# Technical Papers

## Metabolic Oxidation of Phenobarbital to *p*-Hydroxyphenobarbital

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Numerous aromatic compounds have been found to undergo oxidation to phenolic products in the mammalian organism. This type of reaction might be expected to occur in the phenyl group of phenobarbital (5-ethyl-5-phenyl barbituric acid), but it has not hitherto been described. This report (*1*) concerns the discovery in dog urine of a product of the metabolism of phenobarbital and its identification as the *p*-hydroxy derivative of phenobarbital.

The compound as present in urine is largely conjugated and can be released by acid hydrolysis. An equal volume of concentrated hydrochloric acid is added to urine, and the mixture is refluxed for 3 hr. Phenobarbital and *p*-hydroxyphenobarbital are stable under these conditions. Both compounds are extracted from the acid urine with ether, and their separation and purification are accomplished by a systematic procedure of partitions between ether and buffers and benzene and buffers and finally by crystallization from water. The physical properties on which the isolation procedures are based are shown in Table 1. From urine collected for 3 wk from a dog receiving daily doses of phenobarbital, there were isolated in this way 1.54 g of *p*-hydroxyphenobarbital and 0.29 g of unchanged phenobarbital.

The structural identification of the urinary product was established by synthesis. The synthesis of *p*-hydroxyphenobarbital, which has not previously been described, was carried out by the following procedure. *p*-Nitrophenobarbital was prepared by the method of Pierce and Rising (*2*), and the position of the nitro group was confirmed by their procedure of hydrolysis and oxidation to *p*-nitrobenzoic acid. *p*-Nitrophenobarbital was converted to the phenolic derivative by catalytic reduction with hydrogen, diazotization of the amine, and hydrolysis of the diazonium salt. *p*-Hydroxyphenobarbital crystallizes from water with 1 mole of water. It melts at 222° to 223°C, corrected. The ultraviolet absorption spectrum as it is influenced by *p*H is indicative of three dissociations, corresponding to the loss of the protons from the two nitrogen atoms and the phenolic group. Identity of the urinary product with the synthetic compound was demonstrated by the method of mixed melting points. Analysis of hydrate (urinary origin): C, 54.01, 54.20; H, 5.09, 4.95; N, 10.42, 10.52 percent. Calculated for $C_{12}H_{14}O_5N_2$: C, 54.12; H, 5.30; N, 10.52 percent.

Table 1. Physical properties of phenobarbital and *p*-hydroxyphenobarbital furnishing the basis for the isolation procedures.

| Compound | Partition coefficient of acid form | | $pK_1'$* |
|---|---|---|---|
| | Ether/water | Benzene/water | |
| Phenobarbital | 60 | 1 | 7.23 |
| *p*-Hydroxyphenobarbital | 5 | 0 | 7.30 |

* At 38°C and total ionic strength of 0.1.

The other isomeric hydroxyphenobarbitals have not yet been found in urine, but a search for traces of these compounds continues. The conjugated form or forms in which *p*-hydroxyphenobarbital is excreted are still unidentified. Conjugation might be expected to occur with both glucuronic acid and sulfuric acid.

In doses as high as 1 g/kg, *p*-hydroxyphenobarbital is not anesthetic in mice. It appears that oxidation of phenobarbital to the phenolic derivative is a mechanism of major importance in the pharmacologic inactivation of the drug.

### References and Notes

1. This investigation was supported in part by a research grant (B-384) from the National Institute of Neurological Diseases and Blindness, National Institutes of Health, U.S. Public Health Service.
2. A. E. Pierce and M. M. Rising, *J. Am. Chem. Soc.* **58**, 1361 (1936).

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## Determination of Isotopic Carbon in Organic Compounds

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The determination of isotopic carbon in organic compounds has been simplified by development of a procedure in which the sample is heated with copper oxide in a sealed tube and the carbon dioxide produced is isolated by fractional condensation in vacuum. The method is applicable to a wide variety of compounds and yields results that are reproducible to 1 percent and agree with values obtained by a more elaborate procedure (*1*) based on Pregl combustion. Operations required to obtain a sample of gas for isotopic analysis can be performed in 30 min without elaborate equipment. The similarity of the procedure to the zinc fusion technique (*2*) for tritium assay renders it particularly attractive where research with tritium, as well as with $C^{14}$, is anticipated.

A convenient system for application of this procedure to the determination of $C^{14}$ is shown in Fig. 1. In combustion tube *A*, made from a 17-cm length of 11-mm OD Pyrex 1720 glass tubing (*3*) by drawing out one end to form a break tip, are placed 0.75 g of

60-mesh copper oxide and 0.25 g of 60-mesh reduced copper. A weighed sample, 1 to 10 mg, of the organic compound, in either a sealed ampoule or a porcelain boat, is added, and the tube is constricted, evacuated, and sealed. The tube is then agitated to break any ampoule present and to mix the contents. Afterward it is heated (*4*) in a horizontal position for 30 min at (640 ± 10) °C.

After being cooled, the tube is placed in a larger tube *B*, which is subsequently evacuated, closed off, and inverted to break the tip of the combustion tube. The combustion products are then condensed at −195°C in the first tube, *D*, of a series of U-tubes. In a single distillation, carried out by removing the cooling bath, water and less volatile combustion products are condensed at −95°C (*5*) in the second tube, *E*, and carbon dioxide is condensed at −195°C in the third tube, *F*. The carbon dioxide is expanded from tube *F* into the constant-volume manometer *M* and measured manometrically if a value for the carbon content of the sample is desired. For determination of radioactivity, the carbon dioxide is expanded from tube *F* into an evacuated ionization chamber *C* and diluted to atmospheric pressure with tank carbon dioxide; the transfer of radioactive gas can be made quantitative if the tank carbon dioxide is added through inlet *G* and tube *F*. The ionization current is measured, as in tritium assay (*6*), with a vibrating reed electrometer (*7*).

The use of the water condensed in tube *E* for assay of isotopic hydrogen is not satisfactory, since there is appreciable retention of sample hydrogen in the combustion tube. It is interesting to note, however, that tritium analyses by the zinc fusion technique can be performed in this system if the ionization chamber is mounted on tube *D*.

Analyses of organic compounds for $C^{14}$ by ion current measurements on carbon dioxide (Table 1) show that values obtained via sealed-tube combustion are 100.60 ± 1.1 percent of those obtained (*1*) via Pregl combustion. The standard error of an analysis is 0.46 percent in sealed-tube combustion and 0.64 percent in Pregl combustion. The applicability and reliability of sealed-tube combustion are further indicated by the results (Table 2) of total carbon, obtained by manometric measurement of carbon dioxide, in a variety of compounds; the observed values are 100.67 ± 0.30 percent of those calculated. The correspondence of these results to theoretical values suggests the possible development of the procedure into a satisfactory analytic method for total carbon.

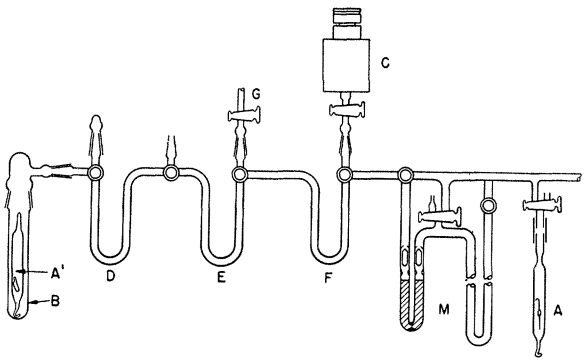


Fig. 1. Apparatus for analysis of carbon dioxide from sealed-tube combustions.

Table 1. Determination of radioactive carbon.

| Compound | Carbon-14 (μc/g) | |
|---|---|---|
| | Pregl combustion | Sealed-tube combustion |
| Sucrose* | 224.0 | 224.7 |
| | 223.1 | 224.8 |
| Dextran* | 5.66 | 5.65 |
| | 5.70 | 5.67 |
| Methyl trityl ether | 54.16 | 54.87 |
| | 54.73 | 55.59 |
| | | 55.74 |
| Tetramethylglucose | 0.791 | 0.782 |
| | .776 | .776 |
| | .775 | |
| Pentothal† | 2748 | 2774 |
| | 2750 | 2781 |
| Ethylenediamine-tetraacetic acid† | 7805 | 7970 |
| | 7812 | 7897 |

* Supplied by N. J. Scully of Argonne National Laboratory.
† Supplied by Abbott Laboratories, North Chicago, Ill.

Table 2. Determination of total carbon.

| Compound* | No. of analyses | Carbon (%) | |
|---|---|---|---|
| | | Calc. | Found |
| Acetanilide | 1 | 71.09 | 71.26 |
| Acetoacetanilide | 2 | 67.78 | 68.44 |
| Acetophenone | 4 | 79.97 | 81.02 |
| Anthracene | 2 | 94.34 | 94.88 |
| Benzene | 2 | 92.25 | 92.67 |
| Benzoic acid | 2 | 68.84 | 69.34 |
| Biphenyl | 2 | 93.46 | 94.38 |
| Bromobenzene | 2 | 45.89 | 45.92 |
| *n*-Butyl alcohol | 2 | 64.81 | 65.58 |
| *n*-Butyl bromide | 1 | 35.06 | 35.19 |
| *n*-Butyl chloride | 1 | 51.90 | 52.30 |
| *n*-Butyl iodide | 1 | 26.10 | 26.69 |
| Chlorobenzene | 2 | 64.02 | 64.74 |
| Cholesterol | 2 | 83.87 | 84.05 |
| Cholesterol acetate | 2 | 81.25 | 81.33 |
| Desoxycholic acid | 2 | 73.43 | 73.90 |
| Di-*p*-tolyl sulfone | 1 | 68.26 | 68.73 |
| Hexane | 2 | 83.63 | 84.08 |
| 8-Hydroxyquinoline | 1 | 74.47 | 74.10 |
| Iodobenzene | 2 | 35.32 | 35.72 |
| Methyl isobutyl ketone | 1 | 71.95 | 72.03 |
| *o*-Nitrobenzoic acid | 2 | 50.30 | 50.47 |
| *n*-Octadecane | 3 | 84.95 | 85.66 |
| *n*-Propyl alcohol | 4 | 59.96 | 60.71 |
| *p*-Toluenesulfonamide | 2 | 63.13 | 63.38 |

* Shelf samples of Matheson Co. chemicals were used in most cases.

### References and Notes

1. K. E. Wilzbach and A. R. Van Dyken, *U.S. Atomic Energy Comm. Doc.*, AECD-2998, Oct. 1950.
2. K. E. Wilzbach, L. Kaplan, and W. G. Brown, *Science* **118**, 522 (1953).
3. Tubing of Pyrex 1720 glass is available on special order from Corning Glass Works, Corning, N.Y. Quartz or Vycor tubing is also satisfactory.
4. A furnace, series 9ADL of the K. H. Huppert Co., Chicago, can be used, with a quartz liner, to heat eight tubes in one loading.
5. This temperature is obtained with a melt, formed by addition of liquid nitrogen, of toluene or di-*n*-butyl ether.
6. K. E. Wilzbach, A. R. Van Dyken, and L. Kaplan, *Anal. Chem.* **26**, 880 (1954).
7. Calibration with standard samples has established that the charge collected at 450 v in a Borkowski-type chamber filled to atmospheric pressure with carbon dioxide corresponds to $1.39 \times 10^{-16}$ coulomb per disintegration of $C^{14}$.


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## Enhancement of Biological Activities of Corticosteroids by Substitution of Halogen Atoms in 9α Position


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The biological actions of hydrocortisone are readily distinguishable from those of desoxycorticosterone. Thus, while hydrocortisone has comparatively little sodium-retaining activity, it has until recently been the most potent known steroid in carbohydrate metabolism, in anti-inflammatory activity, and in depressing the number of circulating eosinophils. Desoxycorticosterone, on the other hand, while practically devoid of carbohydrate effects, anti-inflammatory activity, and eosinopenic activity, has until recently been the most potent known steroid in sodium-retaining activity. It has been of interest to us, therefore, that hydrocortisone, when it bears a fluorine or chlorine atom in 9α position, not only becomes more potent than hydrocortisone itself in carbohydrate and eosinopenic activity but also becomes more potent than desoxycorticosterone in sodium-retaining activity.

Fried and Sabo (*1, 2*) and Borman and Singer (*3*) have reported that substitution of either a fluorine or a chlorine atom in the 9α position of either cortisone or hydrocortisone results in enhancement of the activity of these corticosteroids as measured by glycogen deposition in the livers of fasting adrenalectomized rats.

We have studied, in adrenalectomized dogs, the comparative pharmacology of 9α-chlorocortisone acetate (chloro E Ac), 9α-chlorohydrocortisone acetate (chloro F Ac), 9α-fluorohydrocortisone acetate (fluoro F Ac), 9α-bromohydrocortisone acetate (bromo F Ac), cortisone (E), hydrocortisone (F), hydrocortisone acetate (F Ac), desoxycorticosterone (DOC), and desoxycorticosterone acetate (DOCA) (see Table 1). Several of these steroids have been studied, under a metabolic regimen, in patients with Addison's disease (*4*).

*Effects on excretion of sodium and potassium.* In adrenalectomized dogs, E and F, given intravenously in doses of 2 mg or less, had no appreciable effect on excretion of either sodium or potassium during the 4-hr period following administration. When given in doses of 4 to 20 mg, these steroids induced increases in excretion of both sodium and potassium during the ensuing 4 hr. In no dosage did E or F cause sodium retention. Chloro E Ac, chloro F Ac, and fluoro F Ac, on the other hand, resembled DOC in that each of these steroids in doses of 25 to 100 μg induced sodium retention and potassium loss. The magnitude of these responses was a direct function of dosage. Assays of these activities in adrenalectomized dogs indicated that chloro F Ac had 3.3 (1.9–5.2) (95 percent confidence limits) times the potency of equimolar doses of DOC, while chloro E Ac had 2.1 (1.2–3.8) and fluoro F Ac had 4.7 (2.4–9.2) times the potency of DOC. It was apparent that, insofar as these halogenated steroids caused acute sodium retention in adrenalectomized dogs, they differed qualitatively, as well as quantitatively, from their nonhalogenated analogs.

*Effects on glomerular filtration rate.* DOC apparently exerted its acute effects on electrolyte excretion by acting on renal tubular transport, since it failed to bring about any consistent changes in glomerular filtration rate (GFR) in doses up to 8 mg. Compounds

Table 1. Relative potencies of various steroids during 4-hr periods following intravenous administration to adrenalectomized dogs.

| Steroid | Dose (μg) | Eosinopenia | GFR increase | Sodium retention | Potassium loss |
|---|---|---|---|---|---|
| Cortisone or Hydrocortisone Ac | 100–2000 | 0 | 0 | 0 | 0 |
| | 4000–8000 | + | + | – (Loss) | + |
| 9α-Chlorocortisone Ac or 9α-Chlorohydrocortisone Ac | 25–200 | 0 | 0 | + | + |
| | 500–4000 | + | + | + or – | + |
| 9α-Fluoro-hydrocortisone Ac | 25–100 | 0 | 0 | + | + |
| | 200–800 | + | + | + or – | + |
| 9α-Bromo-hydrocortisone Ac | 25–100 | | | 0 | 0 |
| | 100–400 | | | 0 | + |
| Desoxycorticosterone | 50–8000 | 0 | 0 | + | + |