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STUDIES ON BARBITURATES. XI. FURTHER CONTRIBUTIONS TO METHODS OF BARBITAL RESEARCH.*

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In a previous publication (1), we discussed different methods of extraction of barbiturates from urine, blood and tissues and also colorimetric methods for the estimation of barbiturates. From time to time we have received requests from other laboratories relative to certain difficulties encountered during the process of extraction of barbiturates, and although little difficulty is usually encountered in using the methods described in previous publications it was deemed advisable to improve this part of the procedure. This paper embodies the results of investigations devoted to the problem of the extraction of barbiturates.

URINE.

Methods of Clarification.—Chloroform extracts of pathological and even normal urine specimens are sometimes highly colored with urochrome and other pigments.[2] These materials may interfere with the colorimetric readings as described in the quantitative procedure of Koppanyi, *et al.* (1). Ordinarily, if it is not necessary

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* Scientific Section, A. PH. A., Portland meeting, 1935.

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[2] The interfering colored residues found in the evaporated chloroform extract need not represent urinary pigment, but may be due to some colored impurities present in the chloroform. In order to prevent this, the chloroform used for extraction should be checked for such impurities by evaporating 100 to 200 cc. to dryness. If these impurities are present, the chloroform can be purified by distillation.

to concentrate the chloroform extract, colorimetric determinations may be carried out without removal of the pigments. However, in certain urines so much pigment is extracted that it is impossible to carry out colorimetric readings either directly or after concentration. Also, when the barbital content of the urine is very low and it is necessary to evaporate the extract to dryness, the amount of pigment which is taken up with the small amount of chloroform to carry out the test may be enough to prevent a satisfactory estimation of barbital and its derivatives.

Various procedures have been tried for clarifying highly colored urines before extraction with chloroform. The first requirement of any one of these methods is that it must not change the actual concentration of barbiturates in the urine, *i. e.*, precipitate, adsorb or destroy these compounds. Secondly, it must remove sufficient pigment so that the final color development is not obscured, and it should not yield end-products, such as acetic acid from lead acetate, which have a deterrent action on the subsequent color development.

Our methods of extraction of barbiturates from the urine described previously (1, 3) fulfil these requirements for ordinary purposes, and only highly concentrated urine samples show pigments with the alkaline copper sulphate precipitation method. We believe that the difficulties with this method arise largely from the fact that copper sulphate and sodium hydroxide are not used in sufficient amounts to produce a heavy precipitation of the mixture. The heavily precipitated urines yield little if any pigments. We have endeavored, however, to improve even upon this alkaline copper sulphate precipitation method and have experimented with a number of other methods in an attempt to more completely remove the pigments from the urine without destroying or removing the barbiturates.

In this investigation, sodium barbital was added to pathological, highly pigmented normal urine specimens, and urines which had been concentrated on a water-bath. These urines were cleared by one of the methods described below and aliquots of the filtrate extracted with ten volumes of chloroform, the chloroform extract filtered and evaporated to dryness. Control urines, *i. e.*, urines not cleared or urines cleared by other methods, were similarly treated in each case and the amount of the urinary pigment remaining in the evaporating dish after the evaporation of chloroform noted and compared. This residue was then dissolved in small volume of chloroform and the amount of barbital extracted was determined by the colorimetric procedure. Thus every method was checked not only as to its ability to remove pigment but also as to its efficiency in preserving the original barbital content of the urine.

The following new methods were used to clear the urine for the determination of barbiturates:

A. Sufficient amounts of *bichloride of mercury* are added to urines until heavy white precipitation occurs. The precipitate is filtered off and an aliquot of the filtrate extracted with chloroform. Although considerable pigment was removed by this method, it did not prove to be practical because large amounts of barbital were precipitated and probably destroyed by this method.

B. Enough *zinc sulphate* and *sodium hydroxide* is added to urines to produce heavy precipitation of zinc hydroxide. Although this method did not affect the barbital content of the urine, it was found to be rather ineffective in removing pigments.

C. Two grams of *sodium molybdate* are added to every 25 cc. of urine and sufficient *strong sulphuric acid* (20 to 30 per cent) to produce a heavy green precipitate. The precipitate is filtered off and the filtrate extracted, as usual, with chloroform. This method did not destroy the barbital added to the urine, and was found to be effective in removing pigments from certain concentrated urine specimens. However, it was inferior to the method finally chosen and described below.

D. Five cc. of a 10 per cent *copper sulphate* solution are added to 25 cc. of urine which is then made alkaline with 10 cc. of a 10 per cent *sodium tungstate* solution. After mixing thoroughly, the mixture is filtered and 5 cc. of 5 per cent *sulphuric acid* solution is added to 30 cc. of the filtrate. This is mixed, allowed to stand for about twenty minutes and again filtered. Twenty-five (25) cc. of this filtrate, which is equivalent to 13.39 cc. of the original urine, is extracted with chloroform. More pigment will be removed by this method if the amounts of reagents used for the 25 cc. of urine are doubled. This latter procedure has the disadvantage, however, of requiring a much longer time for filtration.

This method was compared with the original alkaline copper sulphate precipitation method and was found to be its equal in lightly colored urines. However, in highly colored urine specimens this method removed more pigment than any other method. No barbital was removed during this procedure.

Extraction of Diethyl Barbituric Acid from Alkaline Urine.—In a previous publication, we stressed the fact that urines or the cleared filtrates must be acidulated before extraction. This was a necessary precaution because the alkaline salts of barbituric acids are insoluble in chloroform. However, we observed repeatedly that alkaline urines not acidulated before extraction showed the presence of barbituric acids in the chloroform extracts. This led us to suspect that some of the excreted barbiturates are present as acids even in alkaline urines. A typical experiment demonstrating the above fact is described below:

Dog: Female, 11.4 Kg., given by femoral vein, 1650 mg. per Kg. of sodium barbital in divided doses with picrotoxin. Died 1 hour and 25 minutes after the last injection.

The urine excreted during this period was secured by catheterization of the bladder and its p_H was found to be 7.5 by indicators.

Ten cc. of the urine was extracted directly without acidulation with ten volumes of chloroform. The colorimetric determination showed that the extract contained 0.4 mg. of barbital per cc.

Another 10 cc. of the same urine was acidulated with dilute sulphuric acid and extracted with ten volumes of chloroform. The colorimetric test showed in this case the presence of 0.6 mg. of barbital per cc. of the extract.

We endeavored to duplicate these results by adding various amounts of sodium barbital (an alkaline salt) to portions of alkaline rabbit and human urines which had a p_H of 8.0 as determined by the use of indicators. Ten-cc. portions of each concentration were extracted directly, without acidulation with 100 cc. of chloroform and this chloroform was then tested for barbital. The results of one of these experiments on rabbit urine are summarized in Table I.

It can be seen that the amount of barbital recovered is inversely proportional to the amount of sodium barbital added to the urine. Sodium barbital added to

distilled water which was adjusted to p_H 8.0 was not changed into barbital and the chloroform used to extract this solution gave negative tests for barbiturates throughout the experiment.

TABLE I.—THE EXTRACTION OF BARBITAL FROM ALKALINE RABBIT URINE (p_H 8.0) TO WHICH VARYING AMOUNTS OF SODIUM BARBITAL HAVE BEEN ADDED.

Amount of Sodium Barbital Added to Urine. Mg. per Cc.	Amount Recovered as Barbital. Mg. per Cc.	Percentage Conversion of Sodium Barbital to Barbital. Per Cent.
0.5	0.25	50
1.0	0.50	50
2.0	0.80	40
3.0	1.20	40
5.0	1.50	30
10.0	2.50	25
10.0*	Negative	0

* Dissolved in distilled water, p_H 8.0.

BLOOD.

The modified Folin-Wu method for blood precipitation gives such uniform results and clear extracts that no further improvements are deemed necessary. However, we have never extracted volumes of blood larger than 20 to 30 cc. and thus have not tested the pigment removing capacity of the Folin-Wu method for large amounts of blood. Two hundred cc. of freshly drawn oxalated dog blood were precipitated by adding 200 cc. of 10 per cent sodium tungstate and 400 cc. of $^2/_3$ normal sulphuric acid solutions. The filtrate (485 cc.) was evaporated on a water-bath until it reached a volume of 75 cc. and was then extracted with ten volumes of chloroform. This chloroform extract evaporated to dryness showed no pigments.

Incidentally we endeavored to check the specificity of our barium hydroxide and lithium hydroxide tests on this evaporated extract.[1] The evaporated residue of the total blood extract was taken up in 10 cc. of chloroform and tested colorimetrically. Negative results in all three ranges of the micro (lithium hydroxide) and in the first range of the macro (barium hydroxide) tests were noted.

TISSUES.

The alkaline copper sulphate precipitation method and the liquid air method described previously (1) suffice for the study of the barbital concentration of organs. We want to emphasize again, however, that with the alkaline copper sulphate precipitation method the best results are obtained if the proportions of copper sulphate and sodium hydroxide solutions are such as to change the liquefied tissues to a heavy, massive, semi-solid mixture.

The liquid air method, as emphasized elsewhere (1), gives the best yield of barbiturates. However, this method cannot be applied to the extraction of barbiturates from the central nervous system, because as already observed (2) the

[1] The colorimetric test for barbiturates cannot be carried out in ether. The addition of the reagents to an ether extract produces dirty precipitates of miscellaneous hues but no clear cut blue colors. Therefore, if ether is used as the extractive medium, it must be evaporated off and the residue taken up in chloroform for testing.

lecithins and cephalins of the central nervous system are easily soluble in chloroform and interfere with the development of color in the test for barbiturates.

Recently, we found a simple method which allowed us to estimate the barbiturate content of the brain by using the liquid air method. Brain tissue is frozen in liquid air and pulverized. This powdered brain tissue is shaken with chloroform, the chloroform extract is filtered and evaporated to dryness on a water-bath, and the residue taken up again in a small volume of chloroform to which acetone is added drop by drop. A heavy precipitation of phospholipids occurs in the solution. The precipitate is filtered off and acetone is added again to the filtrate to ascertain whether the phospholipids have been completely removed. The solution in which no more precipitation occurs with acetone is again evaporated to dryness, and the residue taken up with chloroform and tested. In properly treated extracts, the test can be carried out without interference.

DISCUSSION.

The additional methods described for the clarification of urine will probably be useful to those who are working with pathological or highly concentrated urines. It is recommended that before discarding a urine sample as unsuitable for colorimetric estimation of barbiturates all three methods for clarification, namely, the copper sulphate, the sodium molybdate, and the copper sulphate-sodium tungstate methods, should be tried. There will be very few urines, indeed, which when appropriately cleared could not be used for the quantitative estimation of barbiturates.

We have shown that the urine has the capacity even at a relatively high p_H of converting sodium barbital into the acid form. The amount of sodium barbital added to alkaline urines and recovered as diethyl barbituric acid is inversely proportional to the amount originally added. It is evident that there is a buffering action of the urine which changes a portion of sodium barbital to barbital, thus making it available for extraction with chloroform. This buffering power of the urine is almost constant since the percentage of barbital formed from the alkaline salt decreases as the amount of added sodium barbital increases. This characteristic of the urine has not been previously observed as far as barbiturates are concerned, but we have described the same phenomenon concerning the blood (3). We conclude, therefore that both blood and urine have the capacity to convert sodium barbital into barbital.

We have shown that even large amounts of blood can be extracted with chloroform without the slightest trace of pigments in the extract. We have also shown that even such large volumes of blood, if the extract is evaporated to dryness and then taken up in a very small volume of chloroform, contain no substance which reacts in our macro or micro tests to give a blue color. These tests, therefore, can be safely used in the diagnosis of barbiturate poisonings.

The liquid air extraction method for other tissues is now extended to the brain, if the phospholipids are removed with acetone. This not only provides a very convenient and rapid method for the extraction of barbiturates from the central nervous system, but also shows that our assumption expressed in the previous communication (2) that lecithins interfere with the colorimetric estimation of barbital, was correct.

SUMMARY.

1. Two practical methods of clearing highly colored urines for the purpose of quantitative estimations of barbiturates are described.

2. The urine has a limited buffering capacity manifested in the conversion of sodium barbital into the acid form even in alkaline urines. The amount of barbital so converted is inversely proportional to the amount of sodium barbital originally added to the urine.

3. Large volumes of blood (after Folin-Wu precipitation) may be extracted with chloroform without obtaining interfering materials in the chloroform extract even after concentration.

4. The liquid air method of direct extraction of barbiturates can now be applied to the central nervous system after removing the phospholipids from the chloroform extract with acetone.

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A PLAN FOR PHARMACY INTERNSHIPS AT THE UNIVERSITY OF MICHIGAN HOSPITALS.*

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Pharmacy as a profession is unquestionably suffering influences that are tending toward pronounced changes. It will not be argued here that pharmacy is or is not wholly conscious of the drift. Certain it is, however, that the authors feel their incapability of intelligent argument or prediction.

Since words have been spoken and written it has been charged that many things are wrong with pharmacy. It has been said that the profession requires fewer and better pharmacists, fewer and better pharmacies and fewer and better schools of instruction. Professional mediocrity does exist within the rank and file of pharmacists and likewise professional morals and business standards are frequently unobserved. This condition reflects to the disadvantage of the public and the physician. Others have claimed that colleges of pharmacy should not and cannot be required to turn out a finished professional and business product. This reflects to the disadvantage of the pharmacist. For comparison it is argued that medical men do not expect such finished products to be graduated from medical schools. However, there are those in the medical profession who recognize this deficiency and consequently make some provision toward a remedy. One needs only mention the general requirement of service as intern before the graduate physician is licensed to practice medicine. It is because the authors sense the same deficiency in pharmacy that the outline of service that follows is proposed for our hospital group.

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