UPTAKE BY RETICULO-ENDOTHELIAL CELLS OF THE RIMINOPHENAZINE B.663 (2-*P*-CHLOROANILINO-5-*P*-CHLOROPHENYL-3 : 5-DIHYDRO-3-ISOPROPYLIMINOPHENAZINE)*

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A PROGRAMME of synthesis of potential new anti-tuberculosis agents in these laboratories led to the production of the riminophenazine B.663 (2-*p*-chloroanilino-5-*p*-chlorophenyl-3 : 5-dihydro-3-isopropyliminophenazine) which has high anti-tuberculosis activity in the experimental animal (Barry, Belton, Conalty, Denneny, Edward, O'Sullivan, Twomey and Winder, 1957 ; Barry and Conalty, 1958).

The compound, which is a red crystalline substance, has been found to be taken up by cells of the reticulo-endothelial system, and some cytological aspects of this have been described in a preliminary communication (Conalty and Jackson, 1960). The gross quantitative distribution of the compound in the tissues of various animals, on various dosage regimens has also been reported (Barry, Buggle, Byrne, Conalty and Winder, 1959 ; 1960). The present paper describes in detail the course of the intracellular uptake and storage, and compares and contrasts the findings with those obtained with other substances stored in cells of the reticulo-endothelial system.

MATERIALS AND METHODS

B.663, which is soluble to the extent of 10 per cent in benzene, 0·7 per cent in olive oil and approximately 1 μg./ml. in water at pH 7·0 and 20°, was administered mixed in a ground meal diet to male albino mice (Schofield) of 25–35 g. weight. The mice were killed at intervals and the presence of drug was studied by means of paraffin sections, frozen sections and direct smears. In paraffin sections the compound was completely removed by the alcohol-xylol processing, and histological changes could be studied, without interference from drug deposits. Frozen sections permitted accurate histological localization of the drug. Direct smears of organs, particularly of the mesenteric lymph node and lungs and smears of peritoneal fluid, were used extensively for the study of the intracellular behaviour of B.663.

Smears were studied, unstained, and stained by the standard Giemsa method for blood smears, or by aqueous toluidine blue. The standard Giemsa method, involving methyl alcohol fixation, removed the drug leaving " ghosts " or outlines of deposited drug. The Giemsa method, modified by fixation by heat or drying, was also used in order to retain the drug in the smear.

In addition, frozen sections and smears from B.663-treated guinea-pigs were examined and also smears of the organs of B.663-treated albino rats. These species, like the mice, were given the drug mixed in the diet.

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RESULTS

Macroscopic findings in the mouse

In mice at the end of the first day's treatment with 50 mg./kg./day a yellow-orange coloration was observed in the fat while the tissues generally had developed a pink tinge of varying intensity. These colours deepened as treatment continued.

By the end of ten days all the fat, visceral and subcutaneous, was a bright orange colour and the ileum and jejunum, the lymph nodes, particularly the mesenteric node, and the caput epididymis were coloured strongly. The normal colour of the liver, spleen and kidneys tended to mask the drug colour but by the end of this period a noticeable darkening of these organs was evident. The pancreas and testes were but faintly coloured. The lungs at this stage showed only a slight pink coloration. The preputial glands took the same orange colour as the fat.

At a later stage, from a month onwards, with continued dosage, the ileum, jejunum and lymph nodes became purplish black, and the liver, spleen and kidneys darkened further. The spleen showed marked enlargement and there was also some enlargement of the liver and kidneys. The lungs showed a mottled appearance of purplish black dots on a pale background. The orange fat became brown in colour and decreased in quantity.

The skin became coloured red from about the end of the first week and after some months on continuing dosage became purplish black. The hair exhibited circumscribed areas of pink coloration symmetrical with reference to the longitudinal axis. The central nervous system was not coloured. From the tenth day or so onwards the peritoneum and the peritoneal fluid became progressively pinker.

Histological findings in the mouse

Examination of histological frozen sections and smears showed that, apart from the fat, the colour was apparently confined to the macrophages. The initial form in the macrophages was as discrete, red-orange, rounded bodies, 1–2 μ in diameter, in the cytoplasm (Fig. 1). After 7–10 days bright red, broad crystals of drug were found within some macrophages with the inclusions (Fig. 2). After about 28 days the inclusions had largely disappeared and the drug was present within the macrophages in the form of crystals only, varying in size (up to 25 μ long and 8 μ broad) and in number (Fig. 3). During the inclusion stage the macrophage nucleus is usually centrally placed. In cells bearing crystals the nucleus or nuclei (the cells are commonly binucleate and occasionally trinucleate or even multinucleate) may be encircled by crystals (Fig. 5), may be displaced to the periphery by them or may be indented or obscured by them (Fig. 4 and 6). The largest crystals were found in macrophages of peritoneal fluid (Fig. 4), lymph nodes and spleen, and the largest aggregations of crystals were found in macrophages of the lungs on prolonged dosage (Fig. 7).

In the liver, it was noteworthy that the Kupffer cells did not take up the drug, which was present in this organ principally in perivascular collections of macrophages. In the lungs, although the development of inclusions and crystals took place more slowly than in the lymph nodes and spleen, a large number of conspicuous macrophages containing drug was to be found.

Findings in the rat and guinea-pig

The rat has not been so extensively studied as the mouse but the uptake appears to be the same as in the mouse. In the guinea-pig, absorption is much less efficient than in the other animals and there were differences in tissue distribution. In this species the bulk of the drug was to be found in the lungs, apart from that which was taken up by the fat. The same sequence of events found in the mouse, *i.e.* inclusions followed by inclusions plus crystals and in turn by crystals only, was observed in both the rat and guinea-pig.

DISCUSSION

The progressive build-up of B.663 in the macrophages, through an inclusion stage to that of crystal-formation, has not been described, so far as we are aware, for other chemical substances given orally. That a crystalline stage follows the inclusion stage is very suggestive that the inclusions are essentially liquid in nature. However, within the inclusions may be seen areas, 1–4 in number, which have a more solid appearance and which become darker as the inclusions generally darken. The massive size and extraordinary packing of the crystals resulting from the progressive concentration of the drug within the macrophages shows that B.663 does not interfere with the phagocytic activity of the cell for B.663, and it will be shown in another publication that such crystal-laden cells have not lost their phagocytic activity for other agents.

The failure of the Kupffer cells to concentrate B.663 exhibits a differentiating property of this compound for cells of the reticulo-endothelial system which is not shared by the usual coloured substances used in the study of this system.

The process of concentration of B.663 intracellularly shows some resemblance to that illustrated by Baillif (1960) in electron micrographs of the storage, within Kupffer cells of rats and mice, of intravenously injected thorotrast particles. The particles are at first scattered at random throughout the macrophage cytoplasm and are secondarily collected into groups. As a greater number of particles become collected within a single group they are forced into association with each

EXPLANATION OF PLATES

FIG. 1.—Macrophage in mouse lung unstained smear showing inclusions of B.663. Fourteen days' treatment at approximately 50 mg./kg. per day. ×2400.

FIG. 2.—Macrophage in mouse lung unstained smear showing inclusions and crystals of B.663. Fourteen days' treatment at approximately 50 mg./kg. per day. ×2500.

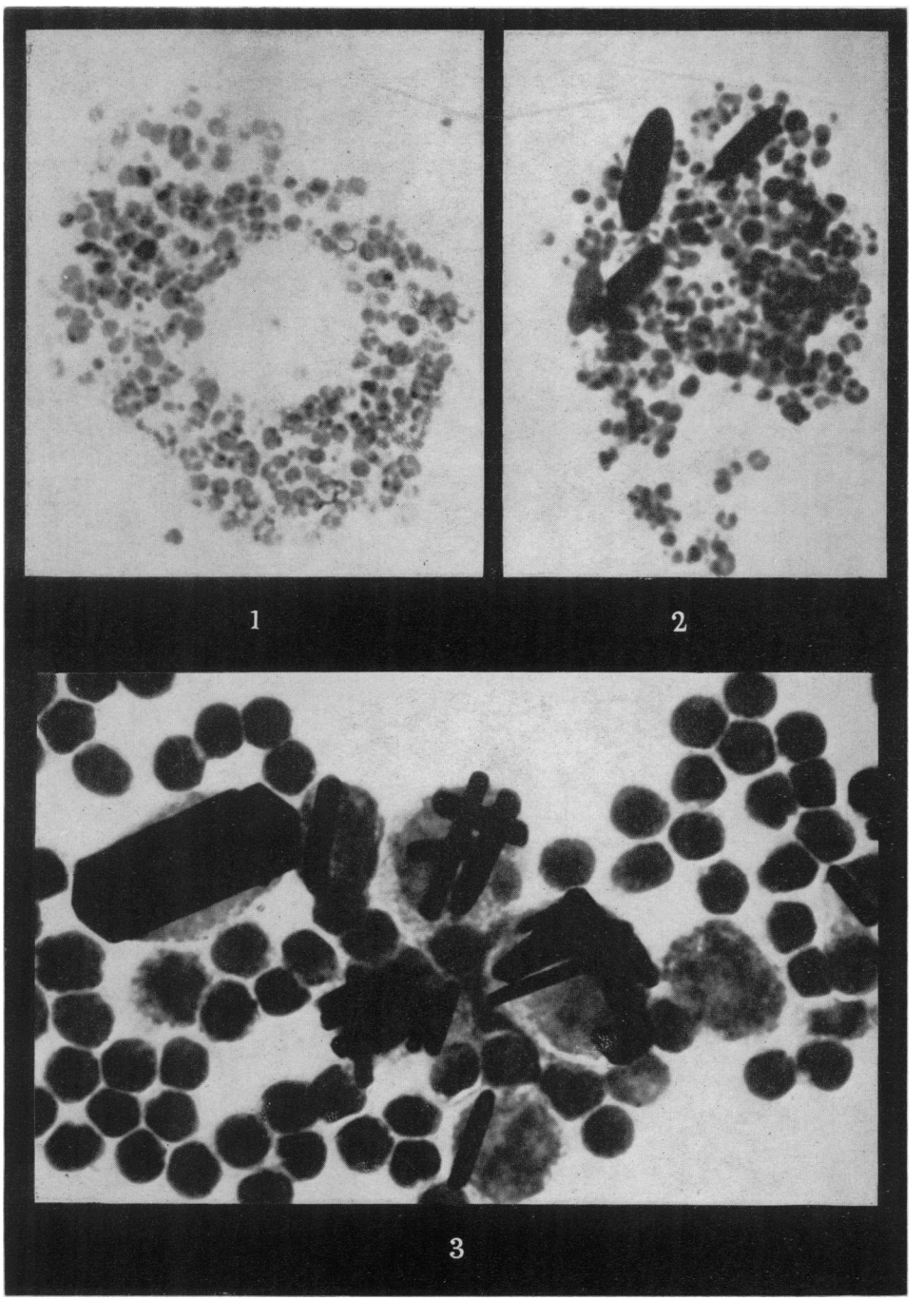
FIG. 3.—Macrophages in mouse peritoneal fluid, stained with Giemsa (fixation by heat), showing the variation in the size and numbers of crystals of B.663 per cell. Forty-eight days' treatment at approximately 50 mg./kg. per day. ×1500.

FIG. 4.—Macrophage in mouse peritoneal fluid, stained with 1 per cent aqueous toluidine blue, showing a large crystal (25 × 7 μ) of B.663 obscuring and displacing the nuclei. Twenty-six days' treatment at approximately 50 mg./kg. per day. ×3500.

FIG. 5.—Macrophage in mouse peritoneal fluid, stained with Giemsa (fixation by heat) showing peripheral arrangement of crystals. Forty-eight days' treatment at approximately 50 mg./kg. per day. ×3000.

FIG. 6.—Macrophage in mouse peritoneal fluid stained with Giemsa, showing displacement of nucleus. The method of fixation has dissolved out the crystals leaving " ghosts ". Forty-eight days' treatment at approximately 50 mg./kg. per day. ×3200.

FIG. 7.—Macrophage in mouse lung unstained smear showing packing of macrophages with a large number of crystals of B.663. One hundred and fifty-five days' treatment at approximately 50 mg./kg. per day. ×2200.



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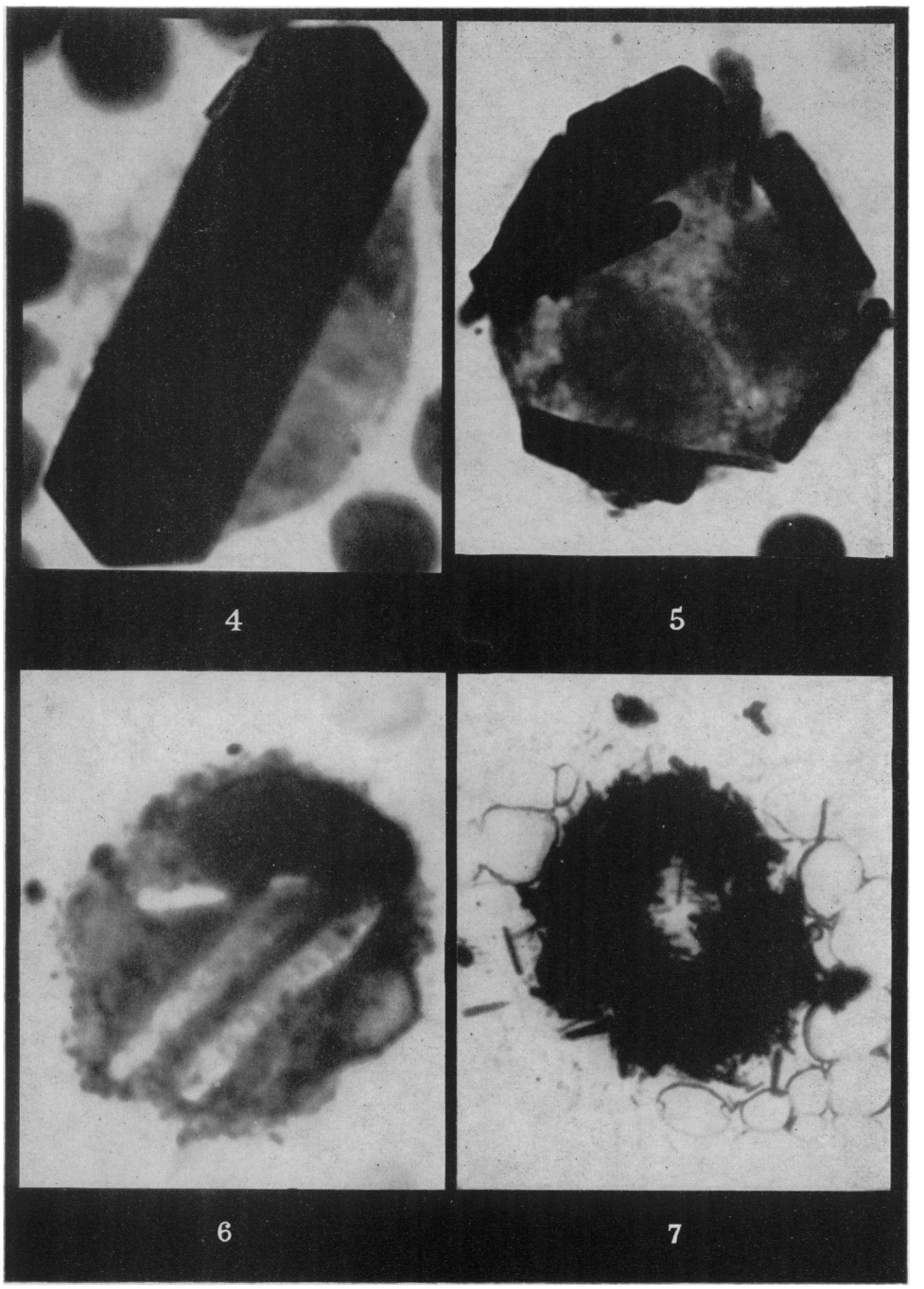

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other to form small lattices and chains. The storage of B.663 differs in that, while it is not taken up by Kupffer cells, in the other drug-containing macrophages intermediate stages were not observed, that is there were no precursors of the inclusions and no intermediates between inclusions and crystals. Karrer (1960), in an electron microscope study of phagocytosis of carbon by lung macrophages, also described formation of inclusion bodies.

A sequence of stained intracellular droplets gradually increasing in intensity of staining to a point at which vital dyes precipitate as granular concentrates, has been described by Rebuck, Boyd and Riddle (1960) for macrophages, neutrophils, monocytes and lymphocytes following topical application of the dyes using a skin window technique. Crystal formation, in macrophages and fibroblasts, " saturation crystals ", accompanied by extensive cell necrosis of the intestinal mucosa, has been reported by Evans and Scott (1921) after intensive oral adminstration of certain soluble acid azo-dyes. These crystals, *e.g.* of trypan blue, were small and were dispersed in the cytoplasm.

Baker (1958) distinguishes two ways in which coloured substances are of assistance in the study of living cells ; (*a*) by being phagocytosed as coloured particles ; (*b*) by colouring pre-existing cellular constituents. It is clear that the special affinity of B.663 for the active phagocytic cells, apart from the affinity for the fat cells, puts it in the first category to which belong the acid disazo and trisazo water-soluble dyes. Its basicity and its broad structural similarity to the phenazine, oxazine and thiazine dyes listed by Baker as producing general vital coloration would put B.663 in the second category. It differs from the other agents in this second category by being water-soluble to the extent of only 1 μg./ml. at 20° and by being soluble in lipid and in lipid-solvents.

We have fed mice sudan black B (Gurr) and sudan 4 (Gurr) as examples of lipid-soluble vital dyes, under the same dosage and conditions as B.663 and have found no coloration of cells, other than the strongly coloured fat cells. Ludford (1950) has described the use of sudan dyes as vital colorants also, and mentions colouring of the fat only. In Baker's (1958) review of vital coloration the importance of solubility in lipid for the promotion of general uptake of the soluble vital dyes by cells, originally discovered by Overton (1890), is discussed. Our findings with B.663 suggest that a high degree of lipid-solubility associated with suitable chemical structure results in its uptake by the macrophages of the reticulo-endothelial system. Also in this connection it is interesting that recent work (Day and French, 1959 ; Day and Harris, 1960 ; Izak and de Vries, 1960) has indicated a role of the reticulo-endothelial system in the transport and metabolism of lipid.

With regard to the suitable chemical structure, the soluble vital dye neutral red has been shown to stain preferentially " round " cells, probably macrophages, in fresh preparations of mouse sarcoma 37 cells (Belkin and Shear, 1937). It is interesting that Vivian (1960) has suggested that the colour base of neutral red which produces this characteristic vital staining, has, like B.663, an iminophenazine structure.

SUMMARY

A study has been made of the uptake by cells of the reticulo-endothelial system of the antituberculosis compound B.663 (2-*p*-chloroanilino-5-*p*-chlorophenyl-3 : 5-dihydro-3-isopropyliminophenazine) following its administration in the diet to

mice, rats and guinea-pigs. The compound, which is a red, crystalline substance, soluble in lipid and practically insoluble in water, coloured the phagocytic reticulo-endothelial cells, with the exception of the Kupffer cells. The fat cells were also coloured.

The initial form in the macrophages was as rounded, red-orange bodies, 1–2 μ in diameter. On continuing dosage intracellular crystals were formed, varying in size up to 25 μ long by 8 μ broad. In the mouse on continuing treatment, the macrophages throughout the body become progressively packed with crystals.

The unique intracellular behaviour of B.663 is discussed in relation to other coloured substances commonly used in the study of phagocytosis by the reticulo-endothelial system.

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