THE HISTOPATHOLOGY OF TRIORTHOCRESYL PHOSPHATE POISONING

THE ETIOLOGY OF SO-CALLED GINGER PARALYSIS (THIRD REPORT) *

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In the first paper of this series 1 there was described a peculiar and new type of multiple neuritis which afflicted thousands of victims during the winter and early spring months of 1930. It appeared that the disease had resulted following the consumption of an adulterated fluidextract of ginger used for beverage or other purposes. In that paper consideration was given to the possible causes of the partial paralysis, and experiments were presented which indicated that a phenolic compound, demonstrated to be present only in samples of the adulterated ginger extract that caused cases of paralysis, was probably the immediate cause of the disease.

Subsequent work 2 fully confirmed these early tentative conclusions. Pharmacologic examination of the available phosphoric esters of the better known phenols, viz., orthocresol and paracresol, and of phenol itself showed conclusively that the phosphate of orthocresol alone had this specific action on the neuromuscular apparatus. Since this was published, the phosphoric ester of metacresol and several other esters of orthocresol have been examined, as shown in figure 1, with the result that the phosphoric acid ester of orthocresol appears to be the only compound so far found capable of reproducing in lower animals the symptom-complex as it occurred in man. The evidence for this, together with a more detailed analysis of the relation of chemical structure to pharmacologic action in so far as it concerns this particular group of phenol compounds, will be described elsewhere.

The clinical manifestations of the paralysis due to the consumption of an adulterated fluidextract of ginger, which were previously described in considerable detail, 1 point to a lower motor neuron involve-

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ment. It does not seem possible, however, to ascertain clinically the exact anatomic seat of the lesion of the neuromuscular apparatus. A systematic study was therefore undertaken on this phase of the problem, and the present report is the outcome of this investigation.

There have appeared recently several papers on the histopathology of paralysis due to adulterated fluidextract of ginger in man, the cases having come to autopsy apparently through causes other than the effects of the specific poison on the neuromuscular apparatus.

Jeter, describing an autopsy on a patient who seemed to have died of cerebral edema and nephritis, gave thickening and edema of the meninges, perineural exudate in the cauda equina, thickening and fibrosis of the perineurium of the peripheral nerves and endarteritis of the vessels of the central nervous system as the essential changes.

More recently, Goodale and Humphreys reported their observations in three cases of paralysis due to adulterated fluidextract of ginger at the Worcester City Hospital. In one case they found acute perineuritis

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of the cauda equina somewhat similar to that in Jeter’s case. The lesions considered characteristic by Goodale and Humphreys, however, were those of the peripheral nerves and of the anterior horn cells of the spinal cord. The nerve lesions were marked by degeneration of the myelin sheaths with occasional fusiform enlargement of the axis cylinders. The changes of the anterior horn cells were characterized by occasional absence of the nucleolus, migration of the nucleus to the periphery and formation of fine fatty granules in the cytoplasm.

The present work is a systematic study of the histopathology of tri-orthocresyl phosphate poisoning in a variety of experimental animals, and a comparison thereof with the pathologic changes in several cases of human paralysis due to adulterated fluidextract of ginger. The material from the human cases was made available to us through the cooperation of Prof. R. S. Austin of the Medical School of the University of Cincinnati. There were in all six cases, all the patients having died at the Cincinnati General Hospital of intercurrent diseases after several weeks of sojourn at the hospital to which they had been admitted for observation and treatment of paralysis of the extremities acquired as a result of drinking adulterated Jamaica ginger extract.

Our experimental material comprised a variety of laboratory animals used throughout the investigation on the etiology of paralysis due to adulterated fluidextract of ginger. Much of this material was obtained before the immediate cause of the paralysis was definitely ascertained. Our material therefore represents a variety of experimental procedures, in addition to the actual production of partial motor paralysis of the extremities by means of the adulterated ginger extract that had given rise to cases of paralysis in man or by the administration of triorthocresyl phosphate, either chemically pure or as isolated from the adulterated ginger extract. Only such material, however, as is likely to have a direct bearing on the present problem, together with what appears to be a sufficient number of controls, is included in this report. Because of the characteristic clinical manifestations of the disease process, the examination of the tissues was usually limited to certain parts of the central nervous system. In many cases some of the viscera and striated muscle were also examined. Attention will be called to these whenever the observations present points of interest.

**TECHNIC**

The routine procedure was to remove the tissues as shortly after death as possible, or immediately after the animal was killed. The tissues were then placed in a 10 per cent solution of commercial formaldehyde and submitted for examination under a key number to the pathologic laboratory, where nothing was known about the treatment or the clinical history of the animals from which the tissues had been removed. Primary fixation of all material in formaldehyde lasted several days.
Blocks were cut routinely, in duplicate, from three levels of the cord, lumbar, thoracic and cervical, and from the medulla, pons, cerebellum and midbrain through the eculomotor nerve roots and anterior colliculi. In the earlier phases of the work, blocks were also taken from the posterior portion of the thalamus, from the corpus striatum and from frontal, parietal, occipital and temporal regions of the cerebral cortex, as well as from the cornu ammonis; this was discontinued in later work as no significant changes were discerned. One set of blocks was hardened further for forty-eight hours in a 2.5 per cent solution of potassium bichromate, followed by 50 and 80 per cent alcohol, completion of dehydration in acetone, clearing in benzene, and embedding in paraffin in vacuo for twenty minutes. These blocks were sectioned at from 5 to 8 microns and stained routinely with toluidine blue for Nissl bodies and with Weigert’s acid iron-hematoxylin and van Gieson’s picrofuchsin for nerve tissues. The second set of blocks was impregnated in several changes of a 2.5 per cent solution of potassium bichromate for seven or eight days, then for a further seven days in two parts of a 2.5 per cent solution of potassium bichromate and one part of a 1 per cent solution of osmic acid, washed overnight, and dehydrated, cleared, embedded and sectioned as already described. These sections were either lightly counterstained by various methods or, more often, were studied unstained.

Material from peripheral nerves was also blocked in duplicate, one set being treated by the Marchi procedure outlined, the second being embedded in a routine manner in paraffin and stained with hematoxylin and eosin, iron-hematoxylin and van Gieson’s method and with other stains as they appeared indicated.

Various gold methods and supravital methylene blue (methylthionine chloride U. S. P.) were tried for the study of terminal ramifications and motor end-plates, but the results proved so uncertain that for the present it was deemed futile to attempt to estimate quantitative or qualitative changes in these structures.

The histologic observations were then reported back to the pharmacologic laboratory and there correlated with the clinical manifestations that resulted from the particular experimental procedure.

**RESULTS**

The results of the investigations are summarized in the accompanying tables. The lesions observed in the human cases, outlined in table 1, were characterized by some involvement of the anterior horn cells.
and in most cases by considerable fatty degeneration of the white substance in the cord, as revealed in Marchi preparations. Changes of the nerve cells, which were most pronounced in case N30279, were marked by some tigrolysis, displacement of the nucleus to the periphery, deposition of clumps of Marchi-positive droplets in the cytoplasm, and distinct evidence of cytoplasmic disintegration in some cells, especially at their periphery (figs. 2 and 3). The changes in the peripheral nerves were, on the whole, perhaps more constant and more pronounced, judging from an examination of the spinal nerve roots. The changes were well marked fragmentation and fatty degeneration of the myelin sheath, which involved groups of fibers throughout the nerve trunks (fig. 4). Unfortunately, the smaller peripheral nerves were available only in two cases. The nerve roots, however, were studied in all cases and were found to present the same picture as the small peripheral nerves.
In table 2 are presented the results of a similar study in the dog. It will be seen from the data in this table that the partial flaccid paralysis of the extremities produced by the subcutaneous injection of 0.5 Gm. or more per kilogram of technical or chemically pure triorthocresyl phosphate resulted in lesions of the nervous system comparable with those seen in the human paralysis due to the consumption of adulterated fluid extract of ginger. Figure 5 shows the fatty degeneration and nuclear displacement seen in some of the anterior horn cells of dog 13 of the series, which compare well with the lesions shown in figure 2. It is to be noted, however, that the lesions of the spinal cord in the dog were rather variable, and in one instance could not be demonstrated at all. The lesions of the peripheral nerves, however, were constant and of a degree entirely comparable with those seen in the human cases.

Attention may also be called to the fact that chronic alcohol or phenol poisoning in the dog produces neither the clinical picture nor,
as a rule, the pathologic lesions produced by the phosphoric acid ester of orthocresol.

Table 3 summarizes the results of our studies in monkeys (Macacus rhesus). The interval between the administration of the drug and the appearance of the clinical symptoms of paralysis is not definite in every instance, since in some of the experiments the specific ester was injected in two or more doses and it is not possible to state whether paralysis resulted from the first or the last injection. In all eleven monkeys that received subcutaneous injections of the chemically pure or technical triorthocresyl phosphate or the ester isolated from the incriminated adulterated fluidextract of ginger, typical motor paralysis of the extremities developed. Lesions of the peripheral nerves were demonstrable in all but one case; well marked tigrolysis or fatty degeneration, or both, of the anterior horn cells in at least seven of the eleven animals, and some fatty degeneration of the white substance in the cord in six
### Table 2.—Histopathology of Triorthocresyl Phosphate Poisoning in the Dog

<table>
<thead>
<tr>
<th>Weight, No. Kg.</th>
<th>Treatment; Gm. per Kg. Body Weight</th>
<th>Nerve Lesions; Tigrolysis or Fatty Degeneration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Spinal Cord</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nerve Cells White Substance</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paralytic Days</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Periperal Nerves</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cell Substance</td>
</tr>
<tr>
<td>13</td>
<td>0.1 10.6 technical triorthocresyl phosphate subcutaneously</td>
<td>10 2 ++ TF* + + +</td>
</tr>
<tr>
<td>12</td>
<td>7.3 1.0 technical triorthocresyl phosphate subcutaneously</td>
<td>5 8 ++ T + + +</td>
</tr>
<tr>
<td>6</td>
<td>10.5 1.5 technical triorthocresyl phosphate subcutaneously</td>
<td>18 30 + F + + Slight</td>
</tr>
<tr>
<td>14</td>
<td>10.9 1.0 technical triorthocresyl phosphate subcutaneously</td>
<td>30 33 + F Slight Slight</td>
</tr>
<tr>
<td>8</td>
<td>12.7 0.4 chemically pure triorthocresyl phosphate subcutaneously</td>
<td>7 1 + F + + +</td>
</tr>
<tr>
<td>9</td>
<td>7.3 0.6 chemically pure triorthocresyl phosphate subcutaneously</td>
<td>20 2 + + +</td>
</tr>
<tr>
<td>17</td>
<td>11.0 1.0 chemically pure triorthocresyl phosphate subcutaneously</td>
<td>7 1 + T + + +</td>
</tr>
<tr>
<td>18</td>
<td>4.6 70% alcohol per os, in 10 doses during an interval of 23 days</td>
<td>14 21 + + TF* ++ T</td>
</tr>
<tr>
<td>19*</td>
<td>5.7 70% alcohol per os, in 10 doses during an interval of 23 days</td>
<td>6 11 + F + + +</td>
</tr>
<tr>
<td>20</td>
<td>7.7 1.9 phenol per os, in aqueous 15% solution, divided in 8 doses over a period of 16 days</td>
<td>10 53 + TF* +</td>
</tr>
<tr>
<td>21</td>
<td>10.0 1.9 phenol per os, in aqueous 15% solution, divided in 8 doses over a period of 16 days</td>
<td>30 33 + + +</td>
</tr>
</tbody>
</table>

* T, tigrolysis; F, fatty degeneration.
† Animal showing meningomyelo-encephalitis and visceral focal lesions suggestive of distemper.

### Table 3.—Histopathology of Triorthocresyl Phosphate Poisoning in the Monkey

<table>
<thead>
<tr>
<th>Weight, No. Kg.</th>
<th>Treatment; Gm. per Kg. Body Weight</th>
<th>Nerve Lesions; Tigrolysis or Fatty Degeneration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Spinal Cord</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nerve Cells White Substance</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paralytic Days</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Periperal Nerves</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cell Substance</td>
</tr>
<tr>
<td>17A</td>
<td>3.7 2.0 technical triorthocresyl phosphate subcutaneously</td>
<td>2 11 2 2</td>
</tr>
<tr>
<td>11C</td>
<td>3.3 1.0 technical triorthocresyl phosphate subcutaneously</td>
<td>2 12 2</td>
</tr>
<tr>
<td>3B</td>
<td>2.9 1.5 technical triorthocresyl phosphate subcutaneously</td>
<td>6 2</td>
</tr>
<tr>
<td>22A</td>
<td>4.0 1.5 technical triorthocresyl phosphate subcutaneously</td>
<td>6 2</td>
</tr>
<tr>
<td>4D</td>
<td>3.3 1.0 technical triorthocresyl phosphate subcutaneously</td>
<td>6 1</td>
</tr>
<tr>
<td>24</td>
<td>2.3 1.6 technical triorthocresyl phosphate subcutaneously</td>
<td>14 1</td>
</tr>
<tr>
<td>20</td>
<td>2.4 2.7 technical triorthocresyl phosphate subcutaneously</td>
<td>2 12 2</td>
</tr>
<tr>
<td>1C</td>
<td>4.6 1.0 chemically pure triorthocresyl phosphate subcutaneously</td>
<td>2 12 2</td>
</tr>
<tr>
<td>29</td>
<td>3.4 1.0 chemically pure triorthocresyl phosphate subcutaneously</td>
<td>10 2</td>
</tr>
<tr>
<td>22A</td>
<td>2.7 0.5 chemically pure triorthocresyl phosphate subcutaneously</td>
<td>5 2</td>
</tr>
<tr>
<td>25</td>
<td>3.3 2.0 specific ester isolated from adulterated fluidextract of ginger, subcutaneously</td>
<td>11 20</td>
</tr>
<tr>
<td>26</td>
<td>2.1 77.0% alcohol, in 11 doses, over a period of 40 days, per os</td>
<td>3 40</td>
</tr>
<tr>
<td>27</td>
<td>3.0 77.0% alcohol, in 10 doses, over a period of 45 days, per os</td>
<td>10 2</td>
</tr>
<tr>
<td>6A</td>
<td>4.7 30.0 U.S.P. fluidextract of ginger in 3 doses, per os</td>
<td>5 2</td>
</tr>
<tr>
<td>24</td>
<td>2.4 0.75 phenol, 5% in 95% alcohol per os in 3 daily doses; acutely fatal</td>
<td>11 70</td>
</tr>
</tbody>
</table>

* T, tigrolysis; F, fatty degeneration.
of the animals were also observed. It is of interest that two animals that received large doses of alcohol and one U. S. P. fluid extract of ginger showed no such changes in the central nervous system, while one animal with acute and fatal phenol poisoning showed a slight involvement of the peripheral nerves and distinct changes in the anterior horn cells. Figures 6 and 7 show the lesions in the anterior roots produced by triorthocresyl phosphate in this animal species.

Fig. 5.—Photomicrograph of a section of the spinal cord in the lumbar region of dog 13 (table 2); Marchi method. Note the fatty degeneration of some anterior horn cells and the nuclear displacement (compare with fig. 2); × 400.

The histologic manifestations of triorthocresyl phosphate poisoning in the chicken are reported in table 4. In this series of experiments the animals were killed at regular intervals following the appearance of symptoms of paralysis in order to ascertain whether the relative degree of involvement of the peripheral nerves and of the spinal cord bears any relation to the duration of the paralysis. The results indicate, however, that while the peripheral nerves showed almost constantly the typical lesions of degeneration of the myelin sheaths, even
as early as the first day of the paralysis, the cellular degeneration in the spinal cord was seldom demonstrable in this species. Figure 8 shows the typical degeneration of the myelin sheaths in one of the small nerves of the leg of chicken 502 that had been paralyzed fifteen days after the oral administration of 0.4 Gm. per kilogram of technical triorthocresyl phosphate. Similar symptoms and lesions resulted from the oral administration of the specific ester isolated from the incriminated adulterated fluidextract of ginger. The oral administration of phenol or the several cresols, in sublethal doses, failed to produce any symptoms referable to the neuromuscular apparatus and gave negative results on histologic examination, as illustrated by fowl 650 in table 4. The negative observations in fowl 594 would have constituted a rather serious exception in this series of experiments were it not for the fact that this fowl had shown definite, though slight, improvement for about fifteen days before it was killed. It is, of course, also possible that the methods used may not be adequate to demonstrate changes in the nerves in the chicken at this later stage.
Our histologic studies of three calves yielded results entirely consistent with those just described. In the first two publications of this series there were described experiments on three calves, all of which developed typical motor paralysis of the posterior extremities, one as a result of the oral administration of an adulterated fluid extract of ginger which had produced paralysis in man, another as a result of the oral administration of U. S. P. fluid extract of ginger diluted with alcohol and adulterated with 2.5 per cent commercial tricresyl phosphate, and the third following the oral administration of 0.2 Gm. per kilogram of chemically pure triorthocresyl phosphate in alcohol. The histologic observations in these three calves, which were killed from three weeks to four months after the onset of the clinical manifestations of the disease, were: calf 1, peripheral parenchymatous neuritis; calf 2, parenchymatous central and peripheral neuritis; calf 3, peripheral parenchymatous neuritis.

Our observations on a small series of cats indicate the same general conclusions, except that in this species the central changes appear to

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be relatively more pronounced than in the preceding species of animals. Our first two reports on this subject did not include any data on the action of triorthocresyl phosphate in the cat. Some experiments performed recently indicate: that the cat reacts typically to this poison; that 0.2 Gm. per kilogram is the minimal surely paralytic dose when administered subcutaneously or intravenously, though as little as 0.1 Gm. per kilogram may produce a moderate degree of motor paralysis of the posterior extremities; that the minimal lethal dose by subcutaneous or intravenous injection is about 0.5 Gm. per kilogram, and that the oral administration of this substance in alcohol is usually ineffective, even in doses up to 3 or 4 Gm. per kilogram. We have

Table 4.—Histopathology of Triorthocresyl Phosphate Poisoning in the Fowl (Plymouth Rocks)

<table>
<thead>
<tr>
<th>No.</th>
<th>Weight, Kg.</th>
<th>Treatment; Gm. per Kg. Body Weight</th>
<th>Effect Interval, Days</th>
<th>Nerve Lesions; Tigrolysis or Fatty Degeneration</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>1.5</td>
<td>1.0 technical triorthocresyl phosphate per os</td>
<td>8 4</td>
<td>- - - +</td>
</tr>
<tr>
<td>408</td>
<td>1.2</td>
<td>0.4 technical triorthocresyl phosphate per os</td>
<td>6 12</td>
<td>+ T* Slight ++</td>
</tr>
<tr>
<td>502</td>
<td>2.1</td>
<td>0.4 technical triorthocresyl phosphate per os</td>
<td>8 15</td>
<td>Slight T + + +</td>
</tr>
<tr>
<td>512</td>
<td>1.7</td>
<td>0.4 technical triorthocresyl phosphate per os</td>
<td>9 13</td>
<td>- - Slight +</td>
</tr>
<tr>
<td>503</td>
<td>1.5</td>
<td>0.2 technical triorthocresyl phosphate per os</td>
<td>10 1</td>
<td>- - + +</td>
</tr>
<tr>
<td>675</td>
<td>1.9</td>
<td>0.1 technical triorthocresyl phosphate per os</td>
<td>10 43</td>
<td>- - - -</td>
</tr>
<tr>
<td>563</td>
<td>1.4</td>
<td>0.5 specific ester isolated from adulterated fluid extract of ginger per os</td>
<td>10 20</td>
<td>- - - -</td>
</tr>
<tr>
<td>562</td>
<td>1.1</td>
<td>0.5 specific ester isolated from adulterated fluid extract of ginger per os</td>
<td>8 14</td>
<td>- - - -</td>
</tr>
<tr>
<td>650</td>
<td>1.5</td>
<td>0.2 phenol in alcohol per os</td>
<td>-</td>
<td>- - - -</td>
</tr>
</tbody>
</table>

* T, tigrolysis.

long suspected that the relative ineffectiveness of triorthocresyl phosphate by oral administration in certain animal species (monkey, dog, cat) was due to difficult and irregular absorption. We have recently secured some results that indicate that, in the cat at least, this substance appears to be absorbed from the gastro-intestinal tract with considerable regularity, though probably not completely, if administered as a fine emulsion with the aid of a 10 per cent aqueous solution of gum acacia. The question of the absorption of this ester is being investigated further, as is the problem of the fate of this substance in the animal body; for the present we offer the evidence summarized in table 5 to illustrate the points enumerated.

The histologic observations in the cat are given in table 6, from which it is evident that the lesions of the peripheral nerves were
uniformly present and the central lesions, though not as uniform, were on the whole perhaps more pronounced than in the other species examined.

Other lesions observed and not included in the tables may be briefly summarized as follows:

1. Man.—Fatty degeneration of the epithelial cells of the ependyma of the central canal of the spinal cord was seen in a greater or lesser degree in every case examined.

2. Monkey.—(a) Striated muscle, taken from the paralyzed extremities in four of the animals, showed some fragmentation, loss of striations and, in one case, hyaline degeneration. No abnormalities were found in three of the seven animals examined.

(b) Tigrolysis, with or without fatty degeneration involving some of the nuclei of the pons and medulla, was seen in several instances. Fatty degeneration of the choroid plexus was also observed once.
### Table 5.—Action of Triorthocresyl Phosphate in Cats as Determined by the Mode of Administration

<table>
<thead>
<tr>
<th>No.</th>
<th>Weight, Kg.</th>
<th>Total Gm. per Kg.</th>
<th>Inter- Day</th>
<th>Paralysis, Days</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Subcutaneous Injection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2.4</td>
<td>1</td>
<td>1.0</td>
<td>5</td>
<td>Died</td>
</tr>
<tr>
<td>9</td>
<td>1.5</td>
<td>1</td>
<td>1.0</td>
<td>4</td>
<td>Died</td>
</tr>
<tr>
<td>8</td>
<td>2.6</td>
<td>1</td>
<td>0.4</td>
<td>12</td>
<td>Paralysis severe; killed</td>
</tr>
<tr>
<td>7</td>
<td>2.3</td>
<td>1</td>
<td>0.3</td>
<td>11</td>
<td>Paralysis severe; died</td>
</tr>
<tr>
<td>4</td>
<td>2.0</td>
<td>1</td>
<td>0.2</td>
<td>18</td>
<td>Slight to moderate paralysis; apparently recovered</td>
</tr>
<tr>
<td>5</td>
<td>2.6</td>
<td>1</td>
<td>0.1</td>
<td>26</td>
<td>Slight paralysis; apparently recovered</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Intravenous Injection, 5 to 10% Suspension in 10% Gum Acacia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>2.5</td>
<td>1</td>
<td>0.5</td>
<td>6</td>
<td>Died in several hours</td>
</tr>
<tr>
<td>25</td>
<td>2.0</td>
<td>1</td>
<td>0.2</td>
<td>32</td>
<td>Moderately severe paralysis; condition unchanged</td>
</tr>
<tr>
<td>26</td>
<td>2.6</td>
<td>1</td>
<td>0.2</td>
<td>19</td>
<td>Slight to moderate paralysis, progressing</td>
</tr>
<tr>
<td>10</td>
<td>2.9</td>
<td>1</td>
<td>0.1</td>
<td>4</td>
<td>Slight paralysis, unchanged</td>
</tr>
<tr>
<td>11</td>
<td>2.0</td>
<td>1</td>
<td>0.1</td>
<td>40</td>
<td>Doubtfully slight paralysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Oral, 10 to 30% in Alcohol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.9</td>
<td>2</td>
<td>3.0</td>
<td>...</td>
<td>No effects</td>
</tr>
<tr>
<td>5</td>
<td>2.8</td>
<td>1</td>
<td>4.0</td>
<td>...</td>
<td>No effects</td>
</tr>
<tr>
<td>22</td>
<td>2.3</td>
<td>3</td>
<td>2.9</td>
<td>13 to 25</td>
<td>Slight paralysis, apparently improving</td>
</tr>
<tr>
<td>23</td>
<td>2.5</td>
<td>3</td>
<td>2.0</td>
<td>3 to 10</td>
<td>Died</td>
</tr>
<tr>
<td>24</td>
<td>3.1</td>
<td>3</td>
<td>3.0</td>
<td>30 to 30</td>
<td>Doubtfully slight paralysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Oral, 10% Suspension in 2% aqueous Solution of Saponin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>1.8</td>
<td>1</td>
<td>1.0</td>
<td>...</td>
<td>No effects</td>
</tr>
<tr>
<td>18</td>
<td>2.6</td>
<td>1</td>
<td>1.0</td>
<td>...</td>
<td>No effects</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Oral, 10% Emulsion in 10% Gum Acacia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>2.5</td>
<td>4</td>
<td>3.0</td>
<td>5 to 38</td>
<td>Moderately severe paralysis; condition unchanged</td>
</tr>
<tr>
<td>14</td>
<td>2.6</td>
<td>1</td>
<td>1.0</td>
<td>4</td>
<td>Severe paralysis; died</td>
</tr>
<tr>
<td>15</td>
<td>2.5</td>
<td>1</td>
<td>1.0</td>
<td>5</td>
<td>Died</td>
</tr>
<tr>
<td>16</td>
<td>1.8</td>
<td>3</td>
<td>2.0</td>
<td>5 to 23</td>
<td>Paralysis severe; died</td>
</tr>
<tr>
<td>19</td>
<td>3.2</td>
<td>3</td>
<td>1.5</td>
<td>5 to 23</td>
<td>Moderately severe paralysis; unchanged</td>
</tr>
<tr>
<td>20</td>
<td>2.5</td>
<td>2</td>
<td>1.0</td>
<td>3 to 13</td>
<td>Severe paralysis; died</td>
</tr>
<tr>
<td>21</td>
<td>3.0</td>
<td>3</td>
<td>1.5</td>
<td>1 to 19</td>
<td>Severe paralysis; died</td>
</tr>
</tbody>
</table>

### Table 6.—Histopathology of Triorthocresyl Phosphate Poisoning in the Cat

<table>
<thead>
<tr>
<th>Weight, Kg.</th>
<th>Treatment; Gm. per Kg. Body Weight</th>
<th>Nerve Lesions: Tigrolysis or Fatty Degeneration</th>
<th>Spinal Cord White Substance Nerve Roots Nerves</th>
<th>Peripheral Nerves</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td></td>
<td>Effect</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>2.3; 0.3 chemically pure triorthocresyl phosphate subcutaneously ...</td>
<td>11 2</td>
<td>— — —</td>
<td>+ —</td>
</tr>
<tr>
<td>6</td>
<td>2.8; 0.4 chemically pure triorthocresyl phosphate subcutaneously ...</td>
<td>12 6</td>
<td>+ — T* —</td>
<td>—</td>
</tr>
<tr>
<td>8</td>
<td>2.6; 0.5 chemically pure triorthocresyl phosphate subcutaneously ...</td>
<td>7 11</td>
<td>— T —</td>
<td>—</td>
</tr>
<tr>
<td>21</td>
<td>3.0; 3.0 ester, 5% emulsion in 10% gum acacia, in 3 doses per os ...</td>
<td>1-19 6</td>
<td>+ + T —</td>
<td>—</td>
</tr>
</tbody>
</table>

* T, tigrolysis.
The kidneys showed a variable degree of parenchymatous degeneration in four animals examined.

Parenchymatous degeneration of the heart muscle was seen in two of the four animals examined.

The liver, adrenals and spleen failed to show any abnormalities in five animals examined.

3. Cat.—Of the four animals reported on in this paper, three presented lesions in the striated muscle of the same character as those already described, and notable multiplication of muscle nuclei. Edema of the cortex of the brain was seen twice; there was also observed tigrolysis with or without cellular fatty degeneration in the red, oculomotor, caudate and pontile nuclei in two animals.

4. Dog.—In two animals of this series in which striated muscle was examined, nuclear multiplication was noted once. Likewise, the brains of two animals of this series showed tigrolysis and some cellular fatty degeneration in the nuclei of the stem.

5. Chicken.—(a) Striated muscle was examined twice; once it appeared normal and once it presented patchy multiplication of muscle nuclei.

(b) The ependyma of the cord presented well marked fatty degeneration twice.

(c) The heart muscle showed edema once, and a normal appearance four times.

(d) The kidney failed to show anything abnormal in the five birds examined.

(e) The liver presented fatty infiltration or degeneration without necrosis twice, and a normal appearance four times.

(f) The lungs and adrenals, examined in three animals, presented a normal appearance. In one bird a sympathetic ganglion appeared normal.

It may be concluded from this brief survey, therefore, that triortho cresyl phosphate is essentially a poison of the central nervous system, affecting primarily the lower motor neuron. The lesions observed in the striated muscle from the affected limbs probably indicate beginning atrophy.

COMMENT

It was hoped that three main points would be elucidated, at least in part, when the present experiments were undertaken: (1) A comparison of the pathologic lesions of paralysis due to the consumption of adulterated fluid extract of ginger in man with those of the experimental disease in animals. This was of particular importance when
this work was begun, at a time when all that was known about the etiology of this paralysis was that it had some relationship to adulterated Jamaica ginger extract. Now that the true nature of the immediate cause of this disease has been definitely established, this particular point has assumed a secondary importance. Nevertheless, it may be said that the questions arising in connection with this part of the problem appear to have been answered satisfactorily, as shown in the foregoing pages. (2) The manner of action of the specific ester. (3) What may be expected as the final outcome of the disease process.

The third point is obviously of immediate and practical importance as it concerns prognosis. Is this peculiar type of multiple neuritis primarily a process of central or peripheral degeneration? In other words, is the phosphoric ester of orthocresol, the acknowledged direct and immediate cause of this paralysis, a poison affecting specifically the nerve cell, the myelin sheath, the axis cylinder, the motor end-plates, or possibly all of these structures. On account of the technical difficulties involved in the histologic work with axis cylinders and motor end-plates and on account of the uncertainties attending the interpretation of such observations, it seemed best to defer such work until better and more certain methods become available. Considering the constancy with which degeneration of the myelin sheaths of the peripheral nerves was observed in practically our entire series of experiments, it seems that it can safely be asserted that the substance in question is probably essentially a myelin poison. The somewhat less certain, though definite, if not uniform, damage found in the motor nerve cells of the spinal cord might indicate one of two possibilities: Either the process is secondary to the axonal degeneration or, what seems perhaps a more likely interpretation, the action of the poison may also extend to the lipoids of the nerve cells, the relative involvement of the myelin sheath and nerve cell being merely a function of the distribution of the poison or its degradation products manifesting a special affinity for the lipins of the nervous system. In support of the first alternative we have the evidence of Ranson and others of retrograde degeneration of groups of anterior horn cells subsequent to section of peripheral nerve trunks. The second alternative, however, seems to be well sustained by the fact revealed in the present work, that the central damage appears to bear little, if any, relation to the peripheral lesions, to the clinical severity of the disease or to the duration of the paralysis, but rather seems to be a characteristic of the species. Thus, in the fowl, in which the disability appears most pronounced, damage of the nerve cells in the spinal cord was seldom

observed, while in the cat, on the other hand, with seeming less disability, the central lesions were well defined in most cases.

It may be recalled here also that the effects of the specific ester in the rabbit appear to be restricted chiefly to the spinal cord, the action being characterized by primary and lasting stimulation followed by depression, with final medullary paralysis. Dr. Smith had previously suggested a possible mode of action of this substance, postulating its accumulation and storage in the lipins of the central nervous system with perhaps subsequent hydrolytic cleavage of the ester in situ, possibly through the influence of certain enzymes. We have as yet no evidence for this, but such a mechanism which is not outside the limits of possibility would fit in with, and also account in some measure for, our present observations.

SUMMARY AND CONCLUSIONS

The histology of the nervous system in paralysis due to adulterated fluid extract of ginger in man has been studied and compared with the effects produced by triorthocresyl phosphate in suitable experimental animals.

The results indicate that the multiple neuritis of this paralysis is essentially a degeneration of the myelin sheaths of the peripheral nerves, with a variable amount of relatively moderate central degenerative changes affecting the anterior horn cells throughout the spinal cord, but more often in the lumbar and cervical regions.

Essentially similar lesions were observed in experimental animals in which partial paralysis was produced by means of triorthocresyl phosphate.
TRANSLATION:


At the beginning of 1930 in the USA thousands of persons developed a peculiar form of paralysis. Due to the epidemic-like occurrence of this condition, it was initially believed to be a new infectious disease. However, thorough anamnestic analysis later revealed that the cause was actually ingestion of certain brands of fluid extract of Jamaica ginger [1, 2], which was consumed in large quantities during Prohibition and, owing to tremendous demand, was adulterated with new, oil-like substitutes for the resinous, aromatic ginger extracts. This unintentional mass experiment, which included at least 20,000 cases according to an official estimate [3], went down in medical history as "ginger palsy" [4, 5].

Smith et al. [6–10] found about 2% tricresyl phosphate in bootleg liquors and, during a new wave of poisonings, about 0.5% tricresyl phosphate, whose paralytic effect had remained unknown, despite the already extensive industrial use of this substance.

Shortly thereafter, there were numerous European cases of "polyneuritis" following ingestion of certain apiol preparations, which were intended as abortifacients; tricresyl phosphate was soon detected as an impurity in these preparations [11–13].

This placed older reports of paralysis after ingestion of phosphoric creosote in a new light. When creosote is treated with phosphorus pentoxide, a portion of the ortho-cresol contained in it esterifies to form toxic com-
pounds. After the first reports of Chaumier [14] and Lorot [15], more than
100 cases of paralysis after ingestion of creosote phosphate had been reported
by 1934 [16–26].

The increasing use of tricresyl phosphate in industry was followed by
additional reports of poisoning.

Subcutaneous injections of tricresyl phosphate, which was mistaken for
paraffin oil, paralyzed several patients [27]. The externally oil-like liquid
also got into edible oils by accident or negligence and in some cases inten-
tionally, causing numerous group and mass poisonings [28–56], one of which
ended fatally [50]. The shortage of fat during the war and the postwar period
led many people who had access to the ester to use it as a frying and baking
oil; in this connection, mainly "torpedo oil" components played a role.
Despite appropriate warnings and official protective measures, the wave of
illness in Germany did not decline until the fat shortage subsided. However,
group illnesses in South Africa have recently been reported [57].

Plastic materials, especially polyvinyl chloride, containing tricresyl
phosphate as a softening additive, have been a special source of poisoning
since the last war.

Fats and fat solvents are able to dissolve the ester out of such plastic
materials relatively easily. Careless handling of articles of daily use
resulted in tricresyl phosphate coming into contact with foods, so that it was
repeatedly ingested and absorbed through the skin [58–64]. A considerable
number of illnesses [56, 65–75] occurred primarily during plastics production
and processing and proceeded with somewhat modified symptoms during chronic
exposure to the poison [69, 76]. Most cases of poisoning by plastics contain-
ing tricresyl phosphate were observed in East Germany.
In a systematic toxicological analysis of the effect of tricresyl phosphates of varied composition, we were able to uncover surprising relationships, which demand a revision of the conventional view of the toxicity of such compounds and at the same time answer some of the questions regarding the etiology and pathogenesis of this poisoning.

I. RELATIONSHIPS OF THE TOXICITY OF TRICRESYL PHOSPHATE TO THE CONTENT OF ortho-CRESOL

Tricresyl phosphate \([C_6H_4(CH_3)O]_3PO\) is formed by reaction of phosphorus oxychloride (POCl₃) with industrial cresol, which is a mixture of the 3 isomers ortho-, meta-, and para-cresol. The first investigators of the toxic effect of tricresyl phosphate [6–10] assumed that the three isomeric triesters of orthophosphoric acid are formed during the synthesis (Formulas I-III).

![Chemical Structure](image)

**tri-ortho-cresyl phosphate**

![Chemical Structure](image)

**tri-meta-cresyl phosphate**
Since symptomatology similar to that observed in cases of human poisoning could be observed in animal experiments only with the tri-ortho compound (I), researchers formed the opinion that the toxicity of an industrial tricresyl phosphate corresponds to its content of tri-ortho-cresyl phosphate, i.e., the toxicity parallels the proportion of ortho-cresol.

In reality, a mixture of ortho-, meta-, and para-cresol with phosphorus oxychloride yields not only the 3 isomers mentioned above (Formulas I–III), but also mixed esters of orthophosphoric acid with different cresyl radicals (cf. Formulas IV–X).

This possibility had already been recognized by Gross and Grosse [77]. Some of these mixed esters had already been described [78]. However, Gross and Grosse investigated only the pure, easily prepared triesters (I, II, III). In this study, they assumed that the different mixed esters would act similarly to the pure isomers, depending on their content of one component or the other, or would be between them with respect to their effect.

We will show, however, that in the case of tricresyl phosphate, the relationships between toxicity and the content of ortho-cresol are actually quite different.
(a) Methods

The methods generally used to determine the toxicity of substances cannot be directly applied to the testing of tricresyl phosphates. Different animal species react very differently to the administration of triaryl phosphates. Researchers have often failed to consider this fact.

To determine toxic effects, we must try to use those species in which the symptoms most closely resemble those observed in cases of human poisoning. Smith, Engel, and Stohlman [10], however, had discovered earlier that in the present case none of the usual laboratory animals is an ideal experimental subject from this standpoint.

Tricresyl phosphate produces a totally uncharacteristic clinical picture in rodents. Rats tolerate even high doses without any signs of illness [10, 80]. Mice regularly develop gastrointestinal symptoms, but they only occasionally develop mild paresis of the legs without the latency period typical in man [81]; rabbits show similar responses [10, 77, 99]. Therefore, experiments conducted with rabbits [82, 83] and mice [84] on the detection of ortho-tricresyl phosphate in plastics seem problematic in advance.

A clinical picture quite similar to that of human poisonings can be produced in cats and, incidentally, in tigers [85]. In particular, we observe the same sphincter insufficiencies that have been reported in recent clinical literature, symptoms which have gained considerable importance in the evaluation of our experiments.

Tricresyl phosphate produces similar symptoms of poisoning in chickens; in particular, in this species there is consistently a dose-dependent asymptomatic interval between ingestion of the poison and the onset of paralysis.

The nervous system of the chicken has been thoroughly studied in
connection with paralysis due to thiamine deficiency [86, 87]. The changes observed in these studies are almost identical with the damage produced by tri-ortho-cresyl phosphate [88-91]. Therefore, chickens have been regarded as the most suitable experimental animals in recent studies on paralytic effects of phosphoric acid esters [89, 91-93]. We can agree with this only with certain reservations. For example, triphenyl phosphate has no effect on chickens*, but it does paralyze cats, although the symptoms are somewhat different from those produced by tri-ortho-cresyl phosphate [10]; such observations are especially important in regard to our specific interests in this study, as we shall discuss at greater length later in the report. Furthermore, oral administration of tricresyl phosphate to chickens causes hardly any gastrointestinal symptoms, but such symptoms, although they do not occur regularly in man, can be anamnestically demonstrated in varied degrees of severity in the majority of cases.

We used chickens for large series of experiments after we had convinced ourselves, by testing the most important members of this class of substances in a small number of cats, that the dose-effect relationships are the same for both species of animals. In this connection, sufficient numbers of animals were used to allow satisfactory determination of the thresholds of effect.

In order to have homogeneous groups of animals at our disposal, we purchased groups of 60-110 young hens of equal age and weighing 600-800 g (purebred, partridge-colored Italians) from uniform broods at a poultry farm. At the beginning of the tests the animals were 12-14 weeks old. Individual sickly animals were culled. After the appearance of paralytic symptoms, the

*Hierholzer, Noetzel, and Schmidt [94] do not consider this fact; we will come back to the evaluation of triphenyl phosphate elsewhere.
affected animals were isolated. Some nonspecific deaths due to a form of can- 
nibalism of stronger animals against weaker and injured animals could not be 
prevented.

The liquid ester preparations were mixed with 1 part by volume of olive 
oil (with 3 parts by volume of olive oil for very small quantities to ensure 
precise dosing). The liquid preparations were administered in 0.5 and 0.75 
cm³ gelatin capsules. The capsules were administered by insertion in the 
craw. High doses (more than 4 capsules) were given on two successive days in 
most cases and on three successive days in rare cases. The dosage was varied 
in an approximately geometric progression, with a few exceptions designed to 
allow more exact determination of the threshold dose. After the threshold of 
effect had been approximately determined in preliminary tests, groups of two 
or occasionally three chickens were used for testing each dosage level. Sev-
eral animals in each experiment remained untreated and were used as a control 
group, since spontaneous epidemic paralysis is occasionally observed in chick-
ens.

Since tricresyl phosphate is unreliably and incompletely absorbed from 
the digestive tract of cats [10], these animals received 2 depot injections of 
the same mixtures in the thigh musculature.

Each day each chicken was individually observed standing and running; the 
observer recorded his observations without knowledge of the previous treatment 
of the animals. The degree of paralysis was rated by the scale shown in Fig-
ure 1.

Mild degrees of weakness are not immediately apparent in cats. We 
allowed them to run along a long corridor with smooth floor tiles; when the 
cats' claws cannot get a firm hold, even slight unsteadiness becomes apparent
Figure 1. Toxicity testing of different tricresyl phosphates in chickens and cats, experimental plan, and results. -- A, B, C, D, E: one closed group. One animal per box, shading according to the severity of the symptoms:
- mild, but definite unsteadiness while walking;
- weakness of the legs;
- severe weakness;
- complete paralysis; f death due to respiratory paralysis following progressive paralysis; f death due to nonspecific cause. KEY: (a) chickens; (b) cats; (c) prep. no.; (d) o-cresol; (e) TOPK = tri-o-cresyl phosphate; and (f) number of animals per dose.

after the animals have been forced to run continuously.

The extensive testing program required several separate groups of tests. Since the sensitivity of the individual groups of animals to tricresyl phosphate varied somewhat (season? early feeding?), within a homogeneous shipment of animals we always ran a series with an ester preparation whose strength of effect, compared to a standard preparation (tri-ortho-cresyl phosphate), was known from an earlier test run. Therefore, in the overall evaluation of the tests, the absolute threshold doses found in the individual series of tests cannot be directly related to one another, but the toxicity of all
preparations can be expressed in relative numbers if the toxicity of the most strongly effective sample is set at unity.

(b) **Strength of Effect of Industrial Tricresyl Phosphates of Old and Recent Production**

To compare the toxicity of industrial tricresyl phosphates with that of pure tri-ortho-cresyl phosphate, we wished to use mainly preparations of the type probably responsible for the cases of poisoning reported in the clinical literature.

In Germany mainly a mixture of aromatic compounds has been used to produce industrial tricresyl phosphate. Such mixtures consisted mainly of varying proportions of the three isomeric cresols commercially available as crude cresol DAB 4 M or tricresol (DAB = German Pharmacopeia); the content of ortho-cresol usually varies between 25 and 40%, depending on origin and processing. Cresol homologues (phenol, dimethylphenols, ethylphenols, etc.) are also present in small percentages. Tricresyl phosphates from cresol mixtures of this type should have about one-third the toxicity of the tri-ortho ester on the basis of the conventional view mentioned above. According to the same literature view, the toxicity of modern preparations with only 3% o-cresol in the aromatic component should amount to no more than 3% of the toxicity of the tri-ortho compound.

The preparations* that were used and the corresponding analytical data are compiled in Table 1.

---

*We would like to thank Farbwerke Hoechst AG for supplying the samples and performing the analyses.
TABLE 1. KEY: (a) preparation; (b) contents in the aromatic component*; (c) -cresol; (d) tri-ortho-cresyl phosphate, pure; (e) tricresyl phosphate, industrial product from crude cresol DAB 4 M; (f) the same; and (g) tricresyl phosphate, modern commercial product.

<table>
<thead>
<tr>
<th>Nr.</th>
<th>Präparat (a)</th>
<th>(d)</th>
<th>α-cresol</th>
<th>m-cresol</th>
<th>p-cresol</th>
<th>Phenol</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Triorthokresylphosphat, rein</td>
<td>99**</td>
<td>0</td>
<td>0.2</td>
<td>0.5</td>
<td>1.7</td>
</tr>
<tr>
<td>II</td>
<td>Trikresylphosphat, technisches Produkt aus Roh(e) kresol DAB 4 M</td>
<td>26.7</td>
<td>34.9</td>
<td>13.8</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>dessel(f)</td>
<td>22.7</td>
<td>37.6</td>
<td>17.3</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>dessel(f)</td>
<td>23.3</td>
<td>30.7</td>
<td>13.2</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>Trikresylphosphat, (g) modern Handelsprodukt</td>
<td>3.3</td>
<td>33.3</td>
<td>20.2</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>dessel (f)</td>
<td>2.5</td>
<td>40.4</td>
<td>23.3</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>VII</td>
<td>dessel (f)</td>
<td>2.3</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td></td>
</tr>
</tbody>
</table>

*After total saponification of the ester preparation, chromatographically determined [95] as % triaryl phosphate, mol. wt. 368 (tricresyl phosphate) and mol. wt. 326 (triphenyl phosphate).

**Calculated by deduction of the proportions of phenol and m- and p-cresol components determined by infrared spectroscopy.

***Not determined.

The results of the tests on 156 chickens and 21 cats are compiled in Figure 1.

In some test series on chickens there is a considerable spread; this was to be expected for a poison with a protracted effect and here remains within limits which do not significantly affect the evaluation of thresholds of effect. The severity of paralysis is approximately parallel to the dosage within a test series; the onset of the first symptoms varied between the 8th and the 15th days after administration of the poison; in chickens the onset of symptoms depended approximately on the amount of poison administered, and in cats there was an exact dependence in this respect.

Surprisingly, tri-ortho-cresyl phosphate (prep. no. I) in experiment A
has only about 10% of the effect of an industrial product (prep. no. II) with 26.7% ortho-cresol. Due to the critical importance of this finding, the comparative determination of the toxicity of these two samples was repeated in experiment B with the same result; this relation was reconfirmed in a later test series' (cf. Figure 3). Other industrial ester mixtures with similar contents of ortho-cresol, such as prep. no. II, have approximately the same effect (experiment C); the sample with the lowest content of ortho-cresol (prep. no. III) thus has a somewhat diminished effect; the animals in this test series reacted much less sensitively to this preparation than to the others.

The tricresyl phosphates with ortho-cresol values around 3% (prep. nos. V, VI, VII) have equal but much weaker effects (experiment E); the toxicity of the tri-ortho compound (experiment D) is about 3–5 times higher.

The results in cats are wholly consistent with the results in chickens.

Very surprisingly, industrial tricresyl phosphates from crude cresol DAB 4 M with an ortho-cresol content of approximately 30%, such as were mainly used in industry a few years ago, are thus about 10 times more toxic than pure tri-ortho-cresyl phosphate. However, preparations of this type are very probably responsible for the cases of poisoning observed in Germany during the war and in the postwar period.

The toxicity of modern commercial preparations with a greatly reduced ortho-cresol content (about 3%) is, contrary to expectations, still about one third the toxicity of tri-ortho-cresyl phosphate. On the other hand, compared to the highly toxic products with about 30% ortho-cresol, its effect is only about 1/30 as great.

As we have already mentioned, our experimental setup allowed us to
express the toxicity of all preparations as a percent of the effect of the most strongly effective sample. The relationships, which we have already briefly reported [96], are summarized in Figure 2.

Contrary to accepted belief, there is no correlation between the content of ortho-cresol and the strength of the effect. Instead, starting from tri-ortho-cresyl phosphate, the toxicity increases with decreasing content of ortho-cresol. 

**Figure 2.** Relative toxicity of tricresyl phosphates with varying proportions of ortho-cresol. KEY: (a) TOCP.

**Figure 3.** Toxicity testing of isomeric tricresyl phosphates in uniform batches in chickens; mono-ortho esters (o-m-p, o-m-m, o-p-p); di-ortho esters (o-o-m, o-o-p); tri-ortho ester (o-o-o). An industrial tricresyl phosphate with 26.7% o-cresol (prep. no. II, cf. Table 1) is shown for comparison. Symbols as in Figure 1. KEY: (a) industrial tricresyl phosphate; and (b) 26.7% o-cresol.
ortho-cresol, reaches a maximum at intermediate values, and falls below the toxicity of the pure tri-ortho compound only at very low concentrations; tricresyl phosphates free of ortho-cresol are most likely nontoxic.

(c) **Strength of Effect of Isomeric Mixed Tricresyl Phosphates**

To clarify the relationships between toxicity and configuration of tricresyl phosphate mixed esters, we tested (together with Bayer [97]) all isomeric tricresyl phosphoric acids (cf. Formulas I–III and IV–X) in chickens by the same experimental setup.

![Chemical structures](image)

**KEY:** TKP = TCP.

All of compounds IV–X are oily liquids. They were systematically
TABLE 2. KEY: (a) formula; (b) name; (c) contents in the aromatic component*; (d) o-cresol; (e) Kp = b.p.; and (f) TCP.

<table>
<thead>
<tr>
<th>Formula</th>
<th>Bezeichnung</th>
<th>Gehalte im Aromatenanteil*</th>
<th>E₃₃</th>
<th>E₃₃</th>
<th>E₃₃</th>
<th>Phenol</th>
<th>Kp 3.10⁻¹***</th>
</tr>
</thead>
<tbody>
<tr>
<td>(f)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I o-o-o-TKP</td>
<td>93</td>
<td>0.2</td>
<td>0.5</td>
<td>1.7</td>
<td>0.3</td>
<td>196</td>
<td>-196.5</td>
</tr>
<tr>
<td>IV o-o-m-TKP</td>
<td>81</td>
<td>33.3</td>
<td>0.5</td>
<td>2.5</td>
<td>0.5</td>
<td>198.5</td>
<td>-199</td>
</tr>
<tr>
<td>V o-o-p-TKP</td>
<td>83</td>
<td>0.3</td>
<td>0.3</td>
<td>4.0</td>
<td>0.8</td>
<td>204</td>
<td>-204.5</td>
</tr>
<tr>
<td>VI o-m-m-TKP</td>
<td>31.8</td>
<td>0.3</td>
<td>0.2</td>
<td>1.2</td>
<td>0.5</td>
<td>209.5</td>
<td>-210</td>
</tr>
<tr>
<td>VII o-p-p-TKP</td>
<td>30.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>209.5</td>
<td>-210</td>
</tr>
<tr>
<td>VIII o-m-p-TKP</td>
<td>29.0</td>
<td>32.0</td>
<td>0.2</td>
<td>1.0</td>
<td>0.5</td>
<td>209.5</td>
<td>-210</td>
</tr>
<tr>
<td>IX m-m-p-TKP</td>
<td>0.2</td>
<td>64.0</td>
<td>0.2</td>
<td>0.5</td>
<td>0.5</td>
<td>211</td>
<td>-211.5</td>
</tr>
<tr>
<td>X m-p-p-TKP</td>
<td>0.2</td>
<td>30.0</td>
<td>64.0</td>
<td>2.0</td>
<td>2.0</td>
<td>214</td>
<td>-215</td>
</tr>
</tbody>
</table>

*Calculated as % tricresyl phosphate (mol. wt. 368) or triphenyl phosphate (mol. wt. 326) after chromatographic determination of the saponified cresols [95].

**Calculated by deduction of the phenol and m- and p-cresol components (dimethylphenols were not present) determined by infrared spectrophotometry.

***Determined in special glass apparatus with a short distillation path. Pressure measurement with thermoelectric ionization manometer. The boiling point of o-o-o-TCP agrees exactly with the value determined from a graph of Burrows [98].

synthesized via the monocresyl dichlorides and dicresyl monochlorides*. The analytical data and boiling points are given in Table 2.

All preparations were administered in a closed test series to a uniform group of animals; for comparison, a highly toxic ester preparation with an ortho-cresol content of about 30% was also tested (prep. no. II, cf. Table 1).

Figure 3 shows the results in 82 chickens; only mixed esters with orthocresyl radicals have a paralytic effect. The o-m-m-, o-p-p-, and o-m-p-

*Farbwerke Hoechst AG also produced and analyzed all of these mixed esters; we would like to thank Hoechst for its generous accommodation of all of our requests for preparations.
phosphates (hereafter called mono-ortho esters) are the most strongly active compounds. There are small differences in effect in the following order \( o\-m\-p \) > \( o\-m\-m \) > \( o\-p\-p \)-cresyl phosphate. The average toxicity of all 3 esters is equivalent to that of an industrial ester mixture with an ortho-cresol content of 26.7%. The isomers with two ortho-cresyl groups (di-ortho esters) have a significantly lower toxicity. The \( o\-o\-p \) compound is more active than \( o\-o\-m \)-TCP; on the average, both are about half as strong as the mono-ortho esters. The toxicity of the tri-ortho compound is calculated to be only 1/10 the toxicity of the strongest mono-ortho esters. A comparison of tri-ortho-cresyl phosphate with the industrial ester preparation (ortho-cresol content 26.7%) confirms the 1:10 effect ratio determined in the first series of tests (see above). The mixed esters without ortho-cresyl radicals (\( m\-m\-p \) and \( m\-p\-p \)-cresyl phosphate) are tolerated without symptoms in doses of 2.5 cm\(^3\)/kg.

The \( o\-o\-o \), \( o\-o\-p \), and \( o\-p\-p \)-esters were recently studied by Hine et al. [92] for their paralytic effect without exactly determining the thresholds of effect; they did not seem to differ significantly. This apparent contradiction of our results may possibly be explained by the fact that the authors did not use uniform animal material. In addition, the two tested mixed esters \( o\-o\-p \)-TCP and \( o\-p\-p \)-TCP may not have been prepared by systematic synthesis, as was the case in our study, but rather from corresponding cresol mixtures; unfortunately, the report contains no information on this question and no analytical data.

Smith et al. [10] were able to show that the specific paralytic effect is due neither to the orthophosphoric acid (or phosphorous acid) nor to the
ortho-cresol alone; therefore, only the molecular group \( p-o-C_6H_4 \cdot CH_3 \) could be the active principle.

To a certain extent, our findings conflict with this theory, according to which the group \( p-o-C_6H_4 \cdot CH_3 \) would be the only determining factor; for in this case the strength of the effect should increase in the following order:

\[
\text{mono-ortho ester < di-ortho ester < tri-ortho ester}
\]

with increasing occurrence of the group.

However, knowledge of the strengths of effect of all isomeric tricresyl phosphates offers the possibility of clarifying the causes of the surprising differences in toxicity among industrial ester preparations with different contents of ortho-cresol.

(d) \textit{ortho-Cresol Component and Toxicity}

In the esterification of orthophosphoric acid with mixtures of the three isomeric cresols, ten isomeric tricresyl phosphates can form (Formulas I–X). Since only the esters with ortho-cresyl groups cause paralysis, and the degree of their paralytic effect depends on the number of \( o \)-cresyl groups in the molecule, we are interested in knowing the given proportions of these toxic isomers in an ester mixture with a known content of bound ortho-cresol. Calculation of these proportions is possible if the isomeric cresols are equivalent with respect to those of their chemical properties which are of interest here (ester equilibria), i.e., they do not affect one another during esterification.

If two substances: \( X \) (orthophosphoric acid) and \( Y \) (cresol) react with one another in such a way that one unit of \( X \) accepts three units of \( Y \), and a
portion of $Y$ ($Y' = \text{ortho-cresol}$) is especially distinguished, compounds of the following types can form:

$$XY_n; X'Y_n; X'Y_n'; X'Y$$

If $n$ = the number of occupied sites of $X$ and $k$ = the number of sites occupied by $Y'$, the following general formula applies to the compounds that are formed:

$$XY_{n-k}Y'^k$$  \hspace{1cm} (1)

Furthermore, if $q$ is the proportion of $Y'$ (o-cresol) in $Y$ ($q = Y'/Y$), and if a fully statistical distribution is assumed, the probability of the formation of such compounds is given by the following general formula:

$$w = \binom{n}{k} q^k (1-q)^{n-k}$$  \hspace{1cm} (2)

For mono-ortho esters ($X Y_2 Y'$):

$$w_1 = 3q - 6q^2 + 3q^3$$  \hspace{1cm} (3)

For di-ortho esters ($X Y Y'_2$):

$$w_2 = 3q^2 - 3q^3$$  \hspace{1cm} (4)

For the tri-ortho ester ($X Y'_3$):

$$w_3 = q^3$$  \hspace{1cm} (5)

Distribution curves for variable o-cresol contents were calculated from the probability functions (3), (4), and (5) and are plotted in Figure 4. Mono-ortho compounds predominate at low ortho-cresol contents, di-ortho compounds predominate in medium and higher ranges, and finally the tri-ortho ester, which, by definition, is present to the extent of 100% in the starting mixture ($q = 1$) in the case of pure o-cresol, predominates in the highest range.

Since $w = f(q)$, the maxima of the distribution curves, i.e., those percentages of o-cresol at which maximum proportions of the given compounds are...
present, are determined by

$$\frac{\tau^*}{\eta} = \eta^* = \eta.$$

![Graph showing statistical distribution of o-cresyl-containing esters and hypothetical toxicity of the total preparation.](image)

Figure 4. Statistical distribution of the o-cresyl-containing esters and hypothetical toxicity of the total preparation. — — mono-ortho esters; — — — di-ortho esters; — — — tri-ortho ester; — — hypothetical total toxicity. KEY: (a) % o-cresol.

Accordingly, the proportion of di-ortho esters is highest at 66 2/3\% o-cresol in the total cresol, and the proportion of mono-ortho esters is highest at 33 1/3\% o-cresol in the total cresol; however, this is the range in which the ortho contents of the highly toxic industrial products fall, which are the basis of most of the cases of human poisoning reported in the German literature during the war years and the postwar period. We will demonstrate this significant fact later.

The distribution curves determined for phosphates containing ortho-cresyl radicals can be used to make purely computational determinations of the toxicity to be expected for tricresyl phosphates with varying ortho-cresol contents.
Assuming that all of the compounds present in the ester mixture act additively, the relationship between total toxicity and ortho-cresol content is obtained by superposition of the 3 probability functions. In this connection, the toxicity of the mono-ortho esters is regarded as 100\%, that of the di-ortho esters as 50\%, and that of the tri-ortho ester as 10\%. With the aid of Equations (3), (4), and (5), we thus obtain the total toxicity:

\[
T = 3q^2 - 4q^3 + 3q^4 \div \frac{3}{2} q^2 - \frac{3}{2} q^2 \div \frac{1}{10} q^2
= \frac{16}{10} q^2 - \frac{9}{2} q^2 \div 3q
\]  

(6)

The toxicity curve calculated from Equation (6) is drawn in Figure 5 over the distribution curves. Its maximum is calculated \(dT/dq = 0\) to be 43.4\% o-cresol in the total cresol. This greatest achievable toxicity would thus be (cf. Figure 4) about 6 times greater than that of the tri-ortho ester. In fact, we found industrial ester mixtures with about 30\% o-cresol 10 times stronger than the tri-ortho compound. This is probably explained primarily by the fact that in the esterification of cresol with phosphorus oxychloride, the ortho isomer is less lightly bound than meta-cresol and para-cresol due to steric hindrance; suitable analyses of the starting cresols and phosphates confirm this. The formation of tri-ortho and di-ortho esters is thus suppressed in favor of the mono-ortho esters. Furthermore, there is the possibility that cresol homologues (phenol, dimethylphenols, ethylphenols, etc.), which occur in small proportions in industrial cresols, sometimes form mixed esters, which are even more toxic than the mono-o-di-(m,p)-cresyl esters. In both cases the result is an increase in toxicity mainly in the intermediate and low ortho-cresol ranges and a shift of the toxicity peak towards lower values (near 30\% o-cresol), so that the curve shown in Figure 5 (broken curve)
Figure 5. Toxicity of tricresyl phosphate mixtures with varied proportions of ortho-cresol. -- actual toxicity curve; -●-●- hypothetical curve for a purely statistical distribution of the mixed esters containing o-cresyl radicals (cf. Figure 4); —— hypothetical toxicity according to the accepted view (proportional to the content of ortho-cresol). KEY: (a) % o-cresol.

can be estimated; in this connection, the values over 100, 25–30, and over 3% o-cresol are confirmed.

At these low ortho contents, the actual toxicity falls below the hypothetical values. Detoxification mechanisms probably play a greater role in relatively less toxic mixtures of this type, which must be administered in high doses in the determination of the thresholds of effect.

The results of our animal experiments make it necessary to revise the previous evaluation of clinical cases of poisoning. Previous calculations of the toxic human dose were based on the amount of ortho-cresol contained in a preparation and related this amount to tri-ortho-cresyl phosphate, in the belief that the bound proportions of meta-cresol and para-cresol have no effect on the toxicity of the total preparation. However, since the meta and para isomers that are present cause the formation of mono-ortho and di-ortho
esters, and since, as we were able to show, the toxicity of these mixed esters is much greater than that of tri-ortho-cresyl phosphate, the old method of calculation, which only recently was again used to obtain an incorrect value for the toxic limiting dose [94], is invalid. In an investigation of a mass poisoning, Staehelin [30] calculated a minimal toxic dose of 0.12–0.15 g of tri-ortho-cresyl phosphate in adults. However, as he explicitly states (cf. also Iselin [100]) the substance actually tested was 0.5 g of industrial tricresyl phosphate with about 30–40% o-cresol in the total cresol; accordingly, 0.5 g of such a preparation must be regarded as the toxic limiting dose.

A comparison with the thresholds of effect determined in our animal experiments (cf. Figure 1) shows that in chickens and cats about 12 mg/kg is the smallest dose of an industrial tricresyl phosphate with about 30% ortho-cresol in the aromatic component that was still able to produce paralysis or weakness of the rear extremities. The threshold doses for man and animals are thus very similar. We will return to this important coincidence in the evaluation of modern tricresyl phosphates [154].

Nevertheless, in possible future poisonings by tricresyl phosphate, chemical analysis of the underlying ester mixtures should definitely be performed. However, it is no longer permissible to relate an analyzed proportion of ortho-cresol to tri-ortho-cresyl phosphate. In evaluating the amount of poison absorbed by an individual, it is necessary, rather, to determine the dose of total tricresyl phosphate and, at the same time, its content of ortho-cresol. When the relationships between toxicity and ortho-cresol content determined in our animal experiments are taken into consideration, it is then possible to draw conclusions about dose-effect relationships in man.
In the course of our experiments it was observed that pure tri-ortho-cresyl phosphate produces only flaccid paralysis in chickens and cats. On the other hand, industrial mixed preparations, whose cresol component contains only 30% o-cresol, and isomers of the mixed ester type produce chiefly spastic paresis; in addition, cats exhibited marked sphincter paralysis. It was to be assumed that the two types of tricresyl phosphate affect the nervous system in different ways. Therefore, we removed the brains, spinal cords, and portions of peripheral nerves from some animals to investigate the question of different types of effect by histological techniques.

Chickens which died of respiratory paralysis were prepared as quickly as possible after death; otherwise, the animals were anesthetized with Pernocton, and then the brain and spinal cord were removed dorsally. The organs were fixed in 5% Formalin solution. The preparations were embedded in paraffin (section thickness 10 µm). The nerve cells were stained with gallo cyanine dye after Einarson; the axis cylinders were stained by the silver impregnation method in the modification of Glees and Marsland [101]. Myelin sheaths with fatty degeneration were made visible by Marchi's osmic acid method.

The histological nervous system damage caused in chickens by tri-ortho-cresyl phosphate was first studied by Smith and Lillie [9] and then by Brouwer [102]. Recent systematic studies were published by Barnes and Denz [89] and by Cavanagh [90]. Myelin sheaths and corresponding axis cylinders of the peripheral nerves are the most severely affected; the damage is greatest in the distal segments and becomes progressively less intense in the proximal direction. Axis cylinders and myelin sheaths simultaneously undergo changes in the
sense of wallerian degeneration [90, 91]. After high doses, three nerve pathways are affected: (1) the analogue of Goll’s fiber in mammals, (2) a laterally situated tract corresponding to the spinocerebellar tract, and (3) the ventral tract; it very probably corresponds to the pyramidal tract in man, but it does not arise in the cortex but rather probably in a control site in the midbrain [90]. Primarily the long fibers with a thick myelin sheath are affected; the lesions are concentrated in the cervical and lumbar intumescentiae. The degenerated fibers of the spinocerebellar tract can be followed all the way into the cerebellum; isolated damaged fibers can also be encountered in the midbrain and in the vestibulocochlear system. The motor cells in the spinal cord are not regularly changed, but when they are, the changes are mild. Moderate chromatolysis becomes apparent only after several weeks and does not differ from the chromatolysis observed after neurotomy. Fat granules in the cytoplasm are occasionally found [9, 90].

With respect to the type and localization of the nervous system lesions, our observations agree with the findings of the earlier researchers cited above. In most of the severely affected animals we also found more or less strongly pronounced signs of damage in many palisade cells in the cerebellum with shrinkage of central apparatus and Golgi apparatus, darkening [?; German Verdämmung -- Tr. Ed.] of the cell body, and neuronophagia; a finding which has never been described before but which is not surprising, since, of course, the fibers of the spinocerebellar tract are damaged, and since, especially in birds, the cerebellum is exceedingly important to motor coordination.

However, clear differences were observed in the distribution of the damage for pure tri-ortho-cresyl phosphate, on the one hand, and for the highly toxic industrial tricresyl phosphates with about 30% ortho content, on the
TABLE 3. DISTRIBUTION OF THE DAMAGE IN THE NERVOUS SYSTEM OF CHICKENS AFTER POISONING WITH DIFFERENT TRICRESYL PHOSPHATES. KEY: (a) animal no.; (b) preparation; (c) dose, cm³/kg; (d) interval, days; (e) duration of paralysis, days; (f) cervical cord; (g) lumbar cord; (h) TOCP; (i) industrial TCP, 26.7% o-cresol; (j) the same; and (k) o-m-p-TCP.

<table>
<thead>
<tr>
<th>No.</th>
<th>Preparat (b)</th>
<th>([C] [K] [L])</th>
<th>Perc.</th>
<th>Lumbar (f)</th>
<th>Lumbar (g)</th>
<th>Nerves ischial.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>T0KP (h)</td>
<td>0.4</td>
<td>11</td>
<td>17</td>
<td>(-)</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>T0KP</td>
<td>0.8</td>
<td>6</td>
<td>10</td>
<td>(-)</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>T0KP (i)</td>
<td>0.3</td>
<td>9</td>
<td>12</td>
<td>(-)</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>(techn. T0K)</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>5</td>
<td>o-cresol</td>
<td>0.2</td>
<td>10</td>
<td>13</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>o-m-p-T0KP</td>
<td>0.03</td>
<td>6</td>
<td>27</td>
<td>-</td>
<td>(-)</td>
</tr>
<tr>
<td>7</td>
<td>o-m-p-T0K</td>
<td>0.2</td>
<td>6</td>
<td>27</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>o-o-m-T0K</td>
<td>0.1</td>
<td>11</td>
<td>21</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>o-o-m-T0K</td>
<td>0.4</td>
<td>6</td>
<td>27</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

On the other hand, the quantitative evaluation (Table 3) showed mainly involvement of the peripheral nerves and relatively minor damage to the central tracts in animals paralyzed by tri-ortho-cresyl phosphate. On the other hand, chickens that had been poisoned by industrial tricresyl phosphates with an o-cresol content of about 30% only occasionally showed damaged fibers in the peripheral nerves but had severe degeneration of the spinal cord. This degeneration also shows clear qualitative differences from the degeneration observed in the case of tri-ortho-cresyl phosphate. Whereas in poisoning by the tri-ortho ester the degenerated fibers are limited to the three tracts mentioned above and to some extent can be recognized as belonging to definite systems, in animals poisoned by industrial tricresyl phosphates the affected fibers appear to be diffusely dispersed through the entire white matter, and assignment to specific tracts is not possible. The type and distribution of the damage caused by mixed esters (animal nos. 6–9, Table 3) correspond to the type and
distribution of the damage produced by industrial tricresyl phosphate mix-
tures.

We presently know as little about the causes of the different effects of the different tricresyl phosphates as about the fine mechanism of action itself. The question of the substrate of the tricresyl phosphate effect seemed at least partially answered when Bloch [103] discovered the inhibition of serum cholinesterase by tri-ortho-cresyl phosphate, and Earl and Thompson [103a] found that the activity of pseudocholinesterase in the nerve sheaths is practically eliminated after poisoning with this substance. The hypothesis based on these findings was quickly shaken by Mendel and Myers [80], who reported that rats are not paralyzed by tri-ortho-cresyl phosphate, despite complete inhibition of the pseudocholinesterase of the brain; the supposed relationship between pseudocholinesterase and paralytic effect was completely refuted by Barnes and Denz [89], who were able to show that only a large number of phosphoric acid esters that act on cholinesterases, only three produce the typical protracted paralysis (tri-ortho-cresyl phosphate, diisopropyl fluorophosphate, and bis(isopropylamino)fluorophosphine oxide). Our studies on relationships between structure and paralytic effect tend to show (especially in regard to the qualitative differences in the pattern of effects) that there are still great difficulties involved with solving the problem of the mecha-
nism of paralysis by this class of substances.

II. EPICRITICAL CONSIDERATION OF TRICRESYL PHOSPHATE POISONING ON THE BASIS OF NEW TOXICOLOGICAL KNOWLEDGE

Our understanding of the nature of tricresyl phosphate poisoning has changed during the last 10 years or so. Whereas purely peripheral paralysis
was reported in connection with the American "ginger" poisonings and the "apiol" poisonings, recent German papers emphasize chiefly involvement of the central neuron. We have critically examined the clinical literature, because our new knowledge about the toxicity of tricresyl phosphates (see Section I) promised clarification of various disputed questions.

There is considerable agreement about the symptoms in the first stage of the poisoning; in this connection, we can refer to earlier review papers [44, 58, 71, 135]. However, the descriptions from the different poisoning epidemics differ greatly with respect to several very important symptoms and especially with respect to the subsequent course of the disease.

(a) Differences in the Clinical Picture

The signs of reduced perfusion in the affected extremities, i.e., cyanosis, lowered skin temperature, chills, cold sweats, and paresthesia, are specially emphasized in recent German papers on "torpedo oil" poisoning and "Igelit" poisoning [30, 33, 38, 44, 58, 71].

Parnitzke [58] speaks of "massive, dripping-wet sweating of the feet". According to Scheid [44], trophic disturbances are among the "most constant and . . . intractable symptoms . . ."; these symptoms were regularly present in the cases reported by Staehelin [30]. On the other hand, vasomotor symptoms of this type are rarely mentioned by American authors, despite the much larger number of observed cases [104]; mild trophic disturbances are mentioned in review works [4, 5]. In the reports on "apiol" poisoning, we found only one mention of such symptoms, namely, by Geithner [105], who noted a feeling of coldness in the legs in one case; Kastan [106] found the appearance and condition of the skin to be completely normal.
In recent years bladder disturbances have been observed with remarkable frequency in poisonings with tricresyl phosphates of the "torpedo oil" type.

Scheid [44] describes bladder problems in one-third of his approximately 100 cases, and Parnitzke [58] describes them in 10% of 125 cases; in severe cases these problems can persist for years [44]. 24 of the 80 patients described by Staehelin [30] suffered from incontinence. The cases described by Mertens [71] included one case of cystoproctoparalysis which persisted for 4 weeks. On the other hand, most authors do not mention these kinds of symptoms in connection with "ginger paralysis". In a critical review Weber [3] speaks of "little or no involvement" of the sphincters. Kiely and Rich [4] found disturbances in only 16 of 201 cases. Kidd and Langworthy [5] point out in their review paper that they observed no cases of urinary or fecal incontinence. The apiol literature contains no mention of disturbances of the bladder or rectum.

The conspicuous differences between these various types of poisoning led to a search for changes in the cerebrospinal fluid.

No abnormalities were found in the "apiol" cases published up to 1934 (that is, in those cases in which the cerebrospinal fluid was tested) [135]; all 11 cases of Ter Braak and Carillo [108] had normal cerebrospinal fluid findings. Large-scale studies were conducted in the American poisoning cases: Of the 120 lumbar punctures that were performed, only two showed a positive globulin reaction [4, 109]; some reports mentioned slightly elevated globulin and cell increases (up to 11 per mm³), but in most cases the fluid was normal [5]. The recently reported findings of German authors are quite different: Mertens [71] found nonspecific elevation of protein in six of eight tested cases; Scheid [44] obtained a slightly positive Pandy reaction in one-third of

27 400901
his more than 100 patients, who all had normal cell values; Parnitzke [58] found mild, uncharacteristic protein elevation in 42% of 125 patients. The changes persist for a long period of time and in some cases are still observed after 2 years [71].

It is especially important for us to consider the further course of the illness.

The flaccid paralysis subsides in a few months in mild cases. In severe cases it largely subsides in 1–2 years, although it may persist even longer in rare cases. Remission of symptoms occurs in the exact opposite order of development.

Mild poisonings by "torpedo oil", "Igelit", machine gun oil, and other industrial tricresyl phosphates produced during the war and in the postwar period in Germany usually ended with complete return of functional normalcy. In the vast majority of severe cases of this group, disappearance of the flaccid paralysis was accompanied or immediately followed by the development of spastic paresis, first in the proximal sections of the extremities and later in the calves and forearms. The knee reflexes, which were often diminished and in some cases completely absent at the height of the paralytic stage, showed an increase at this stage and in pronounced cases caused adductor spasms; ankle clonus could also be provoked in many cases. Spastic scissor gait was observed. An increased patellar tendon reflex and often an increased achilles tendon reflex gave the impression of being permanent symptoms after many years, even in mild cases [44].

Spastic paresis, which is prognostically less favorable than flaccid paralysis, indicates that the central neuron is also involved.
The actual pyramidal signs may be absent. The spinal damage must already occur in the early stage parallel with the involvement of the peripheral motor fibers, since no progression of the spasticity was observed over a period of several years, which would be expected in secondary degenerative processes in the central organ. In fact, fascicular twitching and fibrillatory contractions were observed very early in such cases, i.e., after a few weeks, even in non-paralyzed muscle groups (Scheid [44]). Walthard [110] also reports on, in his opinion, "pseudospastic" symptoms during the first weeks after poisoning. The damage of the central organ is supposedly masked by the motor deficiencies in the peripheral nerves; only after subsidence of the flaccid paralyses, which show a relatively good remission tendency, would spastic signs then become manifest (Scheid [44], Mertens [71], Parnitzke [58]).

This "symptom transformation" (Parnitzke) has already been observed in American follow-up tests of some cases of "ginger paralysis" [3, 107, 109]. Of course, it is overlooked that only the most severe cases, which remained in nursing homes, could be determined here.

The follow-up investigators themselves concede that there could be the impression that only a minority of the victims from 1930/1931 may have been afflicted with such severe residual symptoms; unfortunately, however, there are no exact numerical data, which are undoubtedly difficult to obtain during follow-up studies on lower socioeconomic groups. However, aside from the differences already mentioned above, there is sufficient evidence to suggest the probability of an altered course of the "ginger paralysis" compared to the "torpedo oil" poisonings. The early reports of the American authors extend to an observation period of up to 1½ years in some cases. Nevertheless, signs of involvement of the central neuron were seen only in very isolated cases [111].
Aring [107] gives a detailed report of a case of poisoning with signs of central involvement, which did not occur until 7 years after the great poisoning epidemic by ginger brandy; to be sure, the patient had ingested a very large quantity. If we use the information given by Smith et al. [7, 8], according to whom the ester content of the brandies was 2%, then the 8 U.S. fluid ounces consumed by the man contained 6 g of tricresyl phosphate.

Increased knee reflexes were only occasionally observed in the American cases [107]. In their review paper, Kidd and Langworthy reported no spastic symptoms after observing the patients over a period of 18 months. In his comprehensive study, Weber [3], who was able to perform follow-up examinations of 35 severely ill patients 6 years after the epidemic of poisonings, characterizes the symptoms of the first years as "predominantly [those] of a flaccid paralysis"; examination of his case histories, all of which involve very severe cases, shows that the change from flaccid paralysis to spastic symptoms in these cases cannot have been completed until after several years; some of these patients were bedridden for up to 2 years.

Parnitzke [58], on the other hand, reports that in poisonings by tricresyl phosphates of the "torpedo oil" type, the flaccid paralysis generally disappeared after about 9-10 months. Scheid [44] reports tension and tightening in the thigh muscles and spontaneous clonus only a few weeks or months after the poisoning.

Finally, the reports on "apiol" paralysis [105, 106, 108, 112-134] give hardly any indication of spastic symptoms. One exception is a case reported by Wuite [125], in which ankle clonus interpreted as medullary damage was present after 2 years; some of his contemporaries [135] disputed an etiological connection. The flaccid paralysis was completely cured after 1-14 years in
many cases; even in severe cases the motor functions were later well restored without spastic residual symptoms being superimposed, as a pertinent case reported by Jagdhold [135] shows especially clearly. Follow-up studies after 3 years [136] confirmed previously expressed suppositions regarding a favorable prognosis for a complete cure. There are no reports on the subsequent fate of the patients, supposedly because at that time, after the supposed clarification of the etiology of the illness, there was "apparently very little interest in the further course" (Scheid [44]). On the other hand, as in the later "torpedo oil" poisonings, one probably would have taken the opportunity to make such remarkable findings known if they had actually been present.

The paralysis occurring after creosote phosphate, ginger brandy, and "apiol" was formerly interpreted as "toxic polyneuritis". On the other hand, on the basis of the definite involvement of central tracts in paralysis caused by "torpedo oil", recent authors point out that, as a general rule, the concept of a "toxic polyneuritis" can no longer be accepted for the syndrome of tricresyl phosphate poisoning (Scheid [44], Mertens [71], Parnitzke [58]). However, in a recently published review, Scheller [137] points to the still unexplained discrepancy between apiol paralysis, on the one hand, and torpedo oil and ginger brandy paralysis, on the other hand.

To follow up on the above-described differences in the clinical symptoms, we would like to distinguish three large groups of tricresyl phosphate poisonings: (1) paralysis by certain apiol preparations with almost exclusive involvement of the peripheral pathways; (2) paralysis by tricresyl phosphates of the "torpedo oil" type, which also include "Igelit" poisonings and the Swiss group poisoning, with definite signs of involvement of the central
neuron; and (3) ginger brandy paralysis would fall between the first two types of paralysis; in ginger paralysis there is central involvement only in especially severe cases, so that this type of poisoning is less severe on the whole.

(b) **Etiological and Pathogenetic Characteristics of Different Tricresyl Phosphate Poisoning Epidemics**

As we were able to show above, there are significant differences with respect to both the poisoning symptoms and the histological findings in chickens. Pure tri-ortho-cresyl phosphate affects mainly the peripheral motor neuron and produces flaccid paralysis, whereas industrial tricresyl phosphates containing mainly mono-ortho-cresyl esters cause only slight damage to the peripheral nerves and instead affect predominantly the long tracts of the spinal cord and produce spastic paresis. Similar differences were most likely present in the different poisoning epidemics, although the differences were perhaps not as sharp as in our animal experiments.

Naturally, the various types of damage found in the animals can be related to the illness produced in man only with reservations. A comparative analysis of the nervous system damage in animals and in man is difficult because only a few reports are available on usually pathoanatomically incompletely investigated cases of tricresyl phosphate poisoning.

In addition to the expected damage to musculature and peripheral nerves, Smith and Lillie [9] and various other authors [138-141] found fatty degeneration of the white substance of the spinal cord in occasional patients who died of other causes during the "ginger epidemic", but they failed to provide detailed information regarding localization. Aring [107] was able to conduct
systematic studies on 36 cases after several years and found greater changes in the lateral pyramidal tract and Goll's tract and thickened leptomeninges and considerable damage to the cells of the anterior and lateral horns of the spinal cord; in most cases there were signs of thromboangiitis obliterans. Since the disease had existed for several years in almost all of the cases that he investigated, no definite answer can be given to the question of a relationship between changes in the vessels and nervous elements. In one case there were also changes in the cells in the nucleus dentatus of the cerebellum [142], and in another case there was softening of the nucleus lentis and spongiosis of the cerebral cortex 8 years after "edible oil poisoning" [143]; with respect to the clinical symptoms in tricresyl phosphate poisoning, a causal relationship seems doubtful here.

This small amount of information does not provide any definite evidence of the correctness of our hypothesis that the different poisoning epidemics were based on chemically different tricresyl phosphates. There are absolutely no pathoanatomical findings for the cases of "apiol" paralysis and "torpedo oil" paralysis. We should also keep in mind that the cases investigated by Aring [107] involved especially severe poisoning with ginger brandy and therefore cannot be regarded as a representative cross section.

We felt that the critical finding was that there were any differences at all in the effect of different tricresyl phosphates. We then decided to try to clarify the relationships between the types of paralysis and the tricresyl phosphates causing the paralysis on the basis of experimentally gained knowledge, available published information, and our own follow-up studies.
1. Paralysis by Creosote Phosphate

"Polyneuritides" after therapeutic administration of phosphoric creosote occurred predominantly as group poisonings.

In connection with the illness of 7 pulmonary patients in Haarlem, Huet [20] reports that chemical analysis of the medication that was used revealed only 12.5% phosphate (as $P_2O_5$) instead of the required 20–25%. In addition, it was difficult to saponify, and the boiling range was well above the prescribed range of 190–203°C.

We investigated the question of which toxic component may have been present in the creosote phosphate by preparing such a preparation by an old method [144] and testing it and the residual fraction chemically and in animal experiments.

125 g of officinal creosote was gradually treated with 35 g of phosphorus pentoxide and 5 g of sodium, as the mixture was continuously stirred. The reaction mixture was stirred for another 24 hours and was then fractionally distilled. The major portion passed over between 190 and 202°C as a clear liquid that was insoluble in water; starting at 212°C, a second, pale-yellow fraction distilled, which was immiscible with the first fraction and was more oily in quality. On further distillation, the boiling point rose continuously to 250°C; further reactions probably occurred in the distillation vessel. The two fractions that were obtained were separately dissolved in ethanol and precipitated with water.

In the first fraction, which distilled between 190 and 202°C and supposedly represented the drug in the specified procedure, no phosphate was detectable by the very sensitive molybdate reaction after baking with
magnesia. We also obtained pharmaceutical confirmation of this important finding*. The paralytic effect was tested in 2 chickens by administering a total dose of 5 g/kg, divided into 10 single doses given on 10 consecutive days; except for transient signs of local irritation of the mucous membranes in the crop and esophagus, the animals remained free of symptoms for 4 weeks. The second, higher-boiling fraction contained 1.44% phosphate (calculated as P₂O₅; determination by Wurzschmitt [145]). We fed 2 chickens with 1 or 3 g/kg of this sample, whereupon they promptly became affected with spastic paresis 12 days and 9 days, respectively, after administration of the last dose.

These observations place the etiology of the paralysis by creosote phosphate in a totally different light. Presumably, this preparation has been produced not only by large companies, but also occasionally in laboratories (cf. Huet). Here and there, the distillation may have been performed incorrectly, so that a portion of the toxic, high-boiling fraction got into the preparation.

A similar assumption was made by Martinius [146], who believed that the extremely nonvolatile tri-ortho-cresyl phosphate cannot get into the medication if the specifications for preparing phosphoric creosote are followed exactly. According to the above discussions, however, the tri-ortho ester cannot arise in such reactions or at most can form in only extremely small quantities; instead, mixed esters form, which can contain not only other phenol derivatives, but also ortho-cresyl radicals. The statement by Tiffeneau

*We would like to thank Dr. List of the Pharmaceutical Department of the University of Würzburg for preparing an analogous preparation and analyzing it for phosphate.
that up to 15% tri-ortho-cresyl phosphate could be present in creosote phosphates (this statement is cited in many later papers) has not been analytically demonstrated and thus must be regarded as purely speculative. The amount of ortho-cresyl-substituted phosphoric acid esters contained in such preparations can hardly have been high. In our second fraction we found only 1.44% \( P_2O_5 \); if we convert this to mono-ortho-cresyl phosphates, their content (with respect to moles of tricresyl phosphate) would be a maximum of about 7.5% in the total preparation. We believe that this is confirmed by the data on the amounts actually ingested in such cases of poisoning; in the cases reported by Huët, amounts of up to 120 g of creosote phosphate had been consumed in short periods of time.

2. "Ginger Paralysis"

On the basis of the available clinical reports, we classified the type of paralysis observed in the American ginger brandy cases as hypothetically between "apiol" paralysis and "torpedo oil" poisoning. Involvement of the central motor tracts was demonstrated only in the severe cases of this poisoning epidemic. A comparison with our animal experimental findings suggests that these poisonings were based on a tricresyl phosphate intermediate between the preparations that were thoroughly tested by us (pure tri-ortho-cresyl phosphate and industrial products with 25–30% o-cresol), i.e., a tricresyl phosphate that must have had a high content of ortho-cresol. In fact, according to reports in the technical literature in the USA, an industrial tri-ortho-cresyl phosphate was used as a plasticizer of celluloid and lacquers. Long before the toxic effects of the tri-ortho ester became known, tri-o-tolyl phosphate was being discussed as a camphor substitute in pertinent
published reports and patent documents [148]. In all probability, pure ortho-
cresol was not being produced for such industrial products because its prepa-
ration is very expensive; the starting material that was used probably still
contained a certain amount of the isomers and homologues of ortho-cresol.

Our search of the literature uncovered certain information pointing in
this direction that is important to our problem. Smith et al. [6-10] used an
"industrial tri-ortho-cresyl phosphate" for their first animal experimental
studies on the etiology of ginger paralysis. In a review report on the ginger
brandy cases, Kidd and Langworthy [5] state that a commercial tricresyl phos-
phate was sold under the name Lindol or Lyndol, which consisted mainly of tri-
ortho-cresyl phosphate with only small percentages of meta and para compounds.
After the paralytic effect of the tri-ortho compound became known, the non-
toxic meta and para esters were used to produce Lindol. According to Sampson
[32], a "Lindol" was the cause of the illnesses in 1937/38 in Durban. Accord-
ing to "careful analysis", a contaminated soybean oil, which was responsible
for the mass poisoning, contained "0.28% tricresyl phosphates", which con-
sisted "of the 3 isomers in the following proportions: tri-ortho- 58.2%, tri-
para- 23.7%, and tri-meta- 18.1%.

According to these reports, a tricresyl phosphate with a high content of
ortho-cresol and therefore a high content of tri-ortho ester was very probably
present in the toxic ginger brandies. According to our present discussion,
such preparations produce predominantly peripheral paralysis. Consistent with
this, spastic symptoms are not reported in the victims of the poisoning epi-
demic in Durban, even after an observation period of more than 3 years [32].
3. "Apiol" Paralysis

Samples of apiol, which had been demonstrated to produce paralysis, were reported to contain 28–50% tri-ortho-cresyl phosphate; it is explicitly stated in these reports that chemical analysis showed the presence of tri-ortho-cresyl phosphate [11–13]. This seems to confirm our hypothesis that a pure or almost pure tri-ortho-cresyl phosphate was present in the toxic varieties of apiol on the basis of the clinical picture and the effect in the animal experiment.

However, critical review of those analytical reports shows that the phosphoric acid content was probably quantitatively determined, but cresol was determined only by qualitative methods. In particular, the presence of ortho-cresol was determined only on the basis of a positive Melzer sample [11, 12]; a quantitative ortho-cresol determination was not performed, and apparently there was also no testing for the presence of the meta- and para-isomers.

Since the only pure triester that is active is the tri-ortho compound, the analytical results were automatically related to tri-ortho-cresyl phosphate under the influence of the old conception, according to which only the three specified compounds exist in industrial tricresyl phosphates. There was thus no firm foundation for the statements that were then repeatedly cited, to the effect that the paralyzing apiol samples contained 28–50% tri-ortho-cresyl phosphate.

Despite comprehensive follow-up studies, including studies by official testing institutes and by manufacturers of tricresyl phosphate, we were unable to procure reliable documentation about the composition of the ester preparation that had been added to some apiols. In addition to the agreement between clinical symptomatology and animal experiments with tri-ortho-cresyl
phosphate, there is further evidence that the "apiol" paralysis very probably involved the tri-ortho ester. At the beginning of the 1930s, there was a strong increase in demand for the abortifacient apiol as a result of the economic depression, especially since just prior to this the specific effect of this preparation had been clinically confirmed [149–151]. In isolated cases the increased demand may have induced unscrupulous individuals to replace parsley extracts by a substance with similar physical properties; it is possible that someone with this intention thought to use the likewise bluishly opalescent, oily substance tricresyl phosphate. The intensity of the opalescence depends partly on the content of ortho-cresol; therefore, it seems understandable that one would use tri-ortho-cresyl phosphate to adulterate apiol.

4. Paralysis by Tricresyl Phosphates of the "Torpedo Oil" Type

Reliable information is available on those tricresyl phosphates that caused a large number of poisonings in Europe and especially in Germany during the war and the postwar years. Commercial crude cresol or tricresol, whose o-cresol content can vary between 25 and 40%, was used as the starting material for producing tricresyl phosphates.

The group poisoning described by Staehelin was based on an industrial phosphate whose ortho content is given as 40% [30, 100]. The tricresyl phosphate produced at that time in Bitterfeld and used in East German soft Igelit products contained about 30% o-cresol in the aromatic component, as is apparent from official comments [152] and analyses [82]. The aryl phosphates that were added to the torpedo oil were produced from crude cresol of the exact same type.
By analyzing the clinical symptoms in man and the poisoning symptoms and pathoanatomical changes in experimental animals and by examining the chemical literature, we have been able to show that three different types of poisoning based on chemically different tricresyl phosphates can be distinguished in the observed tricresyl phosphate poisonings in man. Depending on the content of ortho-cresol in an ester mixture, damage to the peripheral motor fibers is accompanied by more or less strong involvement of the central nervous pathways in the degeneration process. This is most markedly the case in paralysis caused by tricresyl phosphates of the "torpedo oil" type, which, with an ortho-cresol content of about 30%, contain predominantly mono-ortho-substituted mixed esters and were identified in the toxicity test as the relatively strongest preparations. Central involvement is less prominent in the case of tricresyl phosphates with high ortho values, as was the case with the ginger brandies; according to available records, the o-cresol contents were at least twice as high as in the phosphates of German production during the war and postwar years, and the content of mixed esters was thus significantly lower. Finally, pure or almost pure tri-ortho-cresyl phosphate produces almost exclusively peripheral paralysis. Although final conclusive proof has not yet been produced due to a lack of suitable chemical analytical data, this type of paralysis appears to have been present in the "apiol" poisonings. This would be an additional argument in favor of Schaltenbrand [116] (cf. also [58]), who at that time correctly characterized "apiol" paralysis as "elective polyneuritis".

The clinical picture is described as tri-ortho-cresyl phosphate poisoning in almost all relevant papers, textbooks, and reference books. The concept on which this term is based is now outdated. As was shown above, the observed
illnesses were not usually produced by the tri-ortho-cresyl ester of phos-
phoric acid, but rather by a relatively high content of ortho-cresyl radicals
in the preparations and especially by highly toxic mono-ortho-di-(meta,
para)-cresyl esters. However, since industrial mixtures of triphosphates of
isomeric cresols are always involved, we should generally speak of tricresyl
phosphate poisoning.

SUMMARY

According to the conventional view, industrial tricresyl phosphate is a
mixture of the 3 isomers tri-ortho-, tri-meta-, and tri-para-cresyl phosphate,
and its toxicity corresponds to the content of tri-ortho compound or ortho-
cresol.

In comparative toxicological animal experiments, older industrial tricres-
yl phosphates with about 30% ortho-cresol are, surprisingly, 10 times more
toxic than pure tri-ortho-cresyl phosphate, whereas modern preparations con-
taining about 3% o-cresol are about 3 times weaker than the tri-ortho ester
and about 30 times weaker than those highly toxic ester mixtures. Accord-
ingly, there is no correlation between the toxicity and the ortho-cresol con-
tent.

Contrary to the prevailing published opinion, industrial tricresyl phos-
phates contain not only the three uniform triesters specified above, but also
seven other isomeric mixed esters. Only tricresyl phosphates with ortho-
cresyl radicals were found to have toxic paralytic effects. Their toxicity
decreases in the order mono-, di-, and tri-ortho-cresyl esters in the propor-
tions 10:5:1.

A mathematical analysis of the theoretically possible proportions of
these mixed esters in tricresyl phosphates, which could arise from creosol mixtures with variable contents of ortho-cresol with a purely statistical distribution, yields the basis for understanding the differences in toxicity in industrial tricresyl phosphates. The content of the strongest mono-ortho esters is highest at 33 1/3% o-cresol, i.e., the range of ortho-cresol contents of the highly toxic industrial phosphate mixtures that produced most of the poisonings of the war years and postwar period.

The newly gained knowledge requires a revision of the previous toxicological evaluation of tricresyl phosphate poisoning; in particular, the calculation of minimal toxic doses may no longer be related to tri-ortho-cresyl phosphate, but rather must be related to the total preparation.

Comparative consideration of the clinical pictures of tricresyl phosphate poisoning shows that 3 types of poisoning can be distinguished, which were observed during the major poisoning epidemics: certain "apiol" preparations produced almost exclusively peripheral paralysis without demonstrated involvement of central pathways; in the American "ginger epidemic" involvement of the central neuron was reported only in very severe cases; cases of paralysis that occurred during the war and in the postwar period ("torpedo oil poisoning", "Igelit poisoning") were accompanied by spinal damage in the majority of moderately severe and severe cases.

The conclusion that the different types of poisoning were caused by chemically different tricresyl phosphates is supported by our own animal experiments. Industrial tricresyl phosphates with an ortho-cresol content of about 30% and mixed esters containing ortho-cresyl radicals produce predominantly spastic symptoms, whereas pure tri-ortho-cresyl phosphate produces only flaccid paralysis. Histological studies confirm that the two types of tricresyl
phosphates trigger different pathogenetic processes. Pure tri-ortho-cresyl phosphate produces lesions mainly in the peripheral nerves with only slight involvement of certain nervous pathways of the spinal cord; with respect to the highly toxic tricresyl phosphates, the damage is concentrated in the spinal cord, and there is only slight involvement of the peripheral nerves.

This knowledge leads to a new interpretation of the etiology and pathogenesis of the various poisoning epidemics that have occurred. The "ginger paralysis" in America was very probably caused by industrial tricresyl phosphate with a high ortho-cresol content; on the other hand, the poisonings which occurred in Europe during the war and in the postwar period were probably caused by tricresyl phosphates that contained about 30% ortho-cresol and consisted chiefly of mixed esters. The cases of paralysis observed after ingestion of apiol preparations were very probably caused by an almost pure tri-ortho-cresyl phosphate; in this variant of the poisoning, which at the time was justifiably characterized as "motor polyneuritis", it seems that no spastic symptoms were ever observed.

The therapeutic use of creosote phosphate has resulted in numerous cases of paralysis since the turn of the century. Our own experiments lead to the conclusion that the medication is occasionally contaminated by high-boiling phosphoric acid esters containing ortho-cresyl radicals with paralytic properties due to improper preparative technique.

The results that have been presented here show that the term "tri-ortho-cresyl phosphate poisoning" should no longer be used; instead, we should use only the more general and more accurate term "tricresyl phosphate poisoning".
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of different ages, a peak value occurring on the eighth day. Our quantitative results also suggest that the metabolic mechanism may not be the same throughout the whole of the period of growth, since it was found that most of the acetic acid appeared during the first 6 days, while succinic acid was most actively formed during the later stages.

The metabolic pathways by which these substances are produced, and the differences between the metabolism of the flagellate and that of the intracellular stages of the parasite, must remain conjectural until more information is available. In particular, it would be interesting to know the part played by phosphorylation reactions and carbon dioxide fixation, and also the relationship between carbohydrate and protein metabolism which has been claimed by Salle & Schmidt (1928). Our findings, however, indicate the formation of several substances, the presence of which is not inconsistent with metabolic cycles known to occur in some other cells.

SUMMARY

1. A study of the biochemical activities of *Leishmania donovani* has been made *in vitro*.

2. During the period of growth glucose disappears from the culture medium, the pH of which falls in a manner related to the growth of the organisms.

3. Carbon dioxide, acetic, pyruvic, succinic, and probably lactic acids were produced, and their quantitative production during the growth of cultures was followed.

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Tricresyl Phosphates and Cholinesterase

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It has been stated that tri-o-cresyl phosphate (TOCP) is an inhibitor of cholinesterase *in vitro* (Hottiger & Bloch, 1943; Bloch, 1943; Earl & Thompson, 1952a). As a general rule, however, such stable organophosphorus compounds are poor inhibitors. Amongst a series of related compounds, a relationship between stability to hydrolysis and inhibitory power has been demonstrated (Aldridge & Davison, 1952a, b). Since TOCP is a stable substance, it was considered unlikely that it would have the inhibitory activity attributed to it, and this view was reinforced by the demonstration by Myers & Mendel (1953) that the inhibitory activity of TOCP against the hydrolysis of tributyrin by rat serum varied considerably from sample to sample. The experiments described in this paper show that most of the inhibitory activity of at least one specimen of TOCP is due to an impurity.

TOCP has the interesting property of causing weakness and ataxia in the legs of chickens due to the demyelination of certain tracts of the spinal cord (Smith & Lillie, 1931). After feeding TOCP to chickens, a profound lowering of the pseudo cholinesterase at various sites is always obtained (Earl & Thompson, 1952b). Using a purified specimen of TOCP possessing little *in vitro* inhibitory activity,
experiments have now been carried out which show that TOCP is undoubtedly converted into another, more inhibitory compound in vivo in chickens, rabbits and rats. Further work with chickens has shown that the tri-m- and tri-p-cresyl phosphates do not inhibit cholinesterase in vivo, do not produce paralysis, and do not, within the limits of experimental observation produce more than traces of demyelination.

EXPERIMENTAL

Methods of analysis. Cholinesterase activity was determined manometrically using as substrates 0·015 M acetylcholine chloride or 0·03 M butyrylcholine perchlorate, and tributyrinase activity using a suspension of 3·4 mg./ml. tributyrin. All determinations were carried out in a buffered solution containing 0·0357 M NaHCO₃, 0·164 M NaCl and 0·1% (w/v) gelatin, and gassed with 5% CO₂ in N₂.

o-Cresol was determined using the following modification of the method of Gottlieb & Marsh (1946). Reagents. (1) 4-Aminoantipyrine, 0·05% (w/v) in Sörensen’s 0·067 M phosphate buffer, pH 7·4. (2) Potassium ferricyanide, 0·03% (w/v) in Sörensen’s 0·067 M phosphate buffer, pH 7·4. Procedure. To 4 ml. of a solution containing o-cresol (0·0–60 μg.) at approx. pH 7·4 were added 2 ml. 4-aminoantipyrine reagent; the solution was mixed and 2 ml. potassium ferricyanide reagent were added. This too was mixed and left at room temperature for 5 min. 2 ml. ethanol were then added and the colour density was measured at 510 mμ, using a Unicam D.G. spectrophotometer. There is a linear relationship between optical density and concentration of o-cresol. The ethanol was added so that when the hydrolysis of TOCP was being studied, any unchanged TOCP was brought into solution. The method is unaffected by the ethanol or by salts produced by neutralizing up to 1 ml. of 2x ethanol KOH. By this method o- and m-cresols can be estimated, but not p-cresol. The total combined o-cresol was determined by refluxing with 50% (w/v) solution of KOH in methyl cellulose (Haslam & Squirrel, 1962). Total P was determined by the colorimetric phosphomolybdic acid method (King, 1946), after digestion of a sample by a reagent containing molybdate, H₂SO₄, HClO₄, and HNO₃ (Simmons & Robertson, 1960).

Materials used. Tri-o-cresyl phosphate (TOCP) was obtained from Geigy Co. Ltd. (Found: P, 8·3; combined o-cresol 88·0. Calc. for C₁₉H₂₄O₃P: P, 8·4, combined o-cresol, 88·0%). Tri-m- and tri-p-cresyl phosphates (TMCP and TPMP) were obtained from A. Boake, Roberts & Co. As TPMP was recrystallized from absolute ethanol. (M.p. 76°; Verhoek & Marshall (1939) reported m.p. 78.4–78.7°. Found: P, 8·4). The TMCP was a liquid, difficult to solidify when received, but was twice recrystallized at -5° from a solution of 120 g. in 300 ml. 17% (w/v) ethyl ether in light petroleum (b.p. <40°). This material was stored at -5°. (M.p. 25°; Breusch & Keskin (1942) reported m.p. 25–6°. Found: P, 8·5.)

Purification of TOCP. The impurity which gives to specimens of TOCP its in vitro activity against cholinesterase can be differentially hydrolysed by alkali. In view of the insolubility of TOCP in water, the following method had been developed using ethanolic NaOH. TOCP (40 ml.) and 0·2 N ethanolic NaOH (40 ml.) were separately cooled to 5°. The solutions were mixed and left at 5° for 1 hr. CHCl₃ (200 ml.) and water (400 ml.) were added and the aqueous layer was washed with a further 100 ml. CHCl₃. The CHCl₃ extracts were combined and washed four times with 200 ml. portions of water. Any emulsion in the CHCl₃ layer was broken by the addition of a little NaCl. After any droplets of water had been filtered off, the CHCl₃ layer was dried over anhydrous Na₂SO₄ for 2 days. The dry solution was then poured through an alumina column (Peter Spence, Type H) to remove any free o-cresol. This was followed by 100 ml. dry CHCl₃, and then the column was allowed to drain dry. The solvent was further removed in vacuo at 50° and then the last traces at 80° by a stream of air. This purified material contained 8·4% P (theoretical 8·4%) and as a further check that the compound had not been chemically altered, it was shown that the rate of hydrolysis and the turbidity of the aqueous suspensions were, within experimental error, the same for both the purified and unpurified materials.

Solubility test for inhibitory impurities in tri cresyl phosphates. When a substance is pure, a saturated solution will contain the same amount of substance no matter how much solute was present in excess during the preparation of the solution. For the detection of impurities in tri cresyl phosphates, suspensions of 20 μg./ml. and 2 mg./ml. were prepared. The excess of solute was removed by centrifuging and the clear supernatants were incubated with human serum cholinesterase or rat serum tributyrinase for 30 min. and the residual activity was determined.

Table 1. Solubility test for inhibitory impurity in samples of tri cresyl phosphates

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<tr>
<th>Supematant from</th>
<th>Hydrolysis of</th>
<th>Hydrolysis of</th>
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<tbody>
<tr>
<td>(mg./ml.)</td>
<td>acetylcholine</td>
<td>tributyrin by human plasma by rat serum</td>
</tr>
<tr>
<td>Unpurified tri-o-cresyl phosphate</td>
<td>0·02</td>
<td>88, 88</td>
</tr>
<tr>
<td>2·0</td>
<td>36, 36</td>
<td>75, 73</td>
</tr>
<tr>
<td>Purified tri-o-cresyl phosphate</td>
<td>0·02</td>
<td>96, 97</td>
</tr>
<tr>
<td>2·0</td>
<td>103, 98</td>
<td>96, 90</td>
</tr>
<tr>
<td>Tri-m-cresyl phosphate</td>
<td>0·04</td>
<td>99, 101</td>
</tr>
<tr>
<td>2·0</td>
<td>102, 97</td>
<td>—</td>
</tr>
<tr>
<td>Tri-p-cresyl phosphate</td>
<td>0·02</td>
<td>98, 101</td>
</tr>
<tr>
<td>2·0</td>
<td>102, 103</td>
<td>—</td>
</tr>
</tbody>
</table>
Table 2. Comparison of the inhibition of cholinesterases (1) by TOCP administered to living animals and (2) incubated in vitro in blood from the same animals

(For the calculation of the in vivo concentration in blood, blood volumes of rabbit and chicken have been taken as 70 ml./kg. (Levine et al. 1941). It was assumed that all the drug would be in the circulating blood. All compounds were injected intravenously as solutions in absolute ethanol. Cholinesterase activity was determined on whole blood using acetylcholine (AcCh, 0.015 M) and butryrylcholine (BuCh, 0.03 M) as substrates. Heparin was used as an anticoagulant.)

<table>
<thead>
<tr>
<th>Animal</th>
<th>Dose (mg./kg.)</th>
<th>Time after injections or of incubation (min.)</th>
<th>AcCh hydrolysis</th>
<th>BuCh hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>In vivo</td>
<td>In vitro</td>
<td>In vivo</td>
</tr>
<tr>
<td>Rabbit</td>
<td>0.8</td>
<td>30</td>
<td>41</td>
<td>3</td>
</tr>
<tr>
<td>Rabbit</td>
<td>1.61</td>
<td>30</td>
<td>58</td>
<td>0</td>
</tr>
<tr>
<td>Rabbit</td>
<td></td>
<td>103</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Chicken</td>
<td>0.8</td>
<td>30</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>Chicken</td>
<td></td>
<td>80</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RESULTS

The inhibitory activity of TOCP against cholinesterases in vitro

Using the solubility test on an unpurified sample of TOCP, it was found that the inhibitory activity of a saturated solution is dependent upon the concentration of the suspension used to prepare it. This was demonstrated using as the test enzymes both human serum cholinesterase and rat serum tributyrinase (Table 1). Another specimen of TOCP kindly supplied by Dr Myers gave a similar result. These experiments indicated that these samples of TOCP contain an impurity which is an inhibitor of cholinesterase and of tributyrinase. A saturated solution of the Geigy material purified as described above showed a negligible activity (Table 1). It should be noted that this sample might still not have been free from the impurity, but the concentration of the latter was below the threshold of the methods of detection.

The inhibitory activity of TOCP in vivo

An experiment has been described (Aldridge & Barnes, 1952) to show that various organophosphorus compounds are converted into more active inhibitors in vivo. In this method the compound is injected intravenously into the animal and samples of blood are removed at various times for the determination of enzymic activity. Some of the animal's normal blood is incubated with that concentration of substance which would be present if all of the injected substance were circulating in the blood stream (this is clearly a much higher concentration than would actually be present). In this way a comparison may be made of the in vivo and in vitro effects after various time intervals. The results given in Table 2 show that both in the rabbit and the chicken the inhibition of cholinesterase is more than can be accounted for by the in vitro activity of the TOCP. It seems, therefore, that TOCP is converted into a more active inhibitor of cholinesterase in vivo.

Table 3. Inhibitory power of solutions of TOCP after incubation with rat-liver slices

(Approx. 4 g. crude rat-liver slices of thickness approx. 0.5 mm. were added to a suspension of 30 µg. TOCP/ml. in buffer solution containing NaHCO₃, 0.0357 M; NaCl, 0.164 M; and gassed with 5% CO₂ in O₂. After various times, a 5 × diluted supernatant (0.5 ml.) was incubated with 6 × diluted horse serum (0.5 ml.). After a further incubation at 37°C for 30 min. the residual cholinesterase activity was determined manometrically using acetylcholine chloride (0.015 M) as substrate. Incubation of undiluted TOCP suspension (0.5 ml.) or of extract from liver slices (0.5 ml.) with horse serum produced no inhibition of horse-serum cholinesterase.)

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Enzyme activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>19</td>
</tr>
<tr>
<td>60</td>
<td>58</td>
</tr>
<tr>
<td>120</td>
<td>77</td>
</tr>
<tr>
<td>210</td>
<td>90</td>
</tr>
</tbody>
</table>

Conversion of TOCP into a more active inhibitor by rat-liver slices

It has been shown that both octamethylpyrophosphoramide (DuBois, Doull & Coon, 1950; Gardiner & Kilby, 1952) and O0-diethyl O-p-nitrophenyl phosphothioate (formerly called 'thio-phosphate') (Gage & Payton, 1952) are converted into more active inhibitors by rat-liver slices. The results (Table 3) show that TOCP is also converted into a more active inhibitor by rat-liver slices. The concentration of inhibitor produced appears to decrease with incubation times of longer duration than 30 min. From other experiments it would appear that this is due to at least two factors. If, after
15 min. incubation of TOCP with rat-liver slices, the slices are centrifuged off, the inhibitory activity of the supernatant decreases to 20% of the original value after 1 hr. at 37°C. The inhibitor is therefore unstable under these conditions. Another factor is that the ability of the liver to produce inhibitor is not maintained under these conditions. Rat-liver slices which have been incubated at 37°C for 1 hr. are unable to produce inhibitor from TOCP. Considerable variation (approx. five-fold) has also been experienced in the amount of inhibitor produced in different experiments.

**The effect of tricresyl phosphates on the chicken**

It has been shown that after oral administration of TOCP to chickens, there is a marked inhibition of the serum cholinesterase activity (Earl & Thompson, 1952b). Using a purified TOCP, a similar fall is produced after intravenous injection (Table 2). While both impure and purified TOCP produce demyelination in the chicken, the tri-\(\text{m}\)- and tri-\(\text{p}\)-cresyl phosphates are either inactive or only feebly active in this respect (Smith, Engel & Stohlman, 1932; Hunter, Perry & Evans, 1944). Experiments have been carried out to determine if the \(\text{meta}\) and \(\text{para}\) isomers produce a lowering of the cholinesterase activity of the serum. There is a danger of impurities in the \(\text{meta}\) isomer, which is normally received as a liquid difficult to crystallize. Both of these isomers after careful recrystallization have very little activity *in vitro* and appear to be substantially free from active impurities as judged by the solubility test. Since TOCP is obviously absorbed when administered in arachis oil, it has been presumed that both the \(\text{meta}\) and \(\text{para}\) isomers will be absorbed when given in this way. All these isomers have therefore been given orally as a 10% solution in arachis oil. The results in Table 4 show that TOCP produced a large depression of cholinesterase activity which had partially recovered in 4 days. This rate of return agrees with the results of Earl & Thompson (1952b). After administration of both the \(\text{meta}\) and \(\text{para}\) isomers respectively, no fall in enzyme activity was found, even though 10 g. of the compound was given. The chicken after receiving TOCP became definitely ataxic in 14 days, and the spinal cord was found upon histological examination to have extensive demyelination of the fibre tracts of the lateral and anterior columns.

**DISCUSSION**

Most of the *in vitro* inhibitory activity of TOCP against human serum cholinesterase and rat serum tributyrinase is due to an impurity. A saturated solution of a 'purified' specimen possessed negligible *in vitro* activity; this specimen was pure within the limits of a solubility test which has been developed. This finding re-emphasizes the warning previously given (Aldridge & Davison, 1952b): 'Without further evidence of purity statements of inhibitory power (as concentrations to produce 50% inhibition) must be regarded as only applying to the particular specimen of inhibitor being examined.' We have no information about the chemical structure of this active impurity and know only that it inhibits human pseudo cholinesterase more than it does true cholinesterase. It has also been shown (Table 2) that if this purified TOCP is injected into rabbits and chickens, their blood cholinesterases are rapidly inactivated. These results suggest that TOCP is converted into a more

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**Table 4. Effect of oral administration of tricresyl phosphates on the cholinesterase of chicken serum**

(5 ml. of a 10% (w/v) solution of the tricresyl phosphate in arachis oil was given daily by mouth to each of two chickens. Samples of blood were removed 24 hr. after such a dose for the determination of cholinesterase activity using acetylcholine (0.015 ml.) as a substrate.)

<table>
<thead>
<tr>
<th>Daily dose (mg./kg.)</th>
<th>No. of daily doses</th>
<th>Time blood sample taken (day)</th>
<th>Cholinesterase activity of serum (ml. CO(_2)/ml./min.)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tri-o-cresyl phosphate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>1</td>
<td>0</td>
<td>27-6</td>
<td>Ataxia at 14 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>5-8</td>
<td>Histological examination at 21 days showed extensive demyelination in the spinal chord</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>9-7</td>
<td></td>
</tr>
<tr>
<td><strong>Tri-m-cresyl phosphate</strong></td>
<td></td>
<td>0</td>
<td>20-5</td>
<td>No paralysis or ataxia 27 days after last dose</td>
</tr>
<tr>
<td>0.21</td>
<td>20</td>
<td>1</td>
<td>20-3</td>
<td>Histological examination at this time showed traces of demyelination in the spinal cord</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>18-8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tri-p-cresyl phosphate</strong></td>
<td></td>
<td>0</td>
<td></td>
<td>No paralysis or ataxia 43 days after last dose</td>
</tr>
<tr>
<td>0.26</td>
<td>18</td>
<td>0</td>
<td></td>
<td>Histological examination showed traces of demyelination in the spinal cord</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
inhibitory compound in vivo and this view is supported by the fact that rat-liver slices produce such a compound in vitro. In vivo, pseudocholinesterase is inhibited more than true cholinesterase but again we have no information about the chemical structure of the inhibitory compound.

Although the theory involving a simple relationship between the inhibition of the pseudocholinesterase of the central nervous system and demyelination is now untenable (Davison, 1953), it is possible that those few organophosphorus compounds which can produce demyelination (Barnes & Denz, 1953) must have the chemical properties necessary for the inhibition of esterases. If demyelination is brought about by the inhibition of an enzyme, it is important to realize that organophosphorus compounds appear to be general inhibitors of enzymes possessing carboxylic esterase activity (Aldridge, 1953). It is not always clear which enzymes possess this activity, for it has recently been shown that some proteolytic enzymes do hydrolyse carboxylic esters (Schwert, Neurath, Kaufmann & Snoke, 1948). It is possible that the products of the inhibitory reaction between the organophosphorus compounds and esterases in general may be important in the production of demyelination. There is, however, no information about the nature of these products after TOCP administration, because a metabolite is the active inhibitory agent.

**SUMMARY**

1. Using a solubility test, the in vitro inhibitory power of tri-o-cresyl phosphate against cholinesterase (TOCP) has been shown to be due to an active impurity. A purified specimen possessed negligible in vitro activity.

2. The in vivo inhibitory power of TOCP against cholinesterase in rabbit, rats and chickens cannot be accounted for by its in vitro activity.

3. Oral doses of tri-m- and tri-p-cresyl phosphates do not produce inhibition of the cholinesterase of chicken serum and do not produce more than traces of demyelination in the spinal cord.

My thanks are due to Geigy Company Ltd. for a gift of tri-o-cresyl phosphate and to Mr F. H. McKenzie (A. Boake, Roberts & Co. Ltd.) for the tri-m- and tri-p-cresyl phosphates. I am also grateful to Dr F. A. Denz for the histological examinations of the spinal cords of the treated chickens, and to Miss J. E. Cremer and Mr C. R. Kennedy for valuable technical assistance.

**REFERENCES**


Biological Activity of a Tri-o-Cresyl Phosphate Metabolite

Tri-o-cresyl phosphate (TOCP) is metabolized in vitro and in vivo to form potent esterase inhibitors. The nature and biological activity of the metabolites were investigated. Rat administered radiophosphorus-labelled TOCP were found to excrete radioiodinated di-aryl phosphates, mono-aryl phosphates and phosphoric acid in the urine. Three anti-esterase metabolites were present in the liver, intestine and feces. Incubation of TOCP with rat liver microsomes fortified with reduced diposphopyridine nucleotide also yielded three esterase inhibitors of similar chromatographic characteristics to those produced in vivo. The major esterase-inhibiting metabolite formed in vivo was isolated on silicic acid–Celite columns with benzene–ether–ether–ether. Examination of the infra-red spectrum and hydrolysis products suggested structure I for this metabolite. Reaction of o-cresylphosphoryl dichloride with o-hydroxybenzyl alcohol yielded 2-(o-cresyl)-4H-1,3,2-benzodiazaphosphorin-2-one, b.p. 159–161°C/0.08–0.1 mm. mercury, nD45° = 1.5584. This compound was identical with the major metabolite in respect to infra-red spectrum, chromatographic characteristics, and anti-esterase activity. One of the other esterase inhibitors appeared to be the o-hydroxyethyl derivative of I. The mechanism of esterase inhibition was investigated by reaction of chymotrypsin (Xtr) with I and a phenyl phosphate analogue, II. Both appeared to phosphorylate by opening the cyclic phosphate structure at the P-O—aryl bond to yield III.

An ataxia and demyelination is induced in mice and other animals by TOCP. A similar ataxia in mice resulted from subcutaneous administration of 4–8 mg/m. kg. m. whereas 240 mg/m. kg. II failed to yield ataxia. Preliminary studies indicate a demyelination with I similar to that induced by TOCP. The high activity of I relative to that of TOCP in producing neurotoxicity in mice suggests that the anti-esterase metabolites rather than TOCP per se yield the ataxia. TOCP and other ataxia-producing phosphates are known to effect prolonged in vivo inhibition of the pseudocholinesterases of brain, spinal cord and sciatic nerve. The function of this pseudocholinesterase which hydrolyzes carboxylic esters of choline is unknown. Experiments with mice established that it also hydrolyzes carboxylic esters of thiamine, that is, O-acetyl and O-propionyl thiamine, an observation consistent with previous reports on the substrate specificity of cholinesterases. The demyelination in mice produced by TOCP is identical with that with certain other organophosphates, and similar lesions appear in mice on a thiamine-deficient diet. Thiamine has been noted as a neuroactive material in peripheral nerves and as a constituent of myelin in peripheral nerves of rabbits. Inhibition of the pseudocholinesterase might result in an accumulation of carboxylic esters of thiamine to impair localized functions of the nerve requiring free thiamine or other thiamine derivatives.

In accordance with this hypothesis, thiamine and other compounds were tested extensively for their effect on the ataxia in mice from TOCP and/or I. Compounds tested alone or in conjunction with thiamine included O-acetyl thiamine, tocopherol and cortisone and their acetates, pilocarpine, salopin, and several adoxine-type cholinesterase reactivators. With varying dosages and multiple administration of these materials before and after the organophosphate, no relief of the neurotoxic symptoms was evident. TOCP potentiates the toxicity to mammals of malathion (O,O-dimethyl S-(1,2-dicarbethoxyethyl) phosphorodithioate). The intraperitoneal LD50 for mice was 150 mg/kg. for each of I and II. Simultaneous injection of malathion (LD50 alone of 1,500 mg/kg.) in a 10:1 ratio with the cyclic phosphates yielded LD50 values of about 1:4:45th those of either component alone. I and II were similar in this respect, and both yielded marked in vivo inhibition of the esterases of plasma and liver hydrolyzing the carboxyethyl groupings of malathion. The activity of these two compounds in malathion potentiation was greater than that of 120 other di-aryl and tri-aryl phosphates studied which lack the cyclic phosphate structure. The pattern of esterase inhibition in mice resulting from TOCP administration also appeared with I, although the latter acted much more quickly and at a much lower dose.

Metabolic activation occurs with many tri-aryl phosphates containing o-methyl, o-ethyl, o-aminopropyl and p-ethyl groupings. In vivo hydroxylation of the o-methyl and subsequent cyclization to anti-
Isolation of Sterol Esters from Human Fæces

The importance of obtaining more precise information regarding the sterol excretion pattern in human beings has recently become apparent and has been emphasized by Aylward. The normal clinical laboratory techniques for stools, namely extraction of dried material or wet extraction coupled with saponification make impossible the isolation of any sterol esters which may be present. This difficulty in isolating (as distinct from estimating) sterol esters was indeed common to all lipid extracts until the methods developed by Börgström and others, were applied by Fillipre and Mead to extracts from animal tissues. Using silicic acid, they were able for the first time to separate cholesterol esters from triglycerides. We have applied a modification of this technique to the lipids of human fæces and have been able to isolate cholesterol ester fractions; and, using gas-liquid chromatography, we have examined the fatty acids present in the esters.

Because the amount of sterol esters in human fæces is very small, it was necessary to extract large quantities of material. Accordingly, collections from five volunteers were arranged for periods of 6-9 days, and the extraction procedure was commenced immediately on the receipt of each sample. Preliminary experiments indicated that fatty pigments interfere with the chromatographic separations, and it was therefore decided to extract the stools first with 0-9 per cent saline, a procedure which removes some of the bacterial lipids. The solid obtained by centrifugation was then extracted successively by ethanol, ether and petroleum ether; precautions were taken to minimize oxidation by carrying out the procedures, wherever possible, under oxygen-free nitrogen.

In order to increase the proportion of sterol ester in the petroleum ether fraction to be chromatographed, the free fatty acids were first removed by washing with 1 per cent sodium carbonate solution; the extract remaining was dissolved in the minimum amount of hexane and fractionated on a column of Mallinkrodt silicic acid, the ratio of adsorbent: extract being kept at about 100:1.5. Pre-treatment of the silicic acid and the elution system followed Barron and Hanahan. Positive oxygen-free nitrogen pressure was maintained, 25 ml fractions were collected, and elution of the sterol esters was judged to be complete when a negative Liebermann–Burchard test was given. The solvent from the fractions was removed at 40°C. by means of a vacuum oven; the fractions were weighed, and the residues were dissolved in petroleum ether and combined according to the peaks shown when the weighed residues were graphed.

Neither free fatty acid nor free sterol was present in the sterol ester fraction, and the weight of the fraction agreed with the estimations of sterol ester made on the original petroleum ether-soluble fraction by a colorimetric method. Table 1 shows the amount of sterol esters isolated by this method, calculated as a percentage of the total material recovered.

The acids obtained by saponification of the sterol esters from extracts 1, 2 and 3 were methylated and submitted to gas-liquid chromatography, diethyleglycol succinate, ethylene glycol adipate, silicone oil, and dimethylfluorosilane being the stationary phases. The fatty acids present in the extract in the unesterified form, and those from the sterol ester fraction, were markedly similar in type and distribution and ranged from C12-C20, saturated and unsaturated.

We thank Mr. J. Piercy and Drs. Raymond Greene and D. S. Rideout of the New End Hospital for the facilities provided, and the Nuffield Foundation for a research grant (to P. W.).

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