3. Glycine and alanine have no effect on either prostatic or red-cell phosphatases. Cysteine slightly activates both enzymes.

4. The effects of ethanol, acetone, formaldehyde and certain organic acid radicals on acid phosphatase activity have been studied. L-Tartrate,

while not affecting the acid phosphatases of plasma and red cells, inhibits other acid phosphatases particularly that of the prostate, and the nature of this action has been studied and discussed.

5. Stilboestrol has no direct effect on any of the acid phosphatases.

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The Micro- and Ultramicro-determination of Chlorine in Organic Compounds

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To follow the metabolism of 1:1:1-trichloro-2:2-di-(p-chlorophenyl) ethane (DDT) in tissues a method was necessary for determining small amounts of organically bound chlorine. In addition, a technique was required for the estimation of DDT in the presence of possible degradation products. Although the determination of inorganic chloride in minute amounts is possible (Sendroy, 1937, 1939; Conway, 1935), adaptation to the new requirements would not be convenient since in Sendroy's method chloride is precipitated with silver iodate and the concentration of chloride must be at least 0.01 M. The method of Conway is not subject to these limitations, but the dimensions of his microdiffusion unit are such that it would be difficult to make estimations of small amounts of organically bound chlorine in relatively large amounts of tissue.

The basis of the present method is the photometric determination of the intensity of the starch-iodine blue colour after liberation of free iodine from potassium iodide by chlorine formed by the oxidation of the compound under test.

A technique has also been devised for the determination of DDT in tissue extracts in the presence of metabolites. The discovery by White & Sweeney (1945) that 2:2-di(p-chlorophenyl)acetic acid (DDA) is a product of DDT metabolism in animals has made it possible to separate an alkali-soluble from a neutral fraction of organically bound chlorine found in the tissues of animals after the ingestion of DDT.

METHODS AND RESULTS

Oxidation of organically bound chlorine is effected by heating with specially prepared silver dichromate in the presence of concentrated sulphuric acid. Inorganic chloride may be determined after its oxidation in the cold by an acid permanganate mixture (Conway, 1935), organically bound chlorine not being attacked under these conditions. The latter method may be used for the specific determination of DDT since 1 mol. of hydrochloric acid is liberated/mol. of DDT by the action of alcoholic alkali (Gunther, 1945). Chlorine volatilized by the oxidation is carried

by a stream of nitrogen into potassium iodide solution contained in an absorbing vessel. The problem then becomes one of micro-iodometry. For amounts down to $1\text{ }\mu\text{g}$. chlorine a direct determination of the liberated iodine is possible. For amounts below $1\text{ }\mu\text{g}$. a technique has been devised for the separation of the free iodine and its conversion to iodate.

In studies of the distribution and fate of DDT in the body, these methods may be applied to the analysis of tissue extracts. By carrying out determinations of total chlorine, inorganic chlorine before and after incubation with alcoholic alkali and of alkali-soluble organically bound chlorine, it is possible to establish the presence of DDT, other organic compounds containing neutral chlorine and alkali-soluble compounds containing chlorine.

Apparatus

Description. The apparatus (Fig. 1) is constructed of pyrex glass. Oxidation is carried out in the reaction vessel *A*, connected by means of combined still head and dropping funnel *B* to the absorbing vessel *C* which consists of a delivery tube contained in an outer vessel, the walls of which fit closely to the spiral rod wound round the delivery tube. *C* is connected with the absorber *D* of similar construction. The central absorber tube *C* is required only for the determination of Cl in tissues. Its purpose is to remove SO_2 which may be evolved during the reaction and it contains a mixture of saturated KMnO_4 (2 vol.) and 50% (v/v) H_2SO_4 (1 vol.).

A spiral of glass rod is wound round the stem of the dropping funnel *B*; its purpose is made plain in the section on the determination of DDT. The gas inlet *E* is connected by pressure tubing to a N_2 cylinder fitted with a reducing valve, and the gas rate is adjusted by the bubble rate in a wash bottle. The gas inlet consists of a tube fitting by a standard joint into the neck of the dropping funnel *B*. The latter holds H_2SO_4 , which is run into the flask after the apparatus has been assembled.

The purpose of a fine capillary in *C* and *D* just below the level of the joint socket will be made clear in the section on gas flow.

Gas flow. Because of the dimensions of the apparatus, the passage of gas at the rates required leads to irregular flow owing to fluctuations of pressure within the assembly. Regularity was achieved by introducing constrictions just below the joints of the delivery tubes (Fig. 1).

Reagents

Sulphuric acid. The concentrated acid (A.R. grade) has been found to give a zero blank. It should be stored away from direct light and should be shaken before use with a small amount of silver dichromate to remove reducing substances.

Silver dichromate. The commercial salt yields a large blank, owing to traces of nitrate. The following method of preparation yields a product giving zero blank values. Silver oxide is prepared from AgNO_3 by precipitation with 40% (w/v) NaOH . The precipitate is washed with water 12 times by decantation. The moist oxide is then dissolved in the minimum volume of 70% (v/v) H_2SO_4 and an excess of CrO_3 , dissolved in the minimum volume of water, is added. A dark

red precipitate of the dichromate separates at once. The flask is now placed in a boiling water bath, and air drawn rapidly through the mixture for about 3 hr. Nitrate and chloride are thus completely removed. A soda-lime tube should be attached to the air intake. The contents of the flask are then diluted and allowed to stand overnight. The precipitate is washed by decantation at least 10 times. At the end of the washing, the water should be bright yellow in colour from dissolved dichromate. The flask and contents are now dried at 90° and the silver dichromate stored in a dark bottle. The yield is almost quantitative.

Fig. 1. Apparatus for liberation of chlorine and absorption train.

Potassium iodide. A 5% (w/v) KI (A.R.) solution is used as the absorbing fluid. It should be freshly made daily in water redistilled over $\text{Ba}(\text{OH})_2$ in a glass apparatus.

Starch. A 1% (w/v) solution of starch (A.R.) is prepared by suspending 1 g. starch in 10 ml. cold glass-distilled water. About 90 ml. of water is brought to the boil and the starch suspension added rapidly to it. The solution is allowed to boil for 1 min. and then made up to 100 ml.

n-Butanol. Commercially obtainable pure *n*-butanol is suitable. It is stored in a cold room to avoid formation of aldehyde, but discarded after 1 month.

Light petroleum. The A.R. grade, boiling range 40–60°, must be purified by washing 6 times with conc. H_2SO_4 and 6 times with water. It is then dried and redistilled.

Reagents for the Grook (1934) oxidation. These are listed by Salter (1937).

Solvents. Acetone (A.R.) was redistilled over anhydrous K_2CO_3 ; ether (A.R.) was redistilled over Na.

Methanolic KOH is prepared from methanol redistilled over Na; KOH (A.R.) gives a small blank value. Each fresh batch of methanolic KOH should be standardized with respect to chlorine content.

Potassium permanganate (A.R.) is recrystallized until it yields no blank.

Microestimation (1.0–120 μg . chlorine)

Method for pure substances. The compound, in a suitable volatile solvent, is introduced into the oxidation flask (Fig. 1, A) and the solvent is evaporated carefully under reduced pressure so that no traces are left. Failure to observe this precaution will lead to violent reactions when oxidation starts and may give erroneous results. Silver dichromate (0.5–1.0 g.) is introduced and the apparatus is assembled without tube C. The absorber D is filled with KI solution (5 ml.) and water up to the shoulder of the vessel. Conc. H_2SO_4 (8 ml.) is run into the dropping funnel B, the gas flow started at 10 ml./min., and the tap of the dropping funnel turned so that the acid is forced into the flask. The flask is now heated to 110° in a glycerol bath. Gas flow is continued for 30 min. after the correct bath temperature is reached. The liberated I_2 is then estimated. For amounts of 1–100 μg . Cl a direct reading is easily possible using the starch-iodine blue colour and an absorptiometer. For amounts over 100 μg . titration with 0.005 N- $\text{Na}_2\text{S}_2\text{O}_3$ is recommended.

Estimation of the starch-iodine blue colour. The contents of the absorber are transferred completely to a volumetric flask which is filled nearly to the mark with water and 0.5 ml. of 1% (w/v) starch added. The volume is made up, the contents of the flask well mixed, and any blue colour which develops read within 10 min. at 25°. Using a single-cell photoelectric absorptiometer with 2 cm. cells and an Ilford red filter (no. 204), it was found that a 25 ml. final volume gave a readable blue colour within the limits 1–25 μg . Cl; with a final volume of 50 ml. the upper limit is 50 μg . and a final volume of 100 ml. raises this to 100 μg .

Effect of iodide concentration. Bairstow, Francis & Wyatt (1947) have shown that in the presence of a large excess of KI (or indeed of any salt) starch will give a purple-red colour with iodine. Conway (1935) has used this colour in his micro-determination of Cl, but with the absorptiometer used in these experiments the readings obtained were found to deviate from Beer's law. The bearing of this point on the present method is that with a final volume of less than 25 ml. and in the presence of 5 ml. of 5% (w/v) KI, the starch-iodine complex gives a purple-red colour; with the dilutions and quantity of KI recommended in the present paper a satisfactory blue colour is obtained.

Effect of temperature. In accordance with the findings of other workers (Bairstow *et al.* 1947; Sendroy, 1937), the starch-iodine blue colour has been found to vary in intensity with temperature. A working temperature of 25° gives reliable results.

Addition of starch. Starch should be added just before the volume of the solution is finally adjusted, otherwise a purple-

red colour may be obtained instead of the usual blue; further dilution does not necessarily result in the conversion of the former to the latter colour.

Time of reading. In contrast to other work (Bairstow *et al.* 1947; Talbot, Butler, Saltzman & Rodriguez, 1944) it has been found that the blue colour is fairly stable in neutral solution and hence the time of reading is immaterial within wide limits. Fig. 2 illustrates this point. Curve A was obtained from repeated estimations of the starch-iodine blue colour corresponding to 10 μg . chlorine. In contrast, curve B was obtained by the action of acid iodate on KI and indicates a steady deepening of the starch-iodine blue colour with time as was expected, since KI decomposes in acid solution.

Fig. 2. Effect of time on starch-iodine blue colour. Iodine equivalent to 10 μg . chloride. A, starch-iodine blue colour in neutral solution; B, starch-iodine blue colour obtained by action of acid KIO_3 on KI.

Calibration curves. These should be prepared separately for each final volume by carrying out a series of determinations on a standard substance and checked by parallel readings on a standardized iodine solution. The calibration curves obtained by the present method have all been straight lines passing through the origin.

From the results of the analysis of two chlorine-containing organic compounds it will be seen that satisfactory accuracy is obtainable (Table 1).

Ultramicro method

For the ultramicro procedure, certain changes are necessary. While it is possible to use the above-described absorber (Fig. 1, D) with good results one of the same pattern, but of half the volume, is more convenient.

Table 1. *Determination of the chlorine content of two organic substances by the micro method*

Amount ($\mu\text{g.}$)	Calc. ($\mu\text{g.}$)	Cl content			No. of determina- tions
		Found			
		Mean ($\mu\text{g.}$)	S.D. ($\mu\text{g.}$)	Range ($\mu\text{g.}$)	
DDT					
120	60.0	60.25	1.89	57-61	4
100	50.0	50.10	1.57	48-52	6
75	37.5	38.30	1.00	37-39	3
50	25.0	24.80	0.80	24-26	5
25	12.5	12.75	0.27	12-13.5	6
10	5.0	4.80	0.35	4.5-5.3	4
5	2.5	2.63	0.29	2.3-3.0	4
<i>p</i> -Chlorobenzoic acid					
499.0	113.0	114.25	1.89	113-117	4
249.5	56.5	56.75	1.26	55-58	4
99.8	22.60	22.90	1.72	21-24	3
49.9	11.30	11.13	0.713	10.3-11.6	3

In this method the iodine is extracted from potassium iodide solution by an immiscible solvent; the solvent is then washed free from iodide and the iodine converted to iodate. The starch-iodine blue colour is used for its determination. In a final volume of 25 ml. the intensity of the blue colour allows the estimation of chloride as low as $0.25\mu\text{g.}$ With the Rehberg or similar microburette, a titrimetric method can be used.

The technique up to the extraction of the iodine is similar to that already described for the micro procedure. Silver dichromate (250-500 mg.) with 5-8 ml. H_2SO_4 is used as the oxidizing mixture. After the passage of the gas for 30 min., the fluid in the absorber is rinsed into a small separating funnel with two washes of water. Extraction is accomplished with a mixture of light petroleum (45 vol.) and *n*-butanol (55 vol.); vigorous shaking should not be used. The butanol-petroleum layer is transferred to a second separating funnel, and the aqueous layer extracted once more with solvent mixture and the extracts combined. The latter are generally opaque and may be cleared by washing with saturated Na_2SO_4 solution. Seven further washings are carried out, water and Na_2SO_4 solution being used alternately with vigorous shaking. I_2 is next converted to iodide by adding to the separating funnel 0.5 ml. $\text{m-K}_2\text{CO}_3$ and 0.5 ml. 0.1 *M*-sodium metabisulphite. The two layers are mixed and allowed to separate. The aqueous layer is run into a small vessel, to which is added two further washes of 1 ml. portions of water. Increase in the volume of aqueous washes to more than 3 ml. and violent shaking should be avoided since they increase the amount of the organic solvents in the aqueous layer and these may interfere with the subsequent oxidation of iodide to iodate according to Groak (1934).

The iodide-containing solution is now heated on a boiling water bath for 30 min., after which there should be little of the organic solvents present. The vessel is now transferred to a water bath at 80° , and 15-20 drops of N-KMnO_4 are carefully introduced directly into the liquid. The solution should remain pink. The vessel is kept at 80° for 4 min., and then 6 drops of 8 *N*- H_2SO_4 are added down the side of the vessel and the latter replaced in the water bath for 4 min. The solution should remain pink to the end of this step.

NaNO_3 (0.75 *N*) is now added dropwise with shaking until the solution is colourless. The sides of the vessel are rapidly washed down with a stream of water, followed by 3 drops of 5 *M*-urea. The vessel is again heated on a boiling water bath for 30 min. and then put in ice. When cold, the solution is transferred to a 25 ml. volumetric flask and 0.5 ml. of 1% (w/v) starch solution added, followed by water nearly up to volume, 0.5 ml. 1% (w/v) KI and water to 25 ml. The intensity of any blue colour developed is read within 5 min. at 25° . Since the colour is developed in an acid medium, the time of reading is critical.

In carrying out this series of steps it is essential to adhere rigidly to the technique. Where the extraction of I_2 is concerned, for example, it is a matter of convenience what volumes of the petroleum-butanol mixture are used, except that washing free of iodide is facilitated by the use of small volumes; on the other hand, the removal of the I_2 from the organic solvents must be carried out in the minimum of the aqueous solution.

The Groak (1934) reaction must be carried out with the utmost care, the solutions being added according to direction; only the 8 *N*- H_2SO_4 should be run down the side of the vessel, all the other reagents being added directly to the solution. It must be stressed that the solution should remain pink during the first 8 min. of the process: this should be the case if the organic solvents have been removed. The analytical results given in Table 2 illustrate the degree of accuracy obtainable.

Table 2. *Determination of chlorine content of DDT by the ultramicro method*

Amount of DDT ($\mu\text{g.}$)	Calc. ($\mu\text{g.}$)	Cl content			No. of determina- tions
		Found			
		Mean ($\mu\text{g.}$)	s.d. ($\mu\text{g.}$)	Range ($\mu\text{g.}$)	
2.00	1.0	0.92	0.265	0.8-1.3	5
1.50	0.75	0.75	0.234	0.6-0.85	5
1.00	0.50	0.525	0.32	0.4-0.6	4
0.50	0.25	0.25	0.186	0.2-0.35	3

The determination of DDT

Gunther (1945) showed that when DDT is incubated with alcoholic potassium hydroxide, the following reaction takes place:

Wain & Martin (1947) showed that this reaction takes place at room temperature. Following Conway (1935), the inorganic chloride so liberated was oxidized in the cold with acid permanganate; the organic by-product of the reaction and DDT itself are not attacked by the cold mixture. Volatilization of chlorine from the reaction flask depends upon thorough agitation of the acid permanganate mixture by the gas stream. A spiral (Fig. 1, A), wound round the stem of the dropping funnel, ensures this and increases the effective surface. The flask is filled with the oxidizing mixture so that the fluid level is well up the stem.

The technique which follows is also applicable to the determination of inorganic chloride.

Procedure. A solution of DDT is placed in the oxidation flask and the solvent evaporated. Methanolic KOH (0.1 N) is added and the mixture maintained at 30° for 0.5 hr. A little water is then added and the methanol removed under reduced pressure. Where amounts below 25 µg. DDT are analysed, 0.02 N-KOH may be used. When evaporation is complete, 12–15 ml. of a mixture of saturated KMnO_4 (1 vol.) and 25% (v/v) H_2SO_4 (1 vol.) is run in, and the determination carried out as for organically bound Cl but without heating.

The analyses recorded in Table 3 illustrate the applicability of the method.

Table 3. *Determination of alkali-labile chlorine of DDT and of sodium chloride*

Amount (μg.)	Calc. (μg.)	Cl content			No. of determina- tions
		Found			
		Mean (μg.)	s.d. (μg.)	Range (μg.)	
DDT					
100.0	10.0	9.60	0.314	9.0-11.5	6
50.0	5.0	4.97	0.182	4.0-6.3	6
10.0	1.0	0.97	0.22	0.8-1.3	6
5.0	0.5	0.51	0.31	0.4-0.65	6
2.5	0.25	0.24	0.19	0.2-0.35	4
NaCl					
116.92	70.92	69.6	1.7	67.0-72.0	6
58.46	35.46	35.6	1.29	34.0-37.0	6
29.23	17.73	17.7	0.53	17.0-18.7	6
11.69	7.09	6.9	0.118	6.5-7.5	6
5.846	3.546	3.6	0.120	3.0-4.0	6
1.169	0.709	0.70	0.103	0.60-0.85	6

Estimation of DDT and allied substances in tissues

The methods described above can be applied to extracts made from tissues. Certain modifications of the procedure are required. The tissue extracts usually contain a rather large amount of fatty and other substances which react violently with the oxidizing mixture to liberate sulphur dioxide. This interferes with the liberation of iodine in the absorber. To obviate this risk, a central absorber (Fig. 1, C) is placed between the oxidation flask and the absorption vessel containing potassium iodide solution. It contains about 10–15 ml. of a mixture of saturated potassium permanganate solution (2 vol.) and 50% (v/v) sulphuric acid (1 vol.). This absorbs all the reducing agents volatilized, but not the chlorine.

Extractions of tissues. Tissues (1–2 g. wet tissue, or as much as is available) are ground with anhydrous Na_2SO_4 (A.R.) until a freely flowing dry powder is produced. During the grinding, enough 8N- H_2SO_4 is added to bring the pH to 2. The powder is transferred completely to a flask and extracted by three successive 35 ml. portions of boiling ether. Each portion of ether is allowed to cool and filtered into a second flask through a filter paper (Whatman no. 1).

The combined filtrates are transferred to a separating

funnel and shaken with 10 ml. 10% (w/v) NaOH. The aqueous layer is run into a second separating funnel. The ethereal layer is washed with 10 ml. water which is added to the alkaline wash. The combined aqueous washes are extracted with 10 ml. ether and the ethereal extract added to the first separating funnel. The combined ethereal extracts are washed twice with water and evaporated, the residue taken up in acetone, filtered if necessary and adjusted to a convenient volume (10–20 ml.). This solution contains all the neutral organic Cl. Portions are used to determine total organic Cl and DDT (as alkali-labile Cl). A determination of entrained inorganic Cl, i.e. Cl fortuitously extracted from the tissue, is also carried out and the values for the total and alkali-labile Cl are corrected by this amount. The combined alkaline extract, containing alkali-soluble organic Cl, is acidified to pH 2 with 8N- H_2SO_4 and extracted by shaking vigorously with an equal volume of ether. The ethereal solution is evaporated, the residue taken up in acetone, which is filtered if necessary and portions of the filtrate analysed for total and inorganic Cl, the value for alkali-soluble organic Cl being obtained by deduction.

Table 4. *Recovery of DDT and di(p-chlorophenyl)-acetic acid (DDA) added to wet tissues*

(Wt. of wet tissue, 2 g. in each experiment.)

Tissue	Wt. of substance added to tissue		Wt. found	
	DDT (µg.)	DDA (µg.)	DDT (µg.)	DDA (µg.)
Kidney	25.0	50.0	25.0	52.0
	10.0	25.0	11.0	23.5
	5.0	0.0	5.0	0.0
Liver	50.0	22.5	55.0	25.0
	10.0	5.0	11.0	4.5
	25.0	100.0	27.0	103.0
Muscle	40.0	55.0	36.0	52.0
	0.0	25.0	0.0	22.0
	50.0	60.0	54.0	57.0
Brain	20.0	10.0	20.0	8.0

Table 4 illustrates the recovery from tissues of added DDT and DDA. It will be seen that satisfactory results can be obtained.

DISCUSSION

The apparatus described resembles that of Krainick & Zacherl (1937) but the dimensions differ, and the introduction of the intermediate absorber makes it possible to apply the method to the analysis of tissue extracts. The differences in the mode of determination may be put shortly by saying that the Krainick & Zacherl method is intended to determine relatively large amounts of chlorine in a small amount of organic material, while the present method is designed for the determination of small amounts of chlorine in relatively large amounts of organic material.

The accurate application of the present method to tissue determinations depends on the securing of tissue extracts which are relatively free from inorganic chloride. However, a blank is always

obtained and parallel determinations of inorganic chloride must be carried out. Using the micro-method, a direct determination of $1.0\text{ }\mu\text{g.}$ chlorine is possible. The ultramicro method is intended for use with amounts of tissue providing less than $1.0\text{ }\mu\text{g.}$ of chlorine, e.g. tissue slices. It is hoped that, by reducing the size of the apparatus and by modifying the determination of iodine, amounts of chlorine down to $0.1\text{ }\mu\text{g.}$ may be determined. Such a technique might be useful for investigations on the metabolism of DDT in insects.

It should be pointed out here that the application of the method to substances other than those specifically mentioned may be limited by their volatility. For example, consistently low results were obtained when estimations were attempted on chloroacetamide. This was attributable to volatilization of the substance before complete oxidation could take place.

SUMMARY

1. Methods are described for the micro and ultra-micro determination of chlorine in DDT and related compounds, applicable to amounts of $1\text{--}120\text{ }\mu\text{g.}$ and $0.25\text{--}1.0\text{ }\mu\text{g.}$ of chlorine, respectively.

2. The techniques have been successfully applied to the analysis of tissues containing these substances.

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Studies in Detoxication

25. THE CHARACTERIZATION OF PHENYLGLUCURONIDE, AND ITS RATE OF HYDROLYSIS COMPARED WITH THAT OF PHENYLSULPHURIC ACID

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The present work was carried out for two reasons. First, investigations in this laboratory on the metabolism of benzene showed that 11% of orally administered benzene was excreted by rabbits as glucuronides (Porteous & Williams, 1949a), and Schmiedeberg (1881) isolated from the urine of a benzene-fed dog a crystalline substance which was probably phenylglucuronide. We therefore required phenylglucuronide as a reference compound for further investigations on benzene. Secondly, phenylglucuronide is used as a standard substrate for the

assay of β -glucuronidase in tissues (see Kerr, Graham & Levvy, 1948; Mills, 1948). After a search of the literature we felt that phenylglucuronide had not been properly described.

Phenyl- β -D-glucuronide was first isolated by Klz (1890) from the urine of rabbits receiving phenol by injection. His material melted at 148° , but he attributed the wrong formula, $\text{C}_{12}\text{H}_{16}\text{O}_7$, in accordance with his analytical figures. It was synthesized from phenol and acetobromoglucurone by Neuberg & Niemann (1905), who described it as anhydrous, m.p. $150\text{--}151^\circ$, $[\alpha]_D^{17} - 83.3^\circ$ (solvent not mentioned). For material isolated from the urine of a sheep fed with phenol, Salkowski & Neuberg (1906) gave m.p. $148\text{--}150^\circ$, $[\alpha]_D - 81.9^\circ$ (solvent not mentioned) and analytical figures for the

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