Determination of DDA in Urine Using an Ion Exchange Resin

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A routine method for extracting DDA from urine was developed from studies on the fate of DDT in humans and animals. Extractions using organic solvents resulted in the formation of stubborn emulsions. Absorption of the urine on a solid absorbent for continuous liquid extraction was not feasible because of the large samples needed. It was found that an ion exchange resin will adsorb DDA quantitatively from urine under certain conditions. The DDA is then desorbed from the resin and estimated by the Schechter-Haller method.

ANIMALS (*3*, *5*) AND HUMANS (*2*) excrete bis (*p*-chlorophenyl) acetic acid (DDA) upon oral ingestion of 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane (DDT). This fact is potentially very useful as an aid in studies of the fate of DDT in higher animals, and it could also have great practical value in the diagnosis of suspected cases of DDT poisoning in humans or animals. In connection with the extensive program of dietary application of DDT to animals and humans carried on by this laboratory, an opportunity arose to study the excretion of DDA under controlled conditions of DDT ingestion.

Previous work on isolating DDA from urine has utilized a solvent-extraction method. Experience indicated that this method would not be suitable for routine analysis, and other methods were investigated.

DDA responds to the Schechter-Haller method for DDT, giving the same red complex as 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE). Work by this laboratory has failed to show the presence of DDT or DDE in the urine of rats maintained on DDT-fortified diets. Although trace amounts of metabolites other than DDA have been reported in urine (*3*), the red color developed by the Schechter-Haller method can be assumed to arise from DDA and calculations can be made on this basis. Thus the principal problem was the isolation of the DDA from the urine.

Final Method

Reagents. Amberlite IRA-400
Ethyl alcohol, 95%
Benzene, C.P.

Alcoholic acetic acid (200 ml. of glacial acetic acid made up to 1 liter with ethyl alcohol)

Apparatus. Special heating unit designed to heat a 250-ml. Erlenmeyer flask from the sides (Figure 1). Powerstat.

Gas dispersion tube with fritted-glass disk of coarse porosity.

Procedure. A sample of urine (not more than 100 ml.) containing not less than 5 γ of DDA is placed in a 250-ml. Erlenmeyer flask equipped with a $\overline{\$}$24/40 joint. Two grams of Amberlite IRA-400 is added, and the flask is inserted in the special heating unit and then connected to a reflux condenser. The urine is heated at 90° to 95° C. for 1 hour. (This temperature is just below the foaming point—i.e., boiling point.) The samples are then placed on a mechanical shaker while still hot and shaken for 1 hour, removed, the urine is aspirated off using the fritted glass tube, and 50 ml. of the alcoholic–acetic acid solution is added. The samples are then refluxed for 1 hour by means of the special heating units.

The alcoholic solution is decanted and filtered while hot into a 250-ml. glass-stoppered bottle. A minimum of ethyl alcohol (10 to 15 ml.) is used to make the transfer quantitatively. Approximately 100 ml. of water is added and exactly 50 ml. of benzene is pipetted into the bottle, the mixture is shaken vigorously and the phases are allowed to separate. The shaking is repeated three times to ensure complete extraction. The lower layer is removed by aspiration. The benzene phase is washed twice with water, the aqueous layer is removed by aspiration and anhydrous sodium sulfate is added to dry the benzene solution.

A 40-ml. aliquot of this solution is transferred to a test tube, 10 mg. of stearic acid in 1 ml. of benzene is added, and the sample is evaporated to dryness with the aid of a water bath (75° C.) and a gentle stream of air. The test tube is then rinsed down with about 5 ml. of ethyl alcohol and it is again taken down to dryness. A modification (*1*) of the original Schechter-Haller method (*4*) is used to determine the DDA.

Experimental

Solvent Extraction The possibility of extraction of DDA with solvents was investigated. Urine is extremely difficult to handle when extracting with solvents in a separatory funnel because of emulsion formation. Consequently, the use of liquid–liquid extractors and continuous extraction in Soxhlets was tried, after taking up the urine on diatomaceous earth. Both techniques were partially successful, except that high blanks were encountered and the procedures were impractical for routine use. It was necessary to acidify the urine before extraction in order to keep the DDA in the acid form. Extraction of the acidified urine produced colored materials which carried through the Schechter-Haller process and interfered with the colorimetric measurement of the DDA. Thus, amounts of DDA equivalent to 5 γ could not be handled.

Ion Exchange Resin The possibility of recovering DDA from the urine by use of the ion exchange resins appeared feasible. Initial tests indicated that Amberlite IRA-400 (obtained from Rohm and Haas Co., Philadelphia, Pa.), a strongly basic resin, would be suitable. Early attempts were made to adsorb the DDA from the urine using a column. However, gross particles were precipitated from the urine to such an extent as to interfere with the flow of the urine through the column. A batch method using Erlenmeyer flasks was adopted.

Various factors were studied. It was found that the blank values were directly proportional to the amount of resin used and that these values could be kept constant by using a standard amount of resin. The adsorbed DDA was rather difficult to recover from the resin, and a number of desorbants were tried. Quantitative removal was obtained by adding 20% acetic acid in ethyl alcohol and heating to reflux.

Exposure Time. The following method gave excellent recoveries of known amounts of DDA added to urine just prior to analysis.

Fifty milliliters of urine and 2 grams of Amberlite IRA-400 were placed in a 125-ml. glass-stoppered flask and shaken for 0.5 hour on a wrist-action shaker. The urine was then removed by aspiration. After the resin had been washed with 25 ml. of water and the water removed by aspiration, the resin was refluxed for 1 hour with 50 ml. of 20% acetic acid in 95% ethyl alcohol. While hot, the acid solution was filtered through paper and the resin and paper were washed carefully with about 25 ml. of water. The filtrate was diluted with 25 ml. of water, cooled, and then extracted with exactly 50 ml. of carbon tetrachloride. After removal of the acid phase by aspiration, the carbon tetrachloride solution was dried with sodium sulfate and an aliquot taken for Schechter-Haller analysis.

The application of this method to unknown samples of urine from persons ingesting DDT, however, gave evidence of low recoveries, especially in the case of urine containing substantially more than 1 p.p.m. of DDA. Exploratory tests indicated that it was necessary to expose the urine to the resin for long periods of time to ensure complete recovery of DDA. The effect of time of exposure on recoveries is illustrated by Table I.

The three samples utilized were obtained from three men: R was receiving 3.5 mg. of DDT daily, M was receiving 35 mg. of DDT daily and G was receiving no DDT in his diet (G was, no doubt, receiving trace amounts through his normal diet). The data show that the DDA added to the urine was recovered quantitatively with a 0.5-hour shaking. Recovery from the M sample containing approximately 1 p.p.m. was fairly good after 0.5 hour and seemed to be quantitative after 2 hours.

However, with the sample containing larger amounts of DDA (M) recoveries were low after 2 hours of shaking, but increased in general with longer shaking periods to a maximum value of 6.64 p.p.m. after 42 hours. This suggests that part of the DDA is found in a complex from which it is liberated only slowly at room temperature. That this may be the case is shown by a low recovery (6.9 p.p.m.) obtained on the control sample (G) reanalyzed (0.5-hour shaking period) 3 weeks after addition of the DDA to the urine.

Heat. Because such long shaking periods were undesirable, studies were made on the effect of heat on the recovery of DDA from urine. A portion of sample M (Table I) was heated for 5 hours on a steam bath in the presence of 2 grams of Amberlite. A recovery of 6.97 p.p.m. of DDA was obtained. This represents a 5% increase over the value found after 42 hours of shaking at room temperature. The relation of length of heating time to adsorption was studied using another urine sample (W). Two grams of the resin were added to 25-ml. portions of urine in flasks immersed in a boiling water bath. At intervals, flasks were removed and the DDA was recovered from the resin and analyzed by the procedure described earlier. No shaking period was introduced. The results (Table II) show an increase in recovery but also indicate that, after 4 hours of heating, the increase over the 3-hour recovery is rather small.

Results of a study of various conditions in liberating the bound DDA are shown in Table III. Urine sample W was used. In each case, 2 grams of the resin were added to the sample after bringing the sample to boil. Without cooling, the flask was immediately placed on a mechanical shaker for the time indicated. These results illustrate the importance of the shaking operation. With no "hydrolytic" treatment but with 1-hour shaking, a value of 4.80 p.p.m. was obtained; with no shaking but heating on the steam bath, 3-hour heating was necessary to attain a recovery of 4.89 with the same urine sample. However, the effect of heat in making the DDA available for adsorption was shown by marked increased recovery in three cases. In this study, the use of sulfuric acid gave low recoveries. However, later using the method in its final form—heating the urine in the presence of Amberlite—sulfuric acid had no significant effect on recovery.

A special heating unit (Figure 1) was devised which heated the flask and urine uniformly just below the boiling point. By regulation of the power input with a Powerstat, a constant temperature (90° to 95° C.) could be maintained readily without fear of bumping.

The presence of the resin in the urine during the heating produced an alkaline condition which could be beneficial with some samples and, furthermore, the exposure time of the urine to the resin was lengthened. This modification did not increase the blank.

As benzene is a better solvent for DDA, it was substituted for carbon tetrachloride with precautions taken for its complete removal before nitration.

Because recovery of DDA increased with exposure time of the urine to the resin up to 42 hours (Table I) and with heating time up to 4 hours when the urine-resin mixture was heated in a boiling water bath (Table II), these variables were studied using the final method. Four portions of the same urine sample were carried through the final method with variations in the lengths of shaking periods (Table IV). Four other portions of the same sample were processed with variations in the heating periods (Table IV).

Table I. Effect of Exposure Time on Adsorption of DDA by Amberlite IRA-400 from Three Urine Samples

Time, Hours	DDA Found, P.P.M. R	M	G[a]
0.5	0.66	3.53	10.7
2	0.78	4.40	10.9
4	0.76	4.82	11.3
8	0.79	5.10	10.8
16	0.78	6.06	10.7
24		5.52	
42		6.64	
72		6.36	

[a] 10 p.p.m. DDA added immediately before analysis.

Table II. Effect of Heating Time on Recovery of DDA from Human Urine (W)

Sample No.	Heating Time, Hours	DDA, P.P.M.	Increase, %
1	0.5	4.08	
2	2	4.57	12
3	3	4.89	20
4	4	5.00	23

Results

These results indicate that the 1-hour shaking period with the 1-hour heating period are adequate to assure complete recovery of the DDA.

The precision shown is excellent. Calculations using all eight values from Table IV gave a standard deviation of 0.114 and a per cent variation of 2.2.

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Table III. Comparison of Recovery of DDA from Human Urine Sample Subjected to Various Treatments before Addition of Resin

Pretreatment	Shaking, Time, Hours	DDA, P.P.M.
None	1	4.80
10 ml. 1N H_2SO_4, heated to boiling	1	2.60
10 ml. 2% NaOH, heated to boiling	1	5.31
Heated to boiling	1	5.35
Heated to boiling	16	5.61

Table IV. Effect of Shaking and Heating Time on Recovery of DDA from Urine Using Final Method

Shaking Time, Hours	DDA, P.P.M.	Heating Time, Hours	DDA, P.P.M.
0.5	5.33	0.5	5.14
1	5.29	1	5.18
2	5.20	2	5.09
3	5.33	3	5.02
Mean	5.29		5.11
Std. deviation	0.0613		0.0689
% variation	1.2		1.3

form of Amberlite IRA-400 has recently been discontinued. However, the chloride form is satisfactory and may be substituted for the hydroxyl form without any special treatment.

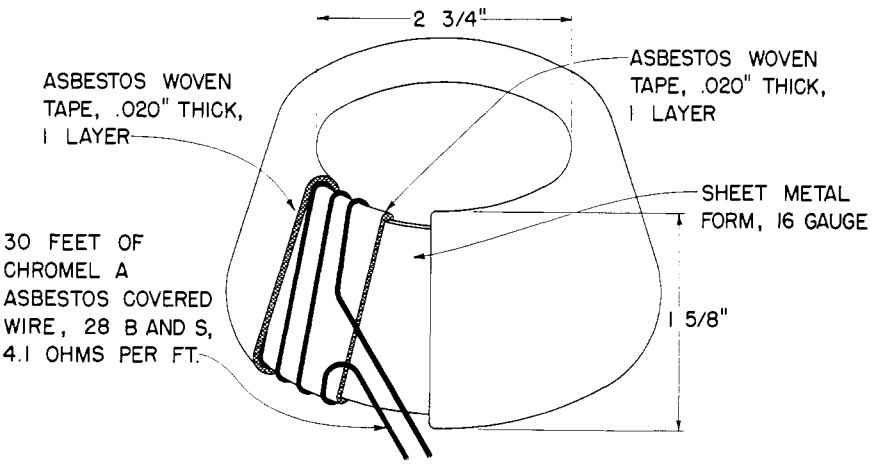

Figure 1. Flask heater for determination of DDA in urine

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BACTERIAL CONTAMINANTS

Effect of Brewer's Yeast Strain on *Flavobacterium proteus* Contaminants of Brewery Fermentations

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The growth of *Flavobacterium proteus* during a brewery fermentation is affected by the strain of brewer's yeast employed. The extent to which *F. proteus* can grow in a brewery fermentation appears to be related inversely to the rate at which the yeast strain employed can deaminate certain amino acids.

THE BREWING INDUSTRY commonly uses the yeast crop from a plant fermentation as inoculum in a subsequent fermentation. This process may be repeated several times before the yeast is replaced with a pure yeast culture and it usually results in a low level of bacterial contamination of the plant yeast.

The types of bacteria that grow sufficiently well during a brewery fermentation to establish a contamination are limited to those that will grow anaerobically, at a low pH level, and at the low temperature of the fermenting beer. It is also possible that the bacteria must be able to compete successfully with the yeast for certain nutrients that are utilized by the yeast during the fermentation (*7*) or that the bacterial growth is dependent on certain nutrients that are synthesized by the yeast and excreted into the fermenting beer (*3*). The bacteria that have been reported to contaminate brewer's yeast and brewery fermentations have been limited to gram-positive lactic acid rods and cocci, and a gram-negative rod *Flavobacterium proteus*.

Microscopic examination of brewer's yeast samples, from approximately 50 breweries, in the authors' laboratory during the past year has revealed that the bacterial contamination of a brewery yeast is usually limited to one morphological type. There are isolated cases where mixtures are found, but, in those cases, one type definitely predominates. That the contaminating bacteria found in any given yeast sample are not only of one morphological type, but also of a single physiological type is indicated by the data presented by Russell, Bhandari, and Walker (*4*). They found a tendency for bacterial strains isolated from any given brewer's yeast to have the same vitamin requirements for growth.

The variation in bacterial contaminants from one brewery to another and