A New Manometric Method for Determination of Respiratory Quotients

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The Estimation of Trichloroethylene in Blood

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A review of the literature on the estimation of chloroform, carbon tetrachloride and trichloroethylene is given by Daroga & Pollard (1941) and by Habgood & Powell (1945). The methods employed depend upon (i) pyrolysis of the halogen compound and absorption of the resulting gases in alkali or alkaline reducing agents, or (ii) hydrolysis by boiling alkali—followed in each case by the estimation with silver nitrate of the halide ion produced—or (iii) the measurement of the colour formed in the upper layer on heating solutions of these substances in an inert solvent with pyridine and a solution of sodium hydroxide of such a concentration as will form a two-phase system, a reaction first described by Fujiwara (1914). Of these the third has been most extensively used in recent years. Daroga & Pollard (1941), after a very thorough investigation of the conditions, developed the method for the estimation of chloroform and carbon tetrachloride in acetone solution, whilst Habgood & Powell (1945) used it to estimate these and also trichloroethylene dissolved in toluene.

Since 1944 further papers have appeared which have, however, added little to the technique of the colour development process devised by these authors. Webb, Kay & Nichols (1945) defined optimum conditions for colour development with standard acetone solutions of various halogen compounds, and recorded minimum concentrations of each which they could detect, but gave no details of their method. Rogers & Kay (1947) recorded the use of a single-phase reagent for carbon tetrachloride prepared by

shaking pyridine with 15 % (w/v) sodium hydroxide, separating the upper layer, and diluting somewhat with water. Colour development was brought about by heating 10 ml. of this reagent with 5 ml. of an acetone solution of carbon tetrachloride at 70° for 15 min. The reagent deteriorated on keeping and the method appears to offer no special advantage.

In connexion with an investigation of the use of trichloroethylene in midwifery by one of us, it was necessary to determine as accurately as possible the concentration of this drug in maternal and foetal blood when only at an analgesic level, bearing in mind that, as the volume of foetal blood available for estimation would be no more than a few millilitres, the amount of drug to be estimated would be extremely small. To this end the method of Habgood & Powell has been reinvestigated to determine its applicability, using only 1 ml. blood samples at low trichloroethylene concentrations. Unexpected difficulties arose and the method of colour development has consequently been considerably modified.

Three methods have been recorded for the recovery of chlorohydrocarbons from blood for the purpose of estimation. Habgood & Powell (1945) steam-distilled the blood sample and collected trichloroethylene in toluene; Kulkarni (1944) swept out chlorohydrocarbons into ice-cold pyridine by means of a stream of air, and, since the present work was concluded, Burgen (1948) has claimed considerable advantages for a method utilizing the Conway microdiffusion technique with an accuracy of $\pm 5\%$

for chloroform; no recovery figures are quoted for trichloroethylene. We have found the steam-distillation method to give excellent results with a modified form of the apparatus described by Habgood & Powell.

EXPERIMENTAL

In Habgood & Powell's (1945) method of estimation, toluene extracts of trichloroethylene were heated with pyridine and 20% (w/v) NaOH. After cooling and separating the alkali the coloured solution, which was turbid, was cleared by diluting with water to 15 ml.; at times it was necessary to add more water, in which case the final volume was noted and a correction made. The present work has shown that it is not always possible to disperse the turbidity completely in this way, and in any case it is doubtful whether, in a method dependent entirely on arbitrary conditions, such a variation in the composition of the solution is justifiable. Further, whereas the colour obtained was mostly the orange-red described by Habgood & Powell, there appeared not infrequently a bluish red tint which persisted for several hours. Burgen (1948) has also referred to the much smaller extinction given by trichloroethylene compared with CHCl_3 when determined by this method, though the two substances appear to give much the same colours in qualitative tests using the Fujiwara (1914) reaction. Moreover, the method also entailed the quantitative separation of the coloured layer from the alkali, a process at best of doubtful efficiency. The conditions of colour formation have therefore been reinvestigated with the following conclusions.

(1) Increase in the concentration of NaOH decreased the rate of colour formation, but eliminated the turbidity with solutions of concentration at least 7M. With 10M-NaOH no colour resulted unless the two layers were mixed by shaking or stirring during heating. (2) A high ratio of pyridine to toluene, or other organic solvent, led to the rapid formation of the orange-red colour, whereas a low ratio resulted in the slow formation of the blue-red colour. (3) The blue-red colour faded very rapidly in sunlight, but was stable in subdued daylight and in artificial light. (4) Anisole, of a limited number of solvents tried, appeared to be preferable to toluene. (5) Very high alkali concentration in the pyridine, resulting from the use of 2M-tetraethylammonium hydroxide, caused a blue colour of high extinction to appear slowly in the cold. This only attained maximum intensity in about 1 hr. and was rapidly changed to the orange-red on gentle warming.

In accordance with these findings the method of colour development described below was adopted.

Materials

Pyridine. 'Pure anhydrous' or A.R. pyridine was dried over KOH (sticks) and redistilled. Lower grade pyridine should be avoided.

Anisole was washed twice with dilute NaOH, three times with water, dried over anhydrous CaCl_2 and redistilled.

Sodium hydroxide. Approx. 45% (w/v) NaOH was standardized with acid and then diluted to 10M.

Tetraethylammonium hydroxide reagent was prepared by adding to 10M-NaOH sufficient of the quaternary hydroxide solution to make it 0.025M with respect to that base.

Apparatus

In the early stages of the recovery of trichloroethylene from blood by steam distillation, using the apparatus of Habgood & Powell (1945), much air will inevitably escape somewhat rapidly through the absorbing liquid until thermal equilibrium is attained within, and in the present work there

Fig. 1. Apparatus for the estimation of trichloroethylene in blood.

was evidence that a small amount of trichloroethylene could be lost during this period. To overcome this difficulty a somewhat more elaborate absorption apparatus has been devised and incorporated as receiver in place of the calibrated cylinder employed by Habgood & Powell (Fig. 1). The dropping funnel used to contain water with which to wash down the inside of the condenser has also been replaced by a Quickfit B 19 socket and stopper. This apparatus, in which the effluent air was washed through two lots of anisole which could be mixed prior to sampling, had two chambers; A (25 mm. internal diam., 20 cm. long and about 90 ml. capacity) joined by 6 mm. tubing and a stopcock (C, 5 mm. bore) to B (12 mm. internal diam., 12 cm. long and about 20 ml. capacity). A and B terminated in B 19 and B 14 ground sockets respectively. A side tube (D, 3 mm. diam.) left A at a point approx. 5 cm. down the side, passed through

the wall of *B* and was bent and drawn off to a jet of 1 mm. diam. to form a bubbler within *B*. *A* was graduated to indicate when approx. 40 ml. of distillate had collected.

Method for blood analysis

The tap *C* was freed from grease and lubricated with a little Aquadag, the surplus of which was removed by running water through the apparatus whilst rotating the tap until the water discharged was no longer darkened. The dead space between *A* and *B*, comprising the whole of the 6 mm. tube and the tap *C*, was filled via *B* with saturated K_2CO_3 solution and *C* was closed. The volume of this solution should not be less than 5 ml. and any surplus over that required to fill the dead space was allowed to collect in *A*. The K_2CO_3 solution also ensured, by increasing the otherwise very slight difference of density between the aqueous and anisole layers, that the latter remained on top throughout the distillation and facilitated its rapid separation after mixing. Exactly 9 ml. (later reduced to 8 ml.) of anisole were run in, of which roughly 3 ml. went into *B* and the rest into *A*, and the apparatus, surrounded by ice water, was then attached by a rubber stopper to the condenser of the steam-distillation apparatus so that the end of the condenser dipped almost to the bottom of the anisole in *A*.

The blood sample was measured from a 1 ml. blood pipette (which had been calibrated by weighing blood of known density delivered from it) into a 250 ml. round flask with standard *B* 24 ground neck, and diluted with 50 ml. distilled water as described by Habgood & Powell (1945). A few drops of tri-*n*-butyl citrate were added to control frothing and the flask attached to the distillation apparatus. The stopper at the top of the condenser was wetted with water and replaced, and steam was passed slowly through the flask until air ceased to be expelled through the solvent in *B*, after which the distillation was allowed to proceed rapidly until 40 ml. of distillate had collected. The absorption apparatus was then detached and the condenser washed down inside (via the socket at the top) and outside with distilled water which was allowed to drain into *A*. *A* and *B* were stoppered, the tap *C* opened and the contents of *B* run into *A* as completely as possible; with *C* now closed the whole contents of *A* were mixed thoroughly by inversion. Part of the anisole was then transferred to *B* via *D* by loosening the stoppers and tilting the apparatus. *B* was rinsed and the anisole returned to *A* via *C* and remixed. This rinsing operation was repeated twice, and after a final mixing the two layers were allowed to separate completely in *A*.

Colour development

The anisole solution of trichloroethylene (5 ml.) was added to dry colourless redistilled pyridine (5 ml.) and 10M-NaOH (3 ml.) in a 6 in. $\times$ $\frac{3}{4}$ in. Pyrex test tube which was immediately placed in a boiling water bath and the mixture stirred mechanically for 10 min. at such a rate as to ensure thorough mixing of the two layers. This and subsequent operations had to be carried out in subdued daylight or in artificial light since bright sunlight caused rapid fading of the colour. The water bath was then removed and exactly 1 ml. of tetraethylammonium hydroxide reagent added to the mixture. Stirring was continued for exactly 1 min. and the tube removed and thoroughly cooled. The two layers separated rapidly and the upper pyridine layer, which was invariably free from turbidity, was removed by a pipette which had

been swept out with CO_2 -free air, and delivered into a 1 cm. absorptiometer cell by admitting CO_2 -free air to the pipette. Ten minutes prior to use, 0.5 ml. of 10M-NaOH had been placed in the cell and covered with a vaselined slide which was replaced after transferring the solution. The colour intensity was measured immediately with a Spekker photoelectric absorptiometer using a water cell in comparison and Ilford spectrum blue-green filter no. 603. The colour, which was a bright cherry-red (absorption being mainly in the yellow with some general absorption in the green of the spectrum) began to fade in subdued light after about 20 min., allowing ample time for a series of readings to be taken. The first reading of a series was sometimes a trifle low.

Calibration of the absorptiometer

Standard solutions of trichloroethylene were prepared as follows. The mean weight (*W*), of anisole delivered by the 5 ml. pipette reserved for this solvent was determined. A sealed thin-glass ampoule containing about 80 mg. of trichloroethylene, accurately weighed, was broken under a known weight of anisole in a stoppered bottle and the solution thoroughly mixed; this primary standard was then broken down in steps by weight until a solution was obtained containing exactly 0.10 mg. of trichloroethylene/*W* g. solution. From this, substandards were prepared by dilution by volume, using always the same pipette for both trichloroethylene solution and pure anisole. Samples (5 ml.) of each substandard solution were submitted to the colour development process described above, and duplicate Spekker readings so obtained are recorded in Table 1. As these gave good agreement at all concentrations tested a more extended series was not considered necessary. The absorptiometer readings (*S*) and trichloroethylene concentration in mg./5 ml. anisole (*T*) were strictly proportional up to a concentration of at least 0.10 mg./5 ml., as was shown by plotting, and obeyed the relationship $T = 0.1434 S$. The values of *S* calculated from this for each value of *T* are included in the last column of Table 1.

Table 1. Calibration of Spekker photoelectric absorptiometer using standard solutions of trichloroethylene in anisole

Trichloro- ethylene (mg./5 ml. anisole, <i>T</i>)	Spekker readings (<i>S</i>)				Calc. from $T = 0.1434 S$
	Found				
	(i)	(ii)	Mean		
0.100	0.699	0.699	0.699	0.697	
0.075	0.518	0.526	0.522	0.523	
0.050	0.347	0.353	0.350	0.349	
0.040	0.276	0.280	0.278	0.279	
0.025	0.175	0.175	0.175	0.174	
0.013	0.092	0.092	0.092	0.093	
Nil	0.002	0.002	0.002	0.000	

RESULTS

Anticipating a variation of recovery from blood samples, the efficiency of the method was first tested by distilling 1 ml. of standard anisole solutions of trichloroethylene. The results, recorded in Table 2, established that the relationship $T = 0.1434 S$, obtained with dry anisole standards, applied equally well to wet solutions and that recovery was essentially complete.

Table 2. *Recovery of trichloroethylene from steam-distilled standard anisole samples*

(Volume of standard taken, 0.988 ml. Volume of anisole used for absorption, 8 ml.)

Trichloroethylene in sample (mg.)	Spekker readings	Trichloroethylene recovered (mg.)	
			Mean
0.175	0.690	0.178	0.176
	0.673	0.174	
0.087	0.332	0.086	0.087
	0.341	0.088	
0.044	0.163	0.042	0.043
	0.169	0.044	
Nil	0.006	0.002	0.002
	0.006	0.002	
0.173*	0.675	0.174	—

* Distilled in the presence of 5 ml. blood.

Table 3 records the recovery of trichloroethylene from standard solutions in blood in the preparation of which the following routine was observed.

Table 3. *Recovery of trichloroethylene from standard solutions in blood*

(Volume of standard taken, 0.976 ml. Volume of anisole, 9 ml.)

Trichloroethylene concentration (mg./ml.)	Spekker readings		Trichloroethylene recovery (mg./ml.)		
	(i)	(ii)	(i)	(ii)	Mean
0.126	0.483	0.471	0.128	0.125	0.126
0.063	0.242	0.233	0.064	0.062	0.063
0.032	0.121	0.118	0.032	0.031	0.032
0.014	0.058	0.051	0.015	0.014	0.014

A primary standard was prepared by breaking a thin-walled ampoule containing a known weight (30–40 mg.) of trichloroethylene under 250 ml. of blood in a stoppered bottle which was then mechanically shaken for 8 hr. to ensure uniformity. The primary standard was diluted with fresh blood in steps by weight and each newly diluted solution similarly shaken for 2 hr. Each standard was well shaken by hand immediately before sampling and any which had stood overnight were shaken mechanically for 15–20 min.

Table 4. *Recovery of trichloroethylene from blood stored over a period of 4 days*

(Volume of blood taken, 0.976 ml. Volume of anisole, 9 ml.)

Time of estimation	Trichloroethylene concentration (mg./100 ml. blood)
Immediately after drawing	3.89, 3.84
3 days after drawing	3.94
4 days after drawing	3.89
Mean	3.89 ± 0.05

In preparation for future work the keeping power of standard blood solutions was also investigated, and it was shown that such solutions maintained their trichloroethylene concentration over a period of several days if kept in well-stoppered bottles in a cool place. The keeping power is illustrated by the typical values obtained with a sample of blood taken from a patient receiving the drug (Table 4).

DISCUSSION

The addition of tetraethylammonium hydroxide was rendered necessary by the sensitivity of the coloured material to carbon dioxide. The amount of alkali in the pyridine-anisole layer was so much reduced by the use of 10M-sodium hydroxide that, in the process of transferring the coloured layer to the absorptiometer cell, sufficient carbon dioxide was absorbed to neutralize it and the blue-red colour, which is formed only in alkaline solution, changed to a pale yellow. The measures adopted to exclude carbon dioxide were taken as additional precautions for the same reason. Kulkarni (1944) has also commented similarly on the effect of acid fumes.

Since the final method depended for accuracy on the constancy of volume of the pyridine-anisole layer, as did that of Daroga & Pollard (1941), it was necessary that conditions should be exactly reproduced throughout. Care was therefore taken to ensure that there could be no variation in the amount of water taken up from the alkali solution by the pyridine-anisole layer by using always exactly 10M-sodium hydroxide which had been checked by titration.

A somewhat simpler procedure for colour development was also investigated in which the pyridine-anisole solution and alkali were not stirred during heating. This gave reproducible absorptiometer readings proportional to the trichloroethylene concentration up to about 0.05 mg./5 ml. anisole, but above this the absorption varied considerably from one estimation to another and was always too large to fit the proportionality of the lower concentrations. Such solutions were invariably more blue than normal, implying that, with greater amounts of trichloroethylene, some probably escaped reaction until the addition of the tetraethylammonium hydroxide reagent, thus leading in part to the formation of the highly absorbing blue compound mentioned above. For this reason mechanical stirring was adopted.

SUMMARY

1. New conditions have been described for the quantitative production of the coloured compound from trichloroethylene and pyridine in the presence of alkali.

2. A more efficient apparatus has been described for the absorption of trichloroethylene distilled from blood in steam. The apparatus could be used for the recovery of traces of other volatile liquid compounds.

3. It has been shown that the concentration of

trichloroethylene in approximately 1 ml. blood samples can be estimated to the nearest $\mu\text{g.}$ at concentrations between 1 and 12 mg./100 ml.

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Vitamin P. 3

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As a result of some clinical experiments on certain pathological conditions characterized by increased permeability of the capillary walls, Armentano, Bentsáth, Béres, St Rusznyák & Szent-Györgyi (1936) and St Rusznyák & Szent-Györgyi (1936) formulated the theory of the existence of a substance called 'vitamin P' capable of increasing the resistance of the capillaries. They arrived at this conclusion because the permeability either to the whole blood (vascular haemorrhagic purpura) or to plasma protein only (various septic conditions) in their patients was decreased by treatment with extracts of Hungarian red pepper, lemon juice or fractions obtained from these extracts. On the other hand, L-ascorbic acid according to them was ineffective. This hypothesis was next claimed to be substantiated by experiments on guinea pigs by Bentsáth, St Rusznyák & Szent-Györgyi (1936, 1937). As the data obtained with the guinea pigs did not appear convincing, the writer (Zilva, 1937*a, b*) repeated the experiments and showed that the results obtained by Szent-Györgyi *et al.* with these animals did not justify the interpretation placed on them. Moll (1937), Detrick, Dunn, McNamara & Hubbard (1940), McHenry & Perry (1940) and even Szent-Györgyi (1938), on repetition of his own experiments, arrived at a similar conclusion.

In spite of the negative character of the above investigations a claim that a 'vitamin P' deficiency could be demonstrated by the 'capillary resistance' technique in guinea pigs was made by Zacho (1939). This procedure had been tried for a number of years by many workers to demonstrate vitamin C

deficiency in man, but no agreement had been reached as to the reliability of the test for this purpose. Zacho, however, claimed that when negative pressure was applied to the lumbar region on each side of the spine of albino guinea pigs after the removal of the hair, the pressure necessary to produce petechiae in scorbutic animals in the 'latent stage' and more so in the 'manifest stage' of scurvy, was lower than that which produced a similar response in normal guinea pigs. Furthermore, when L-ascorbic acid was administered to the animals in this condition, the 'capillary resistance' rose but did not reach the normal level. The normal 'capillary resistance' could only be attained when 'citrin' (Szent-Györgyi's 'vitamin P') prepared from rose hips was given either orally or intraperitoneally. Several workers (St Rusznyák & Benkó, 1941; Bacharach, Coates & Middleton, 1942; and others), also using the negative pressure technique, arrived at a conclusion similar to that of Zacho. Zacho's work called for repetition, and it is the purpose of this communication to record some representative results obtained by the writer, which demonstrate the difficulty of obtaining reproducible or significant results by these methods.

An experiment was also performed with maclurin because of the claim made by Lavollay (1944), Parrot & Lavollay (1944) and Javillier & Lavollay (1946) that a single dose of any of a number of substances, not all chemically related to 'citrin', is capable of raising the 'capillary resistance' in guinea pigs as manifested by Zacho's negative pressure technique. This, according to them, is due