enzyme studies on only one organism, the ability of amidines to exert toxic effects on different oxidation systems is a factor to be taken into account, especially in view of the fact that a high pH value favours inhibition of growth (Elson, 1945) as well as of respiration. The connexion is not apparently a simple one, since the differences in bacteriostatic power between halogenated derivatives and parent compounds are not paralleled by the same differences in anti-oxidase activity. To give an example, the concentrations required to produce bacteriostasis for *Esch. coli* are $11.8 \times 10^{-5} \text{ M}$ for propamidine and $0.57 \times 10^{-5} \text{ M}$ for dibromopropamidine (Wien *et al.* 1948): the corresponding figures for inhibition of oxidation were 80 and 87 % for alanine, and 68 and 84 % for lactate, using $12.5 \times 10^{-5} \text{ M}$ concentrations of the amidines. It cannot, therefore, be claimed that the anti-oxidase effects reflect the therapeutic efficacy of the compounds.

Regarding the mechanism of the inhibitory effect on respiration, it may be that the amidines exert effects on the particular dehydrogenases concerned with the different substrates, but the fact that so many systems are involved would appear to render this unlikely. It is more likely that an inhibitory effect is exerted on a respiratory mediator common to the various systems. One interesting possibility is that the system governing the direct transfer of molecular oxygen may be depressed; this would partly be supported by the absence of effect on the lactate oxidation system of brain, a cozymase-linked system, and by the slight effects of the amidines in inhibiting the growth of anaerobic organisms. It is conceivable that the cytochrome system peculiar to

Esch. coli may be inhibited; the absence of an effect on the cytochrome oxidase of liver does not conflict with this possibility, in view of the different cytochrome compositions of the two types of cell (Keilin & Harpley, 1941). It was not possible to pursue the point.

Finally, experiments on tissue oxidation revealed an apparently specific inhibition of the choline oxidase of liver. This point is of interest in connexion with the chronic toxicity of the amidines, which produce characteristic fatty changes in the liver. In view of the part played by choline metabolism in liver function, especially in regard to fatty changes, the inhibitory effect of propamidine on choline oxidase *in vitro* may well have some bearing on the degenerative changes produced in the liver *in vivo*.

SUMMARY

1. The respiration of washed cells of *Escherichia coli* in presence of various substrates was strongly inhibited by certain diamidines in concentrations of the order of $1.25\text{--}12.5 \times 10^{-5} \text{ M}$.

2. There was no simple relationship between the bacteriostatic potencies of the amidines and their effects on respiration.

3. Propamidine had no effect on several tissue oxidation systems, but inhibited the choline oxidase of liver.

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The Quantitative Determination of Barbiturates in Tissues by Ultraviolet Absorption Spectrophotometry

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The purpose of this paper is to describe a method for the estimation of very small amounts of barbiturates in mammalian tissues. The problem arose in connexion with work on the intermediary meta-

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bolism of the drugs, when it was found that the methods previously described were inapplicable for various reasons which will be discussed.

The basis for the various spectrophotometric procedures is the fact that barbituric acid and its derivatives of pharmacological importance, including

barbital (5:5-diethylbarbituric acid) and pentobarbital (5-ethyl-5'-pentylbarbituric acid) possess in alkaline solution characteristic absorption bands in the ultraviolet. Changes in the location and intensity of these bands in relation to variations in molecular structure have been discussed by Loof-bourow & Stimson (1940), Stuckey (1940, 1941, 1942), Klotz & Askounis (1947), and Walker, Fisher & McHugh (1948). The facts relevant to the present method are: (a) from c. pH 6.5-c. pH 10, a well-defined absorption maximum occurs at a wave length of 239 m μ .; (b) as the reaction becomes still more alkaline, the position of the maximum shifts in the direction of longer wave lengths; (c) in alkaline solution, the extinction at 239 m μ . is directly proportional to the concentration of barbiturate, in accordance with the Lambert-Beer law (Fig. 1). On the basis, therefore, of spectrophotometric data the concentration of the drugs can easily be determined in pure solutions.

Fig. 1. Relation of extinction to concentrations of barbital and pentobarbital in alkaline solutions; pH 9.4; wave length 239 m μ .

In attempts made to recover barbiturate from tissues, however, other substances are usually extracted at the same time. Some of them absorb radiation in the same region of the ultraviolet as the barbiturates. It has not been practicable to recover the drugs quantitatively in 'pure' solution, and it is therefore evident that any spectrophotometric method for the estimation of barbiturate in the presence of such impurities is valueless unless it allows for the contribution made by these interfering substances to the total absorption.

The present method achieves this by making use of the observation that the intensities of absorption at 239 m μ . of barbiturate and of the interfering substances differ in their dependence on pH. The extinction of barbiturate is characteristically related to pH, being maximal at pH 10 and decreasing steeply towards the acid, less steeply towards the alkaline side (Fig. 2). The ratio of the extinctions at any two values of pH is independent of concentration.

Fig. 2. Relation of extinction to pH for barbital and pentobarbital; concentration of drugs 10 μ g./ml.; wave length 239 m μ .

In contrast, the extinction due to the interfering substances obtained from different tissues by the present procedure has been shown to be practically constant over certain ranges of pH. Preliminary experiments are necessary to determine these ranges and the change in extinction of barbiturate when the pH is altered to the same extent. The results permit calculation of the concentration of barbiturate in impure solutions of this nature.

METHOD

Principle. The tissue is homogenized and the proteins are precipitated by ethanol. The barbiturate in the acidified protein-free filtrate is extracted with ether and passed into alkali. To two samples of this alkaline solution are added phosphate or borate solutions which bring the pH to different known values. The extinctions at 239 m μ . of the resulting solutions are measured, and from these the concentration of barbiturate in the tissue can be determined by calculation.

Apparatus. The extinction measurements were made with a Beckman Model DU photoelectric spectrophotometer with 1 cm. quartz cells. pH was determined electrometrically with a glass electrode.

Reagents. Conc. HCl (A.R.); absolute ethanol; diethyl ether (A.R.); NaCl (A.R.); NaOH solution 0.2M; KH_2PO_4 and H_3BO_3 -KCl solutions as for the preparation of buffer solutions according to Vogel (1944), except that the concentrations of the stock phosphate and borate solutions are 1.0M and 0.5M respectively.

Experimental procedure

Preliminary experiments. About 1 g. of normal tissue is extracted as described below. The extracted interfering substances are highly concentrated in the 5 ml. of alkaline solution obtained; this is therefore made up to 50 ml. with more 0.2M-NaOH solution, and 5 ml. amounts of this are then pipetted into a series of test tubes graduated at 10 ml.

Into another series of test tubes similarly graduated are put 5 ml. volumes of a 0.002% (w/v) solution of the barbiturate in 0.2M-aqueous NaOH (equivalent to 100 μg . drug/tube). Into a third series of test tubes are put 5 ml. volumes of pure 0.2M-NaOH solution.

To corresponding tubes of all three series are added increasing volumes of the phosphate or borate solutions, so that the pH increases in each series by steps of approx. 0.5 of a unit from 6 to 10. The quantities to be added may be calculated from the tables given by Vogel (1944). The solutions are then made up to 10 ml. with water.

The extinctions at 239 $\text{m}\mu$. of the first two series of solutions are measured, using the third series as blanks. Then the pH of each solution in the first two series is accurately determined. Curves are plotted to show extinction-pH relationships. These curves show the pH range over which the extinction of the interfering substances is constant, and they show the constants K and k (defined below).

Extraction. The tissues are thoroughly homogenized (Potter & Elvehjem, 1936). The homogenate is transferred quantitatively into a narrow glass-stoppered measuring cylinder. Absolute ethanol (2 vol.) is added and the mixture is shaken and allowed to stand for 15 min. Water ($\frac{1}{3}$ vol.) is added and the volume of the resulting solution noted. The mixture is then filtered through a Whatman no. 42 paper. The clear filtrate is saturated with NaCl. One half of it is taken into a separating funnel, acidified with a drop of concentrated HCl, and extracted once with an equal volume of ether.

The aqueous layer is discarded. The ethereal layer is extracted once, in the same separating funnel, with approx. 5 ml. of 0.2M-NaOH. The alkaline layer is run off, and most of the ether is removed by a current of N_2 . The solution is then made up to 10 ml. with water.

Spectrophotometry. Portions of this alkaline solution (5 ml.) are placed in each of two test tubes graduated at 10 ml. The pH values of these two samples are adjusted to the different desired values by the addition of appropriate volumes of phosphate or borate solutions; the volumes are adjusted to 10 ml. with water. The extinctions at 239 $\text{m}\mu$. are then determined.

Calculation. The two solutions submitted to spectrophotometry are designated S_1 and S_2 , and their hydrogen ion

concentrations pH_1 and pH_2 respectively, where $\text{pH}_1 > \text{pH}_2$. The following symbols are also used:

- R_1 = Extinction of S_1 .
- R_2 = Extinction of S_2 .
- x_1 = Extinction of impurities in S_1 .
- x_2 = Extinction of impurities in S_2 .
- K = Ratio of extinctions at 239 $\text{m}\mu$. of the barbiturate at pH_1 and pH_2 . This is a constant, the value of which is found by reference to curves obtained from the preliminary experiments (e.g. Fig. 2).
- k = Ratio of extinctions at 239 $\text{m}\mu$. of the barbiturate at pH_1 and pH 10. This is also a constant and its value is obtained from the same curves.
- M = Mol. wt. of the barbiturate (Table 1).
- ϵ_{max} = Molecular extinction at 239 $\text{m}\mu$. of the barbiturate at pH 10 (Table 1).
- W = Weight of tissue (g.).

First, the extinction of the impurities in S_1 and S_2 must be calculated. If, as is usually the case, $x_1 = x_2$, it is clear that

$$K = R_1 - x_1/R_2 - x_1. \quad (1)$$

Therefore $x_1 = KR_2 - R_1/K - 1$. (2)

The concentration of barbiturate in S_1 and S_2 is

$$(kR_1 - x_1) \frac{10^3 M}{\epsilon_{\text{max}}} \mu\text{g./ml.} = \left[kR_1 - \frac{KR_2 - R_1}{K - 1} \right] \frac{10^3 M}{\epsilon_{\text{max}}} \mu\text{g./ml.} \quad (3)$$

In the procedure suggested the tissue barbiturate is diluted 1 in 40; therefore the concentration of barbiturate in the tissue is

$$\left[kR_1 - \frac{KR_2 - R_1}{K - 1} \right] \frac{4 \times 10^4 M}{W \epsilon_{\text{max}}} \mu\text{g./g.} \quad (4)$$

This formula can be simplified if it can be shown that the extinction due to the impurities does not change significantly when the pH is varied even more, i.e. from 10 to 6.5 or lower. At pH 10 barbiturates absorb maximally; below pH 6.5 they cease to absorb altogether. Therefore (3) reduces to

$$(R_1 - R_2) 10^3 M / \epsilon_{\text{max}} \mu\text{g./ml.}, \quad (5)$$

and (4) to $(R_1 - R_2) 4 \times 10^4 M / W \epsilon_{\text{max}} \mu\text{g./g.}$ (6)

If the extinction due to the impurities does not remain absolutely constant between pH_1 and pH_2 , but shows small, determinable, variations, it is possible to correct the error introduced thereby, as follows:

If $x_2 = nx_1$ (1) may be written

$$K = R_1 - x_1/R_2 - nx_1, \quad (7)$$

and $x_1 = KR_2 - R_1/nK - 1$. (8)

Therefore, (4) becomes

$$\left[kR_1 - \frac{KR_2 - R_1}{nK - 1} \right] \frac{4 \times 10^4 M}{W \epsilon_{\text{max}}} \mu\text{g./g.} \quad (9)$$

and (6) becomes

$$(R_1 - R_2/n) 4 \times 10^4 M / W \epsilon_{\text{max}} \mu\text{g./g.} \quad (10)$$

Table 1. Molecular weights and molecular extinctions of barbital and pentobarbital

Compound	Mol. wt. (M)	Concentration		Observed extinction at 239 $\text{m}\mu$. and pH 10	Molecular extinction coefficient ϵ_{max}
		($\mu\text{g./ml.}$)	(mm/l.)		
Barbital	184.2	10	0.054 ₃	0.570	10,500
Pentobarbital	226.3	10	0.044 ₁	0.441	10,000

Table 2. *Experimental data on solutions examined*(Buffers added: S₁, borate (0.5M); S₂, phosphate (1.0M).)

Tissue extracted wet wt. (300–500 mg.)	S ₁			S ₂			K	k
	Vol. of buffer added ml.	pH ₁	Extinction observed (E, 1 cm.)	Vol. of buffer added ml.	pH ₂	Extinction observed (E, 1 cm.)		
Rat liver	2.3	10.0	0.184	1.06	8.0	0.180	—	—
Rat liver	2.3	10.0	0.116	1.06	8.0	0.119	—	—
Rat kidney	2.3	10.0	0.126	3.97	6.4	0.128	—	—
Rat-heart muscle	2.3	10.0	0.104	3.97	6.4	0.102	—	—
Barbital	2.3	10.0	0.572	1.06	8.0	0.377	1.52	1
Pentobarbital	2.3	10.0	0.440	1.06	8.0	0.258	1.71	1

RESULTS

Known amounts of barbital and pentobarbital were added to pieces of rat tissue, re-extracted and estimated according to the method described. Table 2 shows experimental data and Table 3 the results of recovery experiments. It will be seen that recoveries were satisfactorily constant and close to 100 %.

Table 3. *Recovery of barbital and pentobarbital added to various tissues*

Tissue (wet wt. 300–500 mg.)	Drug added	Wt. added (μ g.)	Recovery (%)
Rat liver	Barbital	200	95
	Barbital	100	90
	Pentobarbital	200	95
	Pentobarbital	100	94
Rat kidney	Barbital	200	91
	Pentobarbital	200	84
Rat-heart muscle	Barbital	200	92
	Pentobarbital	200	99

DISCUSSION

Published procedures for the estimation of very small amounts of barbiturates are based either on the cobalt colour reaction or on ultraviolet absorption spectrophotometry.

The cobalt colour reaction (Koppanyi, Murphy & Krop, 1933; Koppanyi, Dille, Murphy & Krop, 1934) depends upon the formation of a reddish colour when barbiturates in chloroform solution are treated with a cobalt salt in the presence of isopropylamine or some other weakly alkaline reagent. The test has been repeatedly criticized for its lack of specificity (Riley, Krause, Steadman, Hunter & Hodge, 1940) and for its relative insensitivity. It was originally claimed that the reaction was sensitive to barbital in concentrations of 10 μ g./ml. of the chloroform solution used for colorimetry, but Cohen (1946) found that the range of concentrations susceptible to accurate estimation was 1–10 mg./ml. The author

has found that the colorimetric estimation of concentrations smaller than 0.1 mg./ml. is difficult and inaccurate; Hellman, Shettles & Stran (1943) stress the fact that the pink colour obtained is transient and difficult to read.

Ultraviolet spectrophotometry has formed the basis of a number of earlier methods for the quantitative determination of micro amounts of barbiturates. The technique of Hellman *et al.* (1943) is limited to thiobarbiturates. Dorfman & Goldbaum (1947) reported experiments with barbiturates and referred to a spectrophotometric method (Jailer & Goldbaum, 1946; Goldbaum & Hildalgo, 1949), but no detailed description of their procedure, other than that applicable to pentothal, is available at the time of writing.

Very recently, and unknown to the author until after the completion of the experimental work, Walker *et al.* (1948) have described a spectrophotometric method which is based on considerations similar to those underlying the method described in this paper. These workers, after measuring the extinction in aqueous NaOH solution at pH 10, due to barbiturate and to interfering substances, abolish the extinction due to barbiturate by acidification with a micro drop of sulphuric acid, and again measure the extinction. They find that the extinction due to interfering substances extracted from blood does not alter appreciably between pH 10 and a strongly acid reaction, and they are thus able to calculate barbiturate concentration from the simple difference in the extinctions measured. Their formula is, therefore, essentially the same as equation (5) above. They suggest that this method might also prove applicable to tissues other than blood. The present author has not been able to confirm this suggestion in the case of liver extracts; indeed he has observed that the extinctions of such extracts, obtained and treated in a manner closely similar to that of Walker *et al.* (1948), show variations of up to 20 %. Only over a relatively narrow range of pH have the extinctions been found constant to within the experimental error. Another difficulty

referred to by Walker *et al.* (1948) is that not infrequently acidification with strong acid brings about clouding of the solution which is thereby rendered useless for further absorptiometry. This has not occurred in our procedure. Moreover, the recoveries obtained by the method of Walker *et al.* (1948) are low, the average being 70%.

Ultraviolet spectrophotometry permits the estimation of concentrations of barbiturates as low as 1–2 $\mu\text{g./ml.}$ in pure solution. Since the sensitivity of a method based on ultraviolet spectrophotometry is limited by the ratio of the extinction by barbiturate to the extinction by interfering matter, attempts have been made to obtain the drugs from tissue extracts in pure solution. Earlier procedures employed charcoal for clearing the extracts (Delmonico, 1939, 1940; Levvy, 1940; Anderson & Essex, 1942), but charcoal adsorbs significant amounts of barbiturate (Hellman *et al.* 1943). Hellman and co-workers washed ethereal extracts of the drug from blood with 0.5M-sodium bicarbonate, but according to Walker *et al.* (1948) this again causes the loss of some of the drug. Purification of the drugs by chromatography on alumina columns was described by Raventos (1946), and their isolation by sublimation was used by Cohen (1946). Various micro sublimation procedures were tried here, but it was not found possible to ensure purification and quantitative recovery simultaneously.

The present method makes a high degree of purification unnecessary, since it permits accurate estimation of barbiturate concentrations in the presence of unknown impurities. In particular, it has the following advantages:

(1) Barbiturates are highly soluble in 50% ethanol, whether the reaction is neutral, acidic or basic. Ethanol extraction appears to promote full recovery of the drugs without increasing the amount of impurities extracted.

(2) Absorption spectrophotometry is affected neither by traces of ether left in solution, nor by the variations in the concentrations of the buffering agents, provided use is made of corresponding blank solutions.

(3) The method has been found to be applicable to blood, naturally without preliminary homogenization, and with the following alterations in the procedure for protein precipitation: To 1 vol. of whole blood in a boiling tube is added one fifth vol. of molar potassium dihydrogen phosphate which brings the pH to between 4.5 and 5.0. The tube is heated on a boiling water bath for 3 min. After cooling, 2 vol. absolute ethanol and 1 vol. water are added, the mixture shaken and filtered and the procedure is then continued as before.

SUMMARY

1. A procedure based on ultraviolet spectrophotometry is described for the quantitative determination of very small amounts of barbiturates in tissues and blood.

2. Complete elimination of contaminants is not attempted. The difficulty due to the simultaneous extraction of barbiturate and impurities absorbing in the same region of the ultraviolet is overcome by the use of differential spectrophotometry, depending on extinction-pH relationships.

3. The procedure is rapid, accurate and sensitive and appears to offer some advantages over methods previously described.

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