selected chemical environment. Of course, the presence of radiation sensitizing micro-environment around a target inside a cell can never be ruled out; for example, Braams *et al.*[27] have shown a two-fold protection of invertase when it is irradiated in yeast cells, which they ascribed to the presence of substances rich in sulphydril groups. But Pollard *et al.*[2] demonstrated that succinic dehydrogenase and cytochrome oxidase have the same target sensitivity in or out of the *B. subtilis* cells. In Fig. 6 are plotted the present results of irradiating, at $-60°$, 1×10^9 plaque-forming units of $\varphi X174$ in bacterial lysate, which prior to irradiation is re-suspended in 30 per cent *E. coli* lysate, 4 per cent tryptone broth and 4 per cent nutrient broth. Following irradiation, the different sample series were diluted a thousand-fold into tryptone broth for assay of survivors. It is seen that the sensitivity of the virus in all media indicate closely similar target molecular weight of $1{\cdot}9$–$2{\cdot}0 \times 10^6$, which is well within the usual error of the assay observed in the foregoing experiments.

It can be demonstrated in all three cases of modification of direct action worked on or mentioned here that the protective or potentiating effect is dependent on the concentration of the added modifying agents[8,27]. The foregoing experiments thus suggest that, regardless of the purity of the initial virus preparation, a simple serial dilution of target samples into such innocuous media (for example, see legend, Fig. 1) as tryptone broth, nutrient broth and GSSG for irradiation would obviate any potential effect of the medium. (These media are not unique in this property since results clearly show that the heavy protein matrices of viruses do not modify the radiation sensitivity of the nucleic acid they envelop.) An empirical step of serial dilution, combined with some biological calibration of target procedure using viruses and nucleic acids of known molecular weight, should be effective in obtaining dependable target molecular weight very simply.

Taking due cognizance of radiation modifying effects, valid radiation target molecular weights can be obtained relatively simply for small viruses and their free nucleic acids with an accuracy expected from conventional physical methods. The combined experimental results obtained here from both γ- and X-irradiation bear out the assumptions that: (1) 60 eV constitute a killing event and this quantum of energy has an efficiency of 1; (2) the number of lethal events does not exceed the number of ionizing events. The results are also consistent with the assumption that an ionized target subsequently reacts in some way, probably with oxygen[25], with high efficiency and only then is the 'hit' target scored as a kill. The independence of target sensitivity with temperature between $-180°$ and $+30°$ fits the expectation for sparsely ionizing radiation that the lethal event in the nucleic acids is a direct result of ionization and not of thermal effects arising from absorption of excitation energy.

I thank Dr. E. C. Pollard for useful discussions. I also thank Drs. Sam G. Wildman and John S. Boyle, who provided assay plants, and Drs. Robert L. Sinsheimer, Richard Boyce and William Paranchych for the supply of bacteriophages.

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QUANTITATIVE ESTIMATION AND IDENTIFICATION OF BARBITURATES IN BLOOD IN EMERGENCY CASES

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ANY method for the determination of barbiturate in a patient's blood must, to be of value to the clinician and, hence, to the patient, fulfil three conditions. First, it must be capable of yielding results sufficiently rapidly to allow the clinician to commence treatment before the patient's clinical condition deteriorates. By rapidly, is meant that the time required for the analysis, that is, quantitative assay of the barbiturate and its identification, should be of the order of 1 h. This would be the time reckoned from receipt of the blood by the laboratory to completion of analysis.

Secondly, the quantitative estimation must be precise to within $\pm$ 10 per cent. Greater variation than this might give misleading results if serial analyses were being performed with short time-intervals.

Thirdly, in order to be able correctly to interpret the results of the quantitative assay, it is necessary to know which particular barbiturate is present in the patient's body. In the past, it has not been possible to identify the barbiturate within 1 h, but the introduction by Street[1–4] of paper-partition chromatography at elevated temperatures has enabled the more commonly used barbiturates to be identified in a blood extract within 20 min.

Specificity is a further criterion which must be satisfied and which may be achieved in high degree by a combination of several factors. By carrying out a classical separation of a blood protein-free filtrate, a fraction can be obtained which contains only barbiturates and other weak acids. If ultra-violet spectrophotometry is used for the quantitative assay of this weak acids fraction, it is possible to arrange the conditions so that an absorption maximum occurs at a wave-length which is peculiar to a particular group of barbituric acid derivatives. For example, the

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5 : 5-disubstituted barbiturates, which incidentally are the group most commonly encountered, display the characteristic phenomenon of exhibiting at *p*H 11 a peak at 240 mμ which disappears when the solution is brought to *p*H 2.

The recent work of Street and McMartin[5] has shown that a further step towards the unequivocal identification of individual barbiturates can be taken by heating at 100° C an aliquot part of the weak acids fraction with concentrated sulphuric acid for 3 min and examining the ultra-violet spectrum of the products.

Chromatography at about 90° C on tributyrin-impregnated paper with an aqueous solvent separates most of the more commonly used barbiturates in 15 min and, together with the ultra-violet spectra at *p*H 11 and *p*H 2, and the results of sulphuric acid treatment, provides a rapid procedure for the identification and assay of blood barbiturates.

The method we recommend involves preparation of a protein-free filtrate of blood. For this purpose we have modified slightly the procedure described by Folin and Wu[6] and have used Curry's[7] suggestion to heat the tungstic acid–blood mixture. The acidic and the neutral compounds are extracted from the acid filtrate by shaking with ether. Strong acids such as salicylic acid are removed by extraction of the ether with a *p*H 7·3 buffer. By shaking the ether with alkali, the neutral compounds are left in the organic phase and the weak acids extracted into the aqueous phase. Acidification of the alkaline medium and re-extraction into ether yields a fairly pure weak acids fraction.

This fraction is then divided into three parts. One portion is used for quantitative estimation by ultra-violet spectrophotometry; another portion is used for elevated-temperature chromatography; a third portion is taken for treatment with sulphuric acid to assist further in identification of the barbiturate.

De-ionized water is used throughout. The following reagents are needed:

(1) 10 per cent (w/v) sodium tungstate solution. Dissolve 100 g of $Na_2WO_4 2H_2O$ in water to 1 litre.

(2) Sulphuric acid solutions, 2 per cent, 10 per cent and 6 N. Pour 20 ml., 100 ml. and 167 ml. respectively of 'AnalaR' concentrated sulphuric acid into water and dilute to 1 litre.

(3) Ether. Shake 2½ litres of 'anæsthetic ether' (diethyl ether) with 50 ml. portions of 10 per cent sodium hydroxide solution until the alkaline extract is colourless. Wash the ether with water until the washings are free from alkali. Store the washed ether in a dark room.

(4) Phosphate buffer, *p*H 7·3 (0·5 M). Mix 80 ml. of 0·5 M $Na_2HPO_4.2H_2O$ (89 g/l.) and 20 ml. of 0·5 M KH_2PO_4 (68 g/l.).

(5) Phosphate buffer, *p*H 7·4 (M/15). Mix 80·8 ml. of M/15 $Na_2HPO_4 \cdot 2H_2O$ (11·9 g/l.) and 19·2 ml. of M/15 KH_2PO_4 (9 g/l.).

(6) Sodium hydroxide solution (2·5 per cent). Dissolve 25 g sodium hydroxide pellets in water and dilute to 1 litre.

(7) Sodium sulphate (anhydrous).

(8) Ammonia solutions (0·5 N and 4 N). Dilute 7·1 ml. and 57 ml. respectively of concentrated ammonia solution (sp. gr. 0·88) to 250 ml. with water.

(9) Tributyrin solution. 10 per cent (v/v). Dilute 25 ml. of tributyrin (glyceryl tributyrate) to 250 ml. with acetone.

(10) Sulphuric acid, 95 per cent. Carefully add 5 ml. of water to 95 ml. of 'AnalaR' concentrated sulphuric acid. Mix thoroughly and store in a well-stoppered glass bottle.

(11) Potassium permanganate solution (0·1 per cent (w/v)). Dissolve approximately 100 mg of potassium permanganate crystals in 100 ml. of water. Prepare freshly as required.

To 65 ml. of water contained in a 40 mm × 240 mm test-tube add 5 ml. of blood. Mix and allow to stand for at least 2 min for the cells to lake. Add 15 ml. of 10 per cent sodium tungstate solution, mix and then run in 15 ml. of 2 per cent sulphuric acid solution. Stopper the tube and shake the mixture until its colour changes from red to reddish-brown or brown. This takes about 1 min. Remove the stopper and immerse the tube in a bath containing vigorously boiling water for 3 min. The contents of the tube must be beneath the water surface. Remove the tube and filter the hot mixture through a Whatman No. 1 paper into a 100-ml. measuring cylinder. Cool the filtrate and measure its volume (usually about 90 ml.).

Extract the cooled filtrate by shaking with 50 ml. of washed ether. Allow the liquids to separate and run off the aqueous layer. Shake the ether phase with 10 ml. of 0·5 M phosphate buffer, *p*H 7·3 and discard the aqueous layer. Extract the organic phase with two separate 3-ml. portions of 2·5 per cent sodium hydroxide solution. Run the alkaline extracts immediately into 5 ml. of 10 per cent sulphuric acid solution contained in a clean separating funnel. Shake this acidified solution with 25 ml. of washed ether. Discard the aqueous phase and dry the ether layer by shaking with about 2 g of anhydrous sodium sulphate. Pour the ether extract into a 25-ml. measuring cylinder and divide into three separate portions in 50-ml. flasks *A*, *B* and *C* as follows: Into each of flasks *A* and *C* place two-tenths of the ether extract (*N.B.*, flask *C* should be round-bottomed). Into flask *B* place six-tenths of the ether extract. Evaporate the contents of each flask to dryness on a boiling-water bath. Remove the flasks from the bath immediately the ether has evaporated and place them on a cold slab of metal to cool.

Flask A. Dissolve the residue in 3 ml. of 0·5 N ammonia solution. Measure 2·5 ml. of the solution into a silica or quartz cuvette (1 cm light path) and plot its absorption curve against 2·5 ml. of 0·5 N ammonia solution from 200 to 350 mμ. Add 0·5 ml. of 6 N sulphuric acid to both 'test' and 'blank' cuvettes. Mix the solutions and replot the ultra-violet spectrum. Let R_{OH} be the absorbance of the ammoniacal 'test' solution at 240 mμ and R_H the absorbance of the acid 'test' solution at 240 mμ, then:

$$\text{blood barbiturate} = \frac{(R_{OH} - R_H)}{S} \times \frac{3}{50} \times \frac{10}{2} \times \frac{100}{F} \times \frac{100}{5} \text{ mg/100 ml.}$$

$$= \frac{(R_{OH} - R_H)}{S \times F} \times 600 \text{ mg/100 ml.}$$

where $S = (R_{OH} - R_H)$ for standard barbiturate solution (see below) and F = ml. of tungstic acid filtrate used.

If the ultra-violet spectrum shows no maximum at 240 mμ, it may be assumed that barbiturate is either not present in the blood sample being analysed or is present in insignificant amount.

Flask B. Dissolve the residue in about 6 drops of chloroform and transfer the solution with a glass capillary tube to the starting line of a previously prepared sheet of tributyrin-impregnated Whatman No. 3 paper. (Dip the papers in a 10 per cent (v/v) solution of tributyrin in acetone, press between sheets of filter paper and allow to dry in air. Papers should be impregnated not more than 2 h before being used) (Fig. 1*a*). As reference standards, put about 2 μl. of 2 per cent ethanolic barbiturate solutions also on the starting line. The size of spot should, preferably, be not greater than 3 mm diameter. A mixture of barbitone, phenobarbitone, butobarbitone and pentobarbitone may be placed on one side of the unknown spot and a mixture of amylobarbitone and quinalbarbitone on the other side. Fold the paper into a cylinder, fasten with a paper-clip as shown in Fig. 1*b* and carry out chromatography for 17 min.

This is done by placing the paper cylinder in a 2-litre beaker containing M/15 phosphate buffer *p*H 7·4. The beaker and buffer have, about 0·5 h previously, been put in an oven maintained at a temperature of about 95° C.

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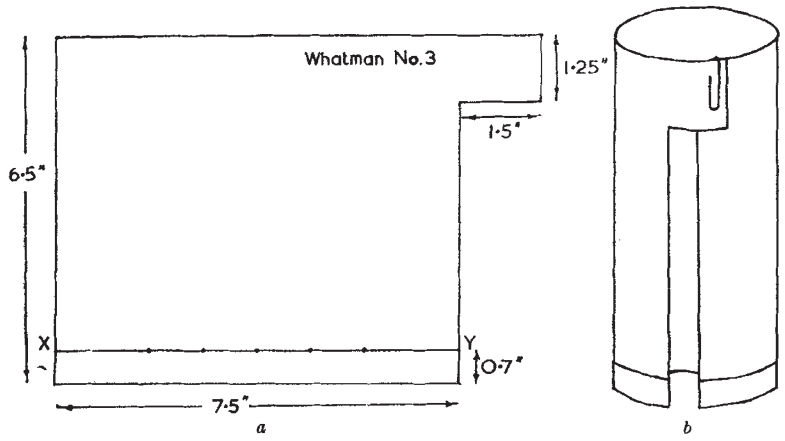

Fig. 1. *a*, Size and shape of Whatman No. 3 paper used for reversed phase paper chromatography at 95° C. After the paper has been impregnated with tributyrin, the compounds to be separated are applied at points on the line *XY*; *b*, paper folded into cylinder and fastened with a paper-clip prior to carrying out reversed phase chromatography at 95° C

A disk of plate glass smeared with silicone grease is used to seal the beaker. The amount of buffer in the beaker is such as to give a depth of about 3/8 in. of liquid. The paper cylinder is placed in the beaker without removing the beaker from the oven.

After 17 min, the solvent will have run about 5 in. up the paper from the starting line. Remove the paper from the beaker and examine in 254 mμ radiation (a Hanovia 'Chromatolite' lamp is suitable for this purpose). Expose the paper to ammonia gas and re-examine under the lamp. Note the position of any absorbing areas (Fig. 2).

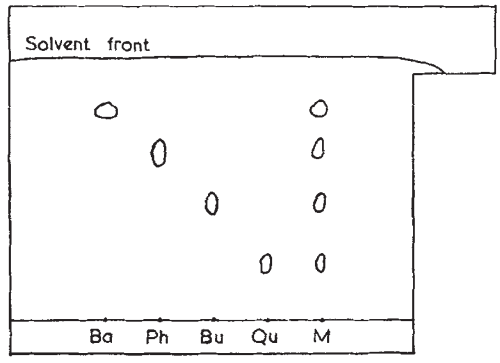

Fig. 2. Separation of four barbiturates by reversed phase chromatography at 95° C on Whatman No. 3 paper impregnated with tributyrin. Solvent, M/15 PO_4, *p*H 7·4; time of run, 17 min

Ba, barbitone; *Ph*, phenobarbitone; *Bu*, butobarbitone; *Qu*, quinalbarbitone; *M*, mixture of these four barbiturates

The paper may now be dried with a hair-dryer and then dipped in 0·1 per cent solution of potassium permanganate. Those barbiturates containing an unsaturated group in their side chain(s) will show up as yellow spots on a pink background.

Flask C. Add 0·2 ml. of 95 per cent sulphuric acid to flask *C*, stopper the flask and make sure that the acid is run over the residue. Heat the flask in boiling water for 3 min, cool and add 4 ml. of water. Pour the solution into a separating funnel, rinse the flask with 1 ml. of water and add this rinsing to the separating funnel. Shake with 25 ml. of ether and allow the layers to separate.

Aqueous phase. Run the aqueous phase into a graduated test-tube and pass a stream of nitrogen through the solution for about 2 min to remove the ether. Then add an equal volume of 4 N ammonia solution, mix and plot the absorption curve from 200 mμ to 350 mμ. Use silica or quartz cuvettes of 4 cm light path. Add 2·0 ml. of 6 N sulphuric acid solution and replot the absorption curve (Fig. 3). The 'blank' in this case is a mixture of 0·2 ml. of 95 per cent sulphuric acid, 5 ml. of water and 5·2 ml. of 4 N ammonia solution.

Ether phase. Dry the ether phase by shaking with about 1 g of anhydrous sodium sulphate, decant, and evaporate to dryness on a boiling-water bath. Remove the flask immediately the last drop of ether has evaporated. Cool the flask and dissolve the residue in 3 ml. of 0·5 N ammonia solution. Plot the absorption curve from 200 mμ to 350 mμ against 0·5 N ammonia solution. To 2·5 ml. of solution, add 0·5 ml. of 6 N sulphuric acid solution, mix and replot the absorption curve (Fig. 3).

A fuller description of the heating of barbiturates with sulphuric acid is given by Street and McMartin[5].

Standard curves should be constructed only from freshly prepared solutions.

Dissolve 20 mg of pure barbituric acid derivative in 200 ml. of 0·5 N ammonia solution. Add 1 ml. of this solution to 4 ml. of 0·5 N ammonia solution and plot the absorption curve from 200 mμ to 350 mμ. Use silica or quartz cuvettes of 1 cm light path. To 2·5 ml. of this solution add 0·5 ml. of 6 N sulphuric acid solution and replot the absorption curve.

The difference, S, between the absorbance in ammonia (R_{OH}) at 240 mμ and that in acid (R_H) at 240 mμ is used for calculation of the amount of barbiturate in blood (see foregoing), that is:

$$S = (R_{OH} - R_H)$$

For the 5 : 5-disubstituted derivatives, $S = 0{\cdot}9 \pm 0{\cdot}1$ (see also ref. 7).

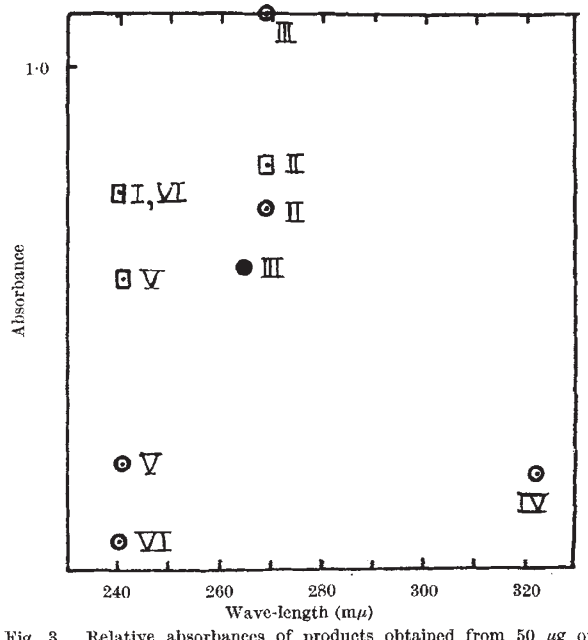

Fig. 3. Relative absorbances of products obtained from 50 μg of barbiturate subjected to treatment with sulphuric acid (see text)

□, absorption maximum of ether-soluble products in alkaline solution;
○, absorption maximum of water-soluble products in alkaline solution;
●, absorption maximum of water-soluble products in acidic solution

I	II	III	IV	V	VI
Products of: Barbitone, Butobarbitone, Amylobarbitone.	Products of: Pentobarbitone, Cyclobarbitone	Products of: Allyl isopropyl barbituric acid. Quinalbarbitone.	Products of: Dial.	Products of: Allyl isobutyl barbituric acid.	Products of: Phenobarbitone Rutonal

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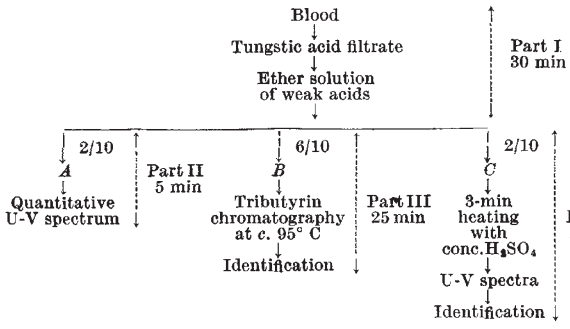
Discussion

A diagrammatic plan of the various steps in the analysis is shown in Table 1. Part I of the analysis, which is to prepare a protein-free filtrate of the blood and produce a partially purified ether extract of this filtrate requires 30 min. Parts II, III and IV need 5, 25 and 20 min, respectively. It is suggested that one worker carry out parts I and IV and another worker do parts II and III. In this way, a complete analysis can be carried out in 55 min.

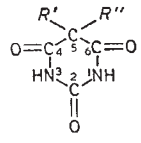
Table 1



There are several steps in the procedure which require amplification and emphasis. Thorough washing of the ether is essential for production of 'low blank readings'; loss of barbiturate can occur if, during evaporation of an ether extract, the flask is not removed from the bath immediately the last drop of ether has disappeared; it is imperative that the sulphuric acid added to flask *C* should come into contact with the whole of the residue; the filters used in the 'Chromatolite' 254 mμ lamp should be tested regularly with a spot of a known barbiturate on tributyrin-impregnated paper in the presence of ammonia gas. The 'life' of some of these filters may be as short as 800 h.

The limitations of the procedure are such that a level of 0·5 mg of barbiturate per 100 ml. of blood can just be measured quantitatively when starting with 5 ml. of blood but this concentration is insufficient for detection by chromatography of the residue in flask *B*. Above this level, no difficulty should be experienced with either the quantitative or qualitative estimation.

In the quantitative assay, it has been assumed that the barbiturates being measured are dialkyl derivatives substituted only on carbon atom 5.

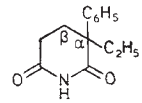


This assumption has been made because the 5 : 5-di-substituted derivatives are in commonest use. However, 1 : 5 : 5-tri-substituted derivatives may occasionally be encountered. The *N*-methyl derivatives and also the thiobarbiturates (which are used exclusively as anæsthetics) may be distinguished by the wave-length of their absorption maxima at different *p*H values (Table 2). Chromatography provides confirmation.

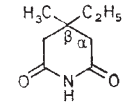
Table 2

Derivative	Wave-length maxima (mμ) at *p*H 2	at *p*H 11	at *p*H 14
1 : 5 : 5-	nil	245	245
5 : 5-	nil	240	255
Thio-	239,288	255,303	303

In the recommended method, no serious interference from substances which are not barbiturates will be encountered, and, in this connexion, only salicylates and glutarimides will, therefore, be considered. Salicylate will be removed by washing with the *p*H 7·3 buffer. If present in large amount, some will escape into the weak acids fraction and will be seen on the chromatogram in 254 mμ light as a light-blue fluorescent spot (R_F about 0·9) and will also distort the ultra-violet absorption curve. It is, therefore, wise to test a portion of the original blood sample with Trinder's[8] reagent. This takes only a few minutes and can be done while the tungstic acid/blood mixture is filtering. If the test is positive, then the excess salicylate can be removed by three washes with the *p*H 7·3 buffer. If Trinder's test is negative, the single wash with *p*H 7·3 buffer should not be omitted as this serves to remove some of the acidic fatty material encountered in all blood/tungstic acid filtrates. Of the glutarimides, only glutethimide and bemegride need be considered.



Glutethimide



Bemegride

In 0·5 N-ammonia solution, glutethimide and bemegride have absorption maxima at 235 mμ and 230 mμ respectively. The glutarimide ring is unstable in alkaline solution and both these compounds may be detected, and also estimated, by observing the decrease in absorbance (at the wave-length maximum) with time.

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HÆMOSTATIC CHANGES ASSOCIATED WITH EXERCISE

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PHYSICAL exercise is known to affect the hæmostatic balance. Even if some hæmostatic indices have been found to behave inconsistently in response to exercise, possibly owing to technical differences, observations on the time of coagulation[1,2], like thromboelastographic results[2,3], have generally yielded evidence suggesting that exercise gives rise to hypercoagulability. Of the coagulation factors of the plasma, the levels of Factor V[2], Factor VIII (AHG)[4] and Factor XII (Hageman factor)[5] have been reported to be raised concurrently. The platelet count

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