Identification of Crystals of the Rimino-Phenazine Compound B663 (Lamprene: Clofazimine) in Mouse Spleen Macrophages by Thin Layer Chromatography and Mass Spectrum Analysis

In 1944, alarm over the increasing incidence of tuberculosis in Ireland, led the Medical Research Council to set up a scientific investigation aimed at developing an effective drug against this disease. Although the riminophenazine compound B663 (Lamprene; clofazimine) did not fulfill this need, its discovery by BARRY in 1957 provided a drug considerable power against the organism of murine leprosy (*Mycobacterium lepraemurium*) and against the bacillus causing human leprosy, *Mycobacterium leprae*. The development, biochemistry, and therapeutic potential of this compound have been fully reviewed[1]. Numerous subsequent papers[2–10] have paid tribute to the action of B663 in the routine treatment of leprosy, and also in the management of various forms of reaction during the course of this disease.

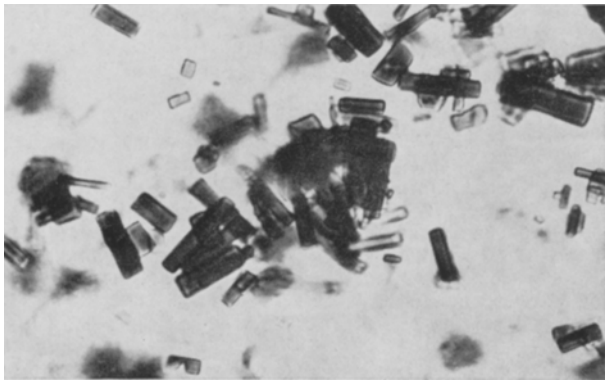
Following absorption from the gut, this drug has the remarkable property of accumulating in the cytoplasm of macrophages, and a simple 'squash' preparation from liver, spleen, peritoneum, or lymph node reveals large numbers of fully-formed crystals under the microscope (Figure). There is experimental work[11] to suggest that the drug enters macrophages in solution, linked to a lipoprotein carrier, '...which is then split off, with the consequent intracellular formation of crystals of B663...'. Electron microscope studies[12,13] have confirmed the location of mature crystals in macrophage cytoplasm and these have more recently been shown[14] to be surrounded by a membrane in situations strongly suggestive of location within the lysosomal-vacuolar system. In both these studies, the fully-formed crystals are represented as spaces containing only the embedding medium, all crystalline substance having been dissolved out during processing. Electron, and X-ray diffraction on these spaces is completely negative. From time to time the acceptance of these spaces as representing the original riminophenazine as administered to the experimental animal has been challenged. For this reason, and also because our previous investigations have occasionally suggested the presence of impurities in the batch of B663 supplied, it was decided to attempt precise identification of the crystals in splenic macrophages of the experimental mouse, and to compare findings with crystals of drug administered in the diet.

Female albino Parkes strain mice were put onto 0.01% of B663 in their diet, and killed $4\frac{1}{2}$ months later. Tail and snout were characteristically pigmented a light reddish-brown by the drug. Subcutaneous tissues and fat were reddish-orange, and this deepened with exposure to air. Internal organs were typically red, and lymph nodes in the abdomen and thoracic cavity contained obvious refractile accumulations of the drug. Spleen, liver and various lymph nodes were taken for examination by light microscopy using a fresh 'squash' preparation under a coverslip, (Figure), and for chemical investigation by thin layer chromatography (TLC) and mass spectrometric analysis. Tissue and crystals for the latter were suspended in chloroform and homogenized prior to analysis. Crystals of the actual drug administered to these animals in their diet were subjected to parallel examinations.

Isolation of pigment. The tissue obtained was blended in chloroform (10 ml) for 3 min (MSE Homogenizer) and the red-orange solution separated by decanting. This extraction was repeated 3 times. The resulting solution was concentrated and examined on TLC (Table). Two major components were present; a very intense lipid zone and an orange red zone at an equivalent Rf to that of B663. Isolation of the pigment by preparative TLC afforded, after removal of the solvent, a small quantity (3 mg) of an amorphous red orange powder which was chromatographically identical to B663.

Mass spectral analaysis. Mass spectra were recorded on an A.E.I. MS9 mass spectrometer and were measured using a direct insertion probe. Spectra of pure compounds were determined at a probe temperature of 210 °C. Those from biological sources were obtained after pre-evaporation, at 100–150 °C, of trace lipid contaminants from the probe in the mass spectrometer.

Results. The thin layer chromatographic behaviour of the purified red-orange pigment isolated from the spleen, liver and various lymph nodes of mice fed on B663 was identical to that of pure B663 (Table). Similarly the mass spectrum was identical in every respect, isotope pattern, and fragmentation pattern to that of pure B663. The mass spectral fragmentation of rimino-phenazines will be discussed in a subsequent paper.



Fully-formed crystals of B663 (Lamprene: clofazimine) expressed from macrophage cytoplasm in a simple 'squash' preparation between coverslip and microscope slide. Mesenteric lymph node of mouse. Original magnification × 500. Unstained.

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TLC behaviour of various rimino-phenazines[a]

Compound		Rf
Isolate		0.39
B663:	3-(*p*-chloroanilino)-10-(*p*-chlorophenyl)-2,10-dihydro-2-(isopropylimino)-phenazine	0.39
B1865:	3-anilino-7-chloro-2,10-dihydro-2-(isopropylimino-10-phenyl-phenazine	0.27
B1911:	3-anilino-7-fluoro-2,10-dihydro-2-(isopropylimino)-10-phenyl-phenazine	0.18
B1811:	3-anilino-7-ethoxy-2,10-dihydro-2-(isopropylimino)-10-phenyl-phenazine	0.02
B1739:	3-anilino-7-methoxy-2,10-dihydro-2-(isopropylimino)-10-phenyl-phenazine	0.02
B1912:	3-anilino-7-chloro-2-(cyclohexylimino)2,10 dihydro-10-phenyl-phenazine	0.55

[a] Silica gel type H plates containing 0.1 NKOH, two elutions in 10% EtOAc/Benzene.

While pigmentation of the skin of leprosy patients taking B663 has been well documented[15,16], the occurrence of crystals in human tissues has received much less attention[17,18]. It has occasionally been suggested that the red crystals seen under light microscopy, and the 'spaces' seen on electron microscopy might represent a metabolite, or an impurity. Our results in the mouse show that crystals obtained from macrophage cytoplasm are in every way identical with those in the drug used for oral feeding of the animals; it is concluded that crystals seen in the tissues represent those of the unaltered drug.

Zusammenfassung. Die Rimino-Phenazin-Verbindung (Präparat B663 – Lampren® Clofazimine) mit starker Wirkung gegen *Mycobacterium leprae* wurde $4^1/_2$ Monate an Mäuse verfüttert. Lichtmikroskopisch, dünnschichtchromatographisch und massenspektrometrisch wurde festgestellt, dass die B663-Kristalle im Cytoplasma der Makrophagen mit den in der Grundsubstanz vorhandenen identisch sind.

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Teratogenicity Study in Rats given High Doses of Pyridoxine (Vitamin B_6) during Organogenesis

The pattern of pyridoxine or vitamin B_6 usage in women has undergone a marked change in recent years. Therapeutic doses of 40–400 mg/person/day are prescribed to counteract depressive conditions arising from schizophrenia[1] and oral contraceptives[2]. A routine administration of high doses of pyridoxine to all pregnant women with a history of depressive disorders has been suggested[3]. Since this megavitamin treatment finds an increasing application in orthomolecular psychiatry, teratogenicity studies are needed to evaluate the safety of high levels of pyridoxine exposure to pregnant women.

This report presents fetal data on rats dosed orally with 20–80 mg pyridoxine/kg on days 6–15 of gestation.

Materials and methods. Randomly bred female (Wistar) rats, 175–200 g body weight, were paired overnight with males, and the morning that a sperm-positive vaginal smear was observed, was noted to be day 1 of pregnancy. The mated females were randomly assigned to experimental groups. Pyridoxine hydrochloride obtained from Nutritional Biochemical Corporation, Ohio, was administered orally in single daily doses on days 6–15 of gestation. The doses given were 0 (distilled water), 20, 40, 60 or 80 mg/kg/day. Solutions for the 4 dose levels were prepared as 0.4, 0.8, 1.2 and 1.6%, respectively, of pyridoxine hydrochloride in distilled water. Pregnant rats were weighed daily in order to permit the volume of solution given to be adjusted to constant dose/body weight ratio for the treatment period.

All female rats were killed on day 22 of pregnancy and their viscera including uteri were examined. The fetuses were removed, weighed and examined for viability and external malformations. Early resorptions and fetuses dying late in development were recorded. One-half of the live fetuses from each litter were studied for skeletal anomalies; the remainder were fixed in Bouin's fluid and were inspected for gross visceral defects.

Normal distribution formed the basis for intergroup comparisons and probabilities were computed for values χ^2. A 5% probability level was chosen to evaluate differences.

Results. There was no evidence of maternal toxicity associated with pyridoxine treatment. Prenatal values for live fetuses, dead fetuses and resorption sites, and fetal

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