighed 125 to 175 g.

| Position of Chlorine Atoms in Phenol | *p*K | Median Lethal Dose | | | Convulsant Activity | Maximal Change in Rectal Temperature (° C.) | Rigor Mortis within 5 min. of Death |
|---|---|---|---|---|---|---|---|
| | | mg./kg. | Limits of Error ($P$=0·05) | m. moles/kg. | | | |
| — | 9·98 | 250 | (188–333) | 2·66 | + | −2·5 | − |
| 4 | 9·37 | 281 | (188–419) | 2·18 | + | −2·5 | − |
| 3 | 9·12 | 355 | (266–473) | 2·75 | + | −2·5 | − |
| 2 | 8·65 | 230 | (—) | 1·78 | + | −2·0 | − |
| 2 : 4 | 7·85 | 430 | (186–997) | 2·64 | (Occasional twitches) | −0·5 | − |
| 3 : 4 : 5 | 7·83 | 372 | (220–630) | 1·88 | − | +0·5 | + |
| 2 : 4 : 5 | 7·07 | 355 | (—) | 1·79 | − | +0·5 | + |
| 2 : 6 | 6·91 | 390 | (—) | 2·39 | + | −0·7 | − |
| 2 : 4 : 6 | 6·62 | 276 | (185–412) | 1·40 | + | +0·5 | + |
| 2 : 3 : 6 | 5·98 | 308 | (161–591) | 1·56 | (Rats sometimes convulsed when handled) | +0·5 | + |
| 2 : 3 : 4 : 6 | 5·46 | 130 | (97–173) | 0·56 | − | +4·0 | + |
| 2 : 3 : 4 : 5 : 6 | 5·00 | 56 | (38–84) | 0·21 | − | +4·5 | + |

eye reflex weakened and there was no withdrawal response to toe pinching. Muscle twitches seldom occurred spontaneously nor could they be evoked by auditory and mechanical stimuli except in the instance of 2:4-dichlorophenol.

*Body Temperature.*—The rectal temperature of control rats ranged from 37.7 to 38.3°. Injection of penta- and tetra-chlorophenols caused marked rises in temperature, while the trichlorophenols were either without effect, or caused a slight elevation. Phenol and the mono- and di-chlorophenols caused either no change or a slight decrease in rectal temperatures. These effects are indicated in Table I.

*Respiratory Effects.*—Respiration was at first accelerated but became slower and dyspnoeic as coma developed, particularly with tetra- and penta-chlorophenols. The extremities became cyanosed and when coma set in poisoning was usually fatal; asphyxial spasms occurred finally about 30 sec. before death. In tetra- and penta-chlorophenol poisoning, respiration always ceased ½ to 2 min. before the heart stopped beating. With the other compounds, respiration ceased either just before the heart or simultaneously with it.

*Rigor Mortis.*—In control rats killed by ether, rigor mortis occurred 50 min. after death. Stiffening always occurred first in the neck; stiffening of the rest of the spinal muscles often did not occur for a further 30 min. Death due to penta- and tetra-chlorophenols or to 2:4:6- or 2:4:5-trichlorophenols was followed by rapid onset of intense rigor mortis within 3 to 5 min. Rapid appearance of a somewhat weaker rigor occurred after fatal poisoning with 3:4:5- and 2:3:6-trichlorophenols. In poisoning due to the former compounds, rigor often preceded death, normally commencing in the jaws which were clamped down upon a deeply cyanosed tongue for about 1 min. before the trunk began to stiffen and the respiratory movements ceased. Fatal poisoning with phenol and with the other chlorophenols was also followed by onset of rigor mortis rather earlier than in controls; this applied particularly to the higher dose groups.

*Other Effects.*—Chromodacryorrhoea, lachrymation, salivation and diarrhoea were sometimes produced. Macroscopic haematuria was observed in no case, although it has been reported to follow subcutaneous injection of monochlorophenols (Binet, 1896).

*Enzyme Experiments.*—The results of the experiments on the oxygen uptake of brain homogenate are shown in Table II. Phenol and *o*-chlorophenol caused no stimulation of oxygen uptake at concentrations from $10^{-6}$ to $10^{-3}$ M; phenol, in fact, caused slight depression of oxygen consumption. At concentrations between $2.5 \times 10^{-5}$ and $10^{-3}$M, *p*- and *m*-chlorophenols and 2:6-dichlorophenol caused slight stimulation of oxygen uptake; it is probable that the peak stimulation concentrations lie above $10^{-3}$ M. 2:4-Dichlorophenol, the four trichloro- isomers, tetra- and penta-chlorophenols all stimulated oxygen uptake at relatively low concentrations while depressing the uptake at higher concentrations.

*Isolated Diaphragm Preparation.*—Five of the compounds tested were found to send the contracting diaphragm into contracture. Minimum doses, which when added to the bath elicited this effect, were of the order of 1 to 5 μ moles for tri-, tetra-, and penta-chlorophenols, but 20 μ moles for 2:4-dichlorophenol. With relatively low doses the contracture was often preceded by a brief potentiation

TABLE II

EFFECT OF CHLOROPHENATES ON OXYGEN CONSUMPTION OF RAT BRAIN HOMOGENATE AND ON THE TWITCH RESPONSE OF ISOLATED RAT DIAPHRAGM TO INDIRECT STIMULATION

+ Indicates potentiation. The numerals in parentheses indicate dose added to 60 ml. bath. The rat brain homogenate was incubated with $10^{-2}$M-glucose at 38°, *p*H 7·4.

| Position of Chlorine Atoms in Phenol | *p*K | % Ionization at *p*H 7·4 | Oxygen Uptake | | | Muscle Twitch Response |
|---|---|---|---|---|---|---|
| | | | Peak Stimulation Conc. (M) | Equivalent Anionic Conc. (M) | Peak Stimulation % above Normal | |
| — | 9·98 | 0·25 | — | — | None | + (10 μ moles) |
| 4 | 9·37 | 1·0 | $<10^{-3}$ | | 47 at $10^{-3}$ M | + (10 μ moles) |
| 3 | 9·12 | 2·0 | $<10^{-3}$ | | 50 at $10^{-3}$ M | + (10 μ moles) |
| 2 | 8·65 | 5·3 | — | — | None | (−) (10 μ moles) |
| 2 : 4 | 7·85 | 26·2 | $2.5 \times 10^{-4}$ | $6.5 \times 10^{-5}$ | 70 | Contracture (20 μ moles) |
| 3 : 4 : 5 | 7·83 | 27·3 | $10^{-4}$ | $2.7 \times 10^{-5}$ | 112 | Not tested |
| 2 : 4 : 5 | 7·07 | 68·2 | $5 \times 10^{-5}$ | $3.4 \times 10^{-5}$ | 120 | Contracture (5 μ moles) |
| 2 : 6 | 6·91 | 75·7 | $<10^{-3}$ | | 26 at $10^{-3}$ M | (+) (10 μ moles) |
| 2 : 4 : 6 | 6·22 | 94·0 | $10^{-4}$ | $9.4 \times 10^{-5}$ | 92 | Contracture (5 μ moles) |
| 2 : 3 : 6 | 5·98 | 96·5 | $5 \times 10^{-5}$ | $5 \times 10^{-5}$ | 38 | Not tested |
| 2 : 3 : 4 : 6 | 5·46 | 99·0 | $5 \times 10^{-5}$ | $5 \times 10^{-5}$ | 154 | Contracture (1 μ mole) |
| 2 : 3 : 4 : 5 : 6 | 5·00 | 99·5 | $5 \times 10^{-5}$ | $5 \times 10^{-5}$ | 200 | Contracture (1 μ mole) |

of the twitch amplitude. Addition to the bath of 10 to 100 $\mu$ moles of *m*- or *p*-monochlorophenols caused powerful potentiation of the twitch which was not followed by contracture. Addition of 10 $\mu$ moles *o*-chlorophenol, on the other hand, caused a slight depression, while 10 to 50 $\mu$ moles phenol caused potentiation.

## Discussion

Progressive chlorination of phenol increases the acidity of the phenolic hydroxyl- group which is demonstrated by the decrease in *p*K value with increasing chlorination as displayed in Table I. All the *p*K values in this table were determined by a potentiometric titration procedure which has been described in detail elsewhere (Gage, 1957); they do not differ greatly from values published in the literature by other methods.

It is evident from Table I that as phenol is progressively chlorinated the molar toxicity shows a consistent tendency to increase when the *p*K value falls below 7.0. These more toxic chlorophenols are more than 90% ionized at physiological *p*H values. The decrease in *p*K value with increasing chlorination is also accompanied by a change in the effects elicited. As convulsions are undoubtedly the most characteristic effect of chlorophenols with *p*K values of 8.65 or higher, it would seem that they can be attributed to the undissociated molecules. Convulsions are not typical of chlorophenols with lower *p*K values, although they are produced by 2:6-dichlorophenol, 2:4:6-trichlorophenol, and sometimes by 2:3:6-trichlorophenol. It is noteworthy that these three compounds are all substituted in the di-*ortho*- position; the significance of this will be mentioned later. Chlorophenols with *p*K value of 7.85 or less appear to be associated with the production of marked hypotonia without convulsions, an increase in body temperature, and an early onset of rigor after death; these effects may, therefore, be attributed to the chlorophenate ion.

The higher chlorinated phenols are also associated with an ability to cause contracture of the isolated diaphragm, and to stimulate oxygen uptake *in vitro*. The manner in which oxygen uptake is stimulated by chlorophenols and dinitrophenols is not fully understood, but it seems likely that these substituted phenols have a common mode of action. As was originally shown by the *in vitro* studies of Loomis and Lipmann (1948) and of Weinbach (1954), the dinitrophenols and pentachlorophenol are inhibitors of oxidative phosphorylation. These compounds dissociate the process of oxygen uptake from the synthesis of high energy phosphate bonds, processes which are normally interdependent. While phosphorylation is inhibited, oxygen consumption may, with the appropriate enzyme system, be shown to be greatly accelerated. If the increase of oxygen uptake observed in our experiments is the outcome of inhibition of oxidative phosphorylation, then it may well be that all the chlorophenols which stimulate oxygen uptake inhibit phosphorylation in corresponding degree, the effect being due to chlorophenate ions.

The results of enzyme experiments indicate that, with the exception of 2- and 2:6-dichlorophenol, the ability to stimulate oxygen uptake increases with progressive chlorination of phenol. Thus, with increasing acidity of the compounds, the molar concentration of chlorophenate required to produce peak stimulation of oxygen uptake decreases, and the actual height of the peak stimulation above normal increases. Taking into account the *p*K values and % ionization at *p*H 7.4 of the buffered medium, the molar concentrations of chlorophenate ions which produced peak stimulation have been calculated. As indicated in Table II, these concentrations lay between $2.7 \times 10^{-5}$ M and $9.4 \times 10^{-5}$ M. This suggests that for the particular enzyme system employed, peak stimulation of oxygen uptake, regardless of its actual height above the normal level, is produced by a fairly constant concentration of chlorophenate ions. The significance of this concentration of anions may be that it corresponds to the concentration of some factor in the homogenate affected by these ions. Using the same enzyme system, Tyler (1950) found that 2:4-dinitrophenol, which has a *p*K value of 4.1, also produced maximum stimulation at a concentration of $5 \times 10^{-5}$ M. It is suggested that the differing degrees of peak stimulation are due to the chemical peculiarities of the chlorophenate (or nitrophenate) ions causing various rates of interaction with the postulated factor. This explanation is in harmony with current theories of the effect of dinitrophenols on oxidative phosphorylation (Lardy and Wellman, 1953; Lee and Eiler, 1953). These theories postulate the existence of a labile, phosphorylated intermediate whose rate of continual synthesis and breakdown determines the rate of oxygen consumption. The substituted phenols are thought to compete for combination with the precursor of this intermediate, forming a compound of greater instability; the synthetic and breakdown processes are thereby accelerated causing an increased rate of oxygen uptake.

The anomalous position of 2:6-dichlorophenol in Table II recalls the observations of Blackman,

Parke, and Garton (1955), who found that chlorophenols substituted in these positions deviate from the linear relationship which they have shown to exist between *p*K value and the ratio of standard bactericidal activity to solubility. It seems probable that the anion produced by ionization of 2:6-dichlorophenol cannot react, presumably with some basic centre in the receptor system, by virtue of the steric or electronic interference by the ortho chlorine atoms.

It is interesting to notice the fairly close negative correlation between median lethal doses and the ability to stimulate oxygen uptake *in vitro*. For the statistical tests of correlation, stimulation of oxygen uptake was taken as the % stimulation above the normal level produced by $10^{-5}$ M chlorophenate. The coefficient of correlation was $-0.86$ on comparison of % increase in oxygen uptake with median lethal dose.

As shown by several authors (Weeks and Chenoweth, 1952; Judah and Magee, 1954; Barnes, Duff, and Threlfall, 1955), 2:4-dinitrophenol sends the isolated rat diaphragm into contracture, this being accompanied by profound falls in the contents of creatine phosphate and adenosine triphosphate in the muscle. This production of contracture may be attributable to the ability of 2:4-dinitrophenol to inhibit oxidative phosphorylation. It is, therefore, not surprising to find that pentachlorophenol, another inhibitor of oxidative phosphorylation, is able to cause the same effect. Production of contracture by other chlorophenols also suggests that these compounds are inhibitors of oxidative phosphorylation which would accord with their ability to stimulate oxygen uptake (see Table II). In the present experiments, contracture was preceded by slight potentiation of the twitch response; an effect which was not reported by Barnes *et al.* (1955) for 2:4-dinitrophenol. Unfortunately, no explanation can be given for the potentiation of the twitch response produced by phenol, *p*- and *m*-monochlorophenols, and by 2:6-dichlorophenol. Mogey and Young (1949), who studied the antagonistic action of phenolic substances on the effect of tubocurarine on the rat diaphragm, found that phenol and *p*-chlorophenol at a concentration of $10^{-4}$ M caused only 1 to 7% inhibition of rat brain cholinesterase activity *in vitro*. It seems highly unlikely, therefore, that potentiation of the twitch is due to the anticholinesterase activity of these compounds.

The appearance of rapid, or even of premature, rigor mortis is also found in dinitrophenol poisoning (Hall, Field, Sahyun, Cutting, and Tainter, 1933; Parker, 1954; Parker, Barnes, and Denz, 1951) and it has been suggested there may be a common mechanism underlying this rapid rigor mortis and contracture of the isolated diaphragm (Barnes *et al.*, 1955). Further evidence for the association between these two effects is provided by the present work since four of the chlorophenols shown to cause contracture of the isolated diaphragm also caused rapid rigor mortis (Tables I and II).

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