dopamine has functionally destroyed an inhibitory system which blocked the behavioral effect of dopa.

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Effect of phenylbutazone breakdown products on drug metabolism assay

Since the publication of a convenient method for the assay of phenylbutazone in biological material by Burns, Rose & others (1953, 1955) the drug has been used to measure the activity of liver microsomal drug metabolizing enzymes. The standardized enzyme preparation is then used to study the kinetics of hydroxylation reactions under various experimental conditions. Phenylbutazone has generally been regarded as a relatively stable compound and it has been normal practice to prepare and to store standard alkaline solutions of phenylbutazone. However, Pawelczyk & Wachowiak (1968, 1969) identified a number of breakdown products in aqueous solutions caused by hydrolysis and oxidation. The decomposition was independent of pH (between 7·0 and 10·6), buffer concentration or ionic strength, and caused a significant change in phenylbutazone concentration. The decomposition products were not the known metabolites of the drug, but appear likely to possess similar chemical characteristics. This raises doubts about the validity of enzyme experiments where freshly prepared solutions of phenylbutazone are not used. Some effects of the breakdown products on the apparent activity of the liver enzymes are now reported.

Solutions of phenylbutazone and its breakdown products, phenylbutazone carboxylic acid, α-hydroxyphenylbutazone carboxylic acid, 4-hydroxyphenylbutazone, n-caproylhydrazobenzene and re-crystallized azobenzene were prepared in acetone. These solutions were used as standards for g.l.c. and t.l.c. analyses. Phenylbutazone solutions in N and 0·1N sodium hydroxide were also prepared and stored at room temperature or −20°.

Both the acetone and aqueous phenylbutazone solutions rapidly developed a yellow-orange colour at room temperature. This also occurred at −20°, although more slowly. After various storage times the alkaline solutions were extracted for azobenzene with ether and for 4-hydroxyphenylbutazone and phenylbutazone with ether after pH adjustment to pH 7·2 and pH 2 respectively using hydrochloric acid. Gas chromatographic analyses of the ether extracts showed a decreasing phenylbutazone

peak and an increasing "unknown" peak. After seven weeks at room temperature, this breakdown product was the major constituent of the solution.

The unknown breakdown product had a g.l.c. retention time the same as 4-hydroxyphenylbutazone. The α-hydroxy compound was thermally unstable and produced three peaks one of which was similar to the unknown. The ultraviolet spectra of these three compounds were too similar to be used for identification purposes. Infrared analysis and t.l.c. supported the suggestion that the unknown compound is 4-hydroxyphenylbutazone. Not enough of the compound could be extracted from the solutions to permit further purification and mass spectrometry.

Large amounts of a yellow-orange compound rapidly precipitated from the alkaline solutions of phenylbutazone when exposed to light. Mixed melting point determinations and g.l.c. indicated that the precipitate was azobenzene.

Drug metabolism studies were made according to Burns & others (1953, 1955) using female Swiss strain mouse liver homogenized in 0·2M phosphate buffer pH 7·4. The 10 000 *g* supernatant was used for the enzyme preparation in amounts equivalent to 60 mg protein. Co-factors employed were those of Kuntzman, Mark & others (1966). Incubation time was 1 h. The contribution of each of the phenylbutazone breakdown products to the ultraviolet absorption assay used in the Burns' method, was determined by incubation of 100 μg of each compound (total volume 3·0 ml) and measurement of absorption at 263 nm after extraction.

All the phenylbutazone breakdown products except n-caproylhydrazobenzene were carried through the extraction procedure of Burns. In the absence of active enzyme during the incubation period, ultraviolet absorption at 263 nm gave amounts equivalent to 10–20% phenylbutazone. In the presence of active enzyme neither α-hydroxyphenylbutazone carboxylic acid nor n-caproylhydrazobenzene showed any apparent metabolism, but phenylbutazone carboxylic acid and 4-hydroxyphenyl butazone showed values of 10 and 58% respectively compared with 26% for phenylbutazone itself. The value for 4-hydroxyphenylbutazone is particularly significant since this appears to be the major breakdown product under the usual storage conditions. Hence the apparent activity of an enzyme system standardized against phenylbutazone will depend on the nature and amount of any breakdown products present. Therefore when using the Burns' assay for estimating the activity of drug metabolizing enzymes, it is important to use freshly prepared solutions of phenylbutazone.

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