From the Institutes of Pharmacology, Universities of Tübingen and Munich

The Isolation of p-Chloronitrosobenzene from the Blood of Dogs injected with p-Chloroaniline

By
MANFRED KIESE and GERHARD RENNER

With 3 Figures in the Text

(Received July 20, 1963)

The N-hydroxylation of aromatic amines in vivo was first demonstrated in 1958, when nitrosobenzene was found in the blood of dogs injected with aniline or N-methylaniline (KIESE 1959a, b, c). In higher animals phenylhydroxylamine, the product resulting from N-hydroxylation, reacts with hemoglobin and oxygen to form nitrosobenzene (DANNENBERG and KIESW; KIESE and REINWEIN). In order to oxidize any phenylhydroxylamine present to nitrosobenzene and in order to facilitate the extraction of nitrosobenzene by splitting nitrosobenzene hemoglobin, ferricyanide was added to the hemolyzed blood before extraction. The nitrosobenzene extracted from the blood was identified by some of its chemical and physical properties, especially its characteristic absorption spectrum at wave lengths of 270 to 330 m\(\mu\).

After injecting other aniline derivatives the products resulting from N-oxidation were also detected by chemical and optical methods (HUSTEDT and KIESE; HERINGLAKE, KIESE, RENNER and WENZ; BAADER, GIRGIS, KIESE, MENZEL and SKROBOT; KIESE 1963a). The isolation of N-hydroxy-2-acetylaminofluorene by CRAMER, MILLER and MILLER from the urine of rats that were fed on a diet containing 2-acetylaminofluorene confirmed our observations on the N-oxidation of aromatic amines in vivo. Further unequivocal proof of the biological N-hydroxylation of aromatic amines was furnished after the experiments reported in this paper had been performed by the isolation of phenylhydroxylamine from suspensions of rabbit liver microsomes incubated with N-ethylaniline in the presence of reduced nicotinamide adenine dinucleotide phosphate (KIESE and RAUSCHER). Although higher concentrations of nitrosobenzene were found in the blood of dogs after injecting N-alkylanilines (KIESE 1959c; HOLZER and KIESE) than after injecting aniline, attempts to purify further the nitrosobenzene extracted from the blood of such dogs were discouraging. Its concentration in the blood was still too low.
A study of the N-hydroxylation of some aniline derivatives in dogs showed that p-chloronitrosobenzene accumulates to a rather high concentration in the blood of dogs injected with p-chloroaniline (Kiese 1963a, b).

p-Chloronitrosobenzene added to blood in vitro in concentrations observed in the blood of dogs after injecting large doses of p-chloroaniline could be isolated and purified until no difference in a series of characteristic physical and chemical properties could be observed. The appearance of p-chloronitrosobenzene in high concentration in the blood of dogs injected with p-chloroaniline and its proven stability during the isolation procedure justified the large expenditure involved in an attempt to isolate p-chloronitrosobenzene from the blood of dogs injected with p-chloroaniline.

By the methods described below, from the blood of dogs injected with p-chloroaniline, a substance was isolated which was found to be identical with authentic p-chloronitrosobenzene in the following physical and chemical properties:

- From the blood the substance was extracted into carbon tetrachloride or into hexane;
- From carbon tetrachloride or hexane it was not extracted into aqueous solutions of sulfuric acid;
- In countercurrent distribution between carbon tetrachloride and aqueous methanol after 800 distributions and in countercurrent distribution between hexane and aqueous ethanol after 240 distributions the maximum concentration was found very close to the tube calculated from the partition coefficient of authentic p-chloronitrosobenzene;
- Absorption of ultraviolet light in various organic solvents;
- Odor of nitrosobenzene;
- Volatile with steam;
- Almost colorless in the solid state;
- Green when dissolved, when adsorbed on silica gel, molten, or steam-distilled;
- Melting point;
- Paper chromatography with two solvents;
- Thin-layer chromatography on silica gel;
- Formation of a color after being diazotized and coupled with α-naphthylethylenediamine.

The discovery of the biological N-oxidation of aromatic amines was claimed first by Ellinger. He described the isolation of a substance from the blood of cats following the administration of acetanilide which he assumed to be acetylphenylhydroxylamine. Thore, Williams and Shearswell as well as Hustedt and Kiese have pointed out that Ellinger did not isolate acetylphenylhydroxylamine. Later Rosenthal and Bauer and Rosenthal (1939a) reported the detection of an aromatic hydroxylamine in the urine of animals and men following the