Effect of 6-Methylpurine on Phosphohexose Isomerase and Lactic Dehydrogenase Activities of Plasma, Erythrocytes, Liver, and Skeletal Muscle*

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Although it has been stated that plasma or serum enzymes are derived from the destruction of cellular elements in the blood or tissues (8), indirect evidence has recently been accumulating that enzymes may pass outward from the cell into the extracellular fluid and thence into the circulation in ways other than those involving necrosis or destruction of the cell (2, 3, 5, 8). It has long been known and is generally well appreciated that elevations in serum alkaline phosphatase, acid phosphatase, amylase, and lipase may reflect certain nondestructive cellular and tissue processes in specific organs (4). A study of the effects of a variety of toxic agents on plasma phosphohexose isomerase activity indicated that, although extensive necrosis might in some instances account for rises in this enzyme activity, it was not the only mechanism by which elevations could be induced (11).

Of the various substances used (11), 6-methylpurine was of particular interest. The minimum lethal dose in dogs receiving ten consecutive daily injections was only 0.35 mg/kg/day, much lower than that for other purines (10). Administered in this dose to those species, 6-methylpurine caused a greatly protracted intoxication characterized by anorexia, progressive weight loss, and, of particular interest, sustained elevations in plasma phosphohexose isomerase activity. In the midst of these striking changes, renal and hepatic function remained essentially normal, and microscopic study failed to demonstrate consistent lesions of sufficient magnitude to account for the enzymatic alterations in the plasma.

The failure to observe any impressive histologic or conventional biochemical alterations in the presence of extreme toxicity made it of interest to determine which tissues contributed to the rise in plasma phosphohexose isomerase activity. Accordingly, it was decided to determine this enzyme activity in the plasma, erythrocytes, liver, and skeletal muscle of rats at suitable intervals after the administration of 6-methylpurine. To gain further information concerning the mechanism involved, it was also decided to perform similar and concurrent determinations of the lactic acid dehydrogenase activities.

MATERIALS AND METHODS

6-Methylpurine1 was injected intraperitoneally in 0.85 per cent NaCl in the constant volume of 1 ml/100 gm. In two preliminary experiments a dose of 8 mg/kg was given to groups of male CFW rats (Carworth Farms Wistar); simultaneously, control animals received isotonic saline. Two or three animals were randomly selected at various times up to 8 days after treatment, and samples of blood and tissues were taken under ether anesthesia. The results obtained were similar to those found in the third definitive experiment to be described below. For this experiment, however, it was decided not to employ the more common Wistar rat, since this animal is susceptible to unpredictable acute exacerbations of pneumonitis caused by endemic infections. Instead, a new strain of Wistar rat (CPN) was selected which had been bred free of murine pneumonitis (7).

In total, 74 male CFN rats were used in the final study. The weights at the beginning of the experiment ranged from 250 to 800 gm. Nineteen rats were given a single intraperitoneal injection of 8 mg 6-methylpurine/kg of body weight; 23 controls received single injections of saline. Two or three animals were randomly selected at various times up to 8 days after treatment, and samples of blood and tissues were taken under ether anesthesia. The results obtained were similar to those found in the third definitive experiment to be described below. For this experiment, however, it was decided not to employ the more common Wistar rat, since this animal is susceptible to unpredictable acute exacerbations of pneumonitis caused by endemic infections. Instead, a new strain of Wistar rat (CPN) was selected which had been bred free of murine pneumonitis (7).

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The rats that were given injections of 6-methylpurine lost considerable weight. The possible effect of starvation and loss of weight per se was therefore evaluated by determin-

1 The investigators are indebted for generous supplies of this compound to Dr. G. H. Hitchings and Miss Gertrude Elion, The Wellcome Research Laboratories, Tuckahoe, N. Y.

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ing the plasma and tissue enzyme activities in a series of nineteen animals that were permitted water but deprived of food, and a control group of eighteen rats given water and food ad libitum. Sacrifices of a suitable number of animals from each of these groups were made at 4, 7, and 11 days.

The rats were anesthetized with ether, and blood was collected from the dorsal aorta into heparinized syringes. The animals were killed by exsanguination. The whole liver and of the gastrocnemius muscle were placed immediately after removal into suitable containers kept in an ice bath and were transferred within 1 hour to the deep freeze. At a convenient time thereafter, usually within about 10 days, the tissues were homogenized with distilled water in a Waring Blender, 1:40 for liver and 1:250 for muscle. No significant decrease in isomerase or dehydrogenase activity of the tissues occurred on storage in the deep freeze for this period. Further dilutions of the homogenates were made as necessary for the determination of enzyme activity. The blood was centrifuged to remove the plasma. The erythrocytes were washed 8 times with an equal volume of saline; the leukocyte layer was pipetted off after each centrifugation. A sample of erythrocytes was hemolyzed in 100-fold its volume of distilled water, and further dilution was made as necessary for the enzyme determinations.

A small sample of the liver of each animal was fixed with Zenker-formol for histopathological study. Representative pieces of all tissues (except from the central nervous system) were also taken for microscopic study from the 7- and 19-day animals given 6-methylpurine. The sections were stained with hematoxylin-eosin.

Determination of enzyme activities.—The method for determining the phosphohexose isomerase (PHI) activity has been previously described, and the units of PHI activity have been defined (1, 2, 3, 5). The determination of LAD activity has also been previously described (5, 9). This enzyme activity was expressed in units, as 103 times the change in optical density at 340 m/µ produced by 0.0167 cc. of plasma or 0.0167 gm. tissue per cc. reaction mixture per minute.2

RESULTS

Toxicity and pathologic changes.—In previous studies with male CFW rats 8 mg/kg of 6-methylpurine was found to be a median lethal dose that caused severe, progressive weight losses eventually in death as late as 3 weeks after injection (10, 11). Animals given twice the median lethal dose died between 4 and 7 days after injection. The CFN rat appeared to be similarly susceptible. In preliminary trials 16 mg/kg killed all animals in less than 7 days. Of the nineteen given 8 mg/kg, one died at 14 days and two at 17 days; the remainder exhibited steady losses in weight (Table 1).

Since most of the pathologic effects in the present series of rats were like those previously observed (10),4 only those parallel changes (or the absence thereof) will be described that seemed particularly pertinent to this study. No gross or microscopic abnormalities were seen in the

1 In a previous publication (5) the lactic acid dehydrogenase units were described erroneously as the changes in optical density produced by 0.006 cc. of plasma or 0.006 gm. tissue. However, correction of this error was made subsequently.

livers of the control rats. In the starved animals the hepatic cells were smaller and more sharply outlined than in the controls, and the basophilic granularity of their cytoplasm was diminished. Though less advanced, similar cytologic changes were evident in the liver cells of all methylpurine rats. Hepatic necrosis was seen in only two animals, and both of these were among the four killed at 19 days after 6-methylpurine. In one, isolated nests of two or three necrotic cells appeared with the frequency of about one in every other high-power field. This change was associated with distortion of the lobular architecture. Similar nests were found in the other rat but in even lower frequency. There were no histologic abnormalities in skeletal muscle and no evidence for in vivo hemolytic effects. Plasma samples were free of visible contamination by hemoglobin.

Several other pathologic alterations occurred which had not been seen previously in rats given 6-methylpurine (10). Half of the 7-day and all the 19-day animals had atrophic prostates with debris in the glandular lumina.

The hematopoietic tissues of the sternal bone marrow, which are usually densely crowded, showed reductions in quantity estimated to be between 10 and 30 per cent in the 7-day rats and between 35 and 80 per cent in the 19-day rats. Finally, in the 19-day animal with the highest incidence of necrotic hepatic cells the stomach showed extensive hemorrhages throughout the muscular coat, submucosa, and mucosa; the hematocrit of this animal was unusually low, 17 per cent (see footnote to Table 1).

Phosphohexose isomerase and lactic dehydrogenase activities.—In both preliminary studies with CFW rats, the activity of PHI in plasma rose above normal during the 1st week after injection. For example, in one of the two trials in which the treated rats were sacrificed in groups of three at 4, 6, and 7 days, the ranges of the plasma PHI values were 44–104, 132–2080, and 174–426 units, respectively. The range of values in six control animals, sacrificed in pairs on each of the same days, was 10–28 units, well within the normal range.

Table 1 shows the mean values for PHI and LAD of plasma, erythrocytes, liver, and muscle at 4, 7, and 19 days after injection of 6-methylpurine into the CFN rats, and the statistical significance of the differences between the mean values for the experimental and control animals. The ratios of the mean value for the 6-methylpurine animals to that of the control animals were calculated for these various parameters and are shown in Chart 1A. This ratio has a value of 1.0 at zero days and/or at any other time when the mean values for the control and experimental animals are the same.

From Table 1 and Chart 1A it is evident that the plasma PHI activity increased markedly by the 4th day and continued to increase until it was about 8 times the control value at 19 days. The plasma LAD activity also increased, but to a somewhat lesser degree—namely, to about 3 times the control value at 19 days.

Examination of the enzyme contents of tissues that might serve as sources indicated doubtful results with regard to erythrocytes and muscle.

Possibly beyond the 7th day after administration of 6-methylpurine, muscle was the source of some plasma PHI and LAD, and the erythrocytes were the source of some PHI but not of LAD. In contrast (Table 1 and Chart 1A), liver showed statistically significant and marked decreases in both LAD and PHI activities within 4 days after the administration of 6-methylpurine. The relative decrease in liver isomerase activity was greater than in dehydrogenase activity. These decreases would readily account for the elevations in plasma enzyme activities, and the liver could therefore be considered as the major, if not the complete, source of the increased enzyme activities in the plasma.

Body and liver weights in 6-methylpurine-injected
rats.—Table 1 shows that, whereas the control animals gained an average of 31 per cent in weight during the course of the 19 days of the experiment, animals given injections of 6-methylpurine showed a steady loss, and their average weight was 39 per cent less than at the beginning. The weights of the livers of the 6-methylpurine-injected animals also showed a marked decrease and were less than half the weights of the livers of the control animals by the 10th day. Nevertheless, it was only at 4 days that the decrease in liver weight was disproportionately greater than the loss of body weight—for at this time the ratio of liver to body weight was significantly less than in the controls (Table 1).

Enzyme activities in starved rats.—Because of the preceding findings, the possibility existed that the rises in plasma PHI and LAD activities in the 6-methylpurine-injected rats might simply reflect the breakdown of tissue as a result of failure to eat. Upon sacrifice, the stomachs of these animals were empty, in contrast to those of the controls, which usually contained much food. The average losses in body weight of the groups of starved rats were as follows: 19 per cent at 4 days, 25 per cent at 7 days, and 37 per cent at 11 days. The average loss in body weight at 11 days was therefore about the same as that of the 6-methylpurine-injected rats at 19 days (Table 1). The average liver weights per 100 gm. body weight in the starved animals at these times were, respectively, 2.36, 2.09, and 1.80, considerably lower than those of the 6-methylpurine-injected animals (Table 1).

The increases in plasma LAD and PHI activities in the starved animals were small and only occasionally statistically significant, as compared with the values in groups of control animals. No significant changes occurred in the erythrocyte or muscle enzyme activity of the starved animals during the course of the experiment. The liver PHI and LAD activities showed no changes at the end of 4 days of starvation. After the 7th day, the liver PHI activity decreased to about 85 per cent of the control value; the LAD dropped to 83 per cent of the control value at 7 days and to 72 per cent at 11 days. These results are summarized in Chart 1B and show that, on the whole, the enzyme changes in the plasma and tissues of the starving rats were insignificant in comparison with the much greater alterations found in the 6-methylpurine-injected rats.

**DISCUSSION**

Widespread and massive hepatic necrosis may, of course, be readily produced by carbon tetrachloride. Bruns and Neuhaus (6) have shown that this type of necrosis in mice is accompanied by sharp rises in serum aldolase and phosphohexose isomerase activities to a maximum at approximately 20 hours after the intraperitoneal administration of the carbon tetrachloride. The activities of these enzymes in the liver decrease to about 60 per cent of the original value at about 50 hours, then rise again to normal levels. We have observed similar rapid increases in plasma PHI to peak values in 48 hours in rats or dogs given carbon tetrachloride, and rapid recession to normal values by 4 days after administration of the carbon tetrachloride (11). Histologic study of the liver showed maximal necrosis at 2 days and almost complete repair by 4 days.

In comparison with the massive hepatic necrosis induced by carbon tetrachloride, the lesions visible in the livers of animals intoxicated by 6-methylpurine are insignificant. Although the prostate and hematopoietic tissue showed histologic changes, as described in the text, these did not resemble in nature or extent the hepatic necrosis caused by carbon tetrachloride. Rises in plasma enzyme activity that occurred in the 7-day rats were not related to the presence of prostatic atrophy. Moreover, chemical substances that produce effects on hematopoietic tissue, similar to or more pronounced than those produced by 6-methylpurine, do not lead to any rises in plasma isomerase activity and indeed may even cause decreases (11). The acutely developing and rapidly receding plasma enzyme rises produced by carbon tetrachloride also differ in pattern from the slowly developing but prolonged and substantial elevations in plasma LAD and PHI activities following the administration of 6-methylpurine. Nonetheless, the association of these enzyme elevations in the 6-methylpurine-injected rats with marked decreases of the enzyme activities in the liver indicates clearly that this tissue is the major source for the elevations in these blood enzymes. The absence of any except delayed, minor, and only occasional decreases of these enzyme activities in the skeletal muscle and erythrocytes supports this conclusion. It would therefore appear that alterations in the cell, other than those that lead to necrosis, may account for the release of enzyme proteins from the liver into the circulation after administration of 6-methylpurine.

In terms of the units as defined in this and preceding papers, the ratios of the isomerase: dehydrogenase activities in the plasma of the groups of control animals averaged 1.37. After the administration of 6-methylpurine, the ratio rose to 2.80 at 4 days, 2.92 at 7 days, and 3.89
at 19 days. This would indicate, in connection with the preceding considerations, that PHI was liberated relatively more rapidly than LDH from the liver or that it left the circulation relatively more slowly. A similar conclusion was drawn previously with respect to the liberation of these two enzymes from growing Walker carcinosarcoma 256 in rats (5).

SUMMARY

Intraperitoneal injections in rats of 8 mg 6-methylpurine/kg of body weight led to constantly increasing elevations in the plasma phosphohexose isomerase and lactic dehydrogenase activities. Nineteen days after the injection, the isomerase and dehydrogenase activities had risen to about 8-fold and 3-fold the control values, respectively. These plasma enzyme elevations were accompanied by substantial and significant decreases of the enzyme activities in the liver, but by minor and generally insignificant decreases in skeletal muscle and erythrocytes. The 6-methylpurine-injected rats showed losses in body and liver weight, but rats starved to points of corresponding or greater loss of weight exhibited only minor and generally insignificant increases in plasma enzyme and decreases in liver enzyme activities.

The absence of any consistent histologic abnormalities in the livers of the 6-methylpurine-injected rats indicated that alterations in the cell other than those leading to cell necrosis accounted for the release of enzyme proteins into the circulation.

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