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EXPERIMENTAL STUDIES ON THE DISTURBANCE OF HEMATOPOIETIC ORGANS DUE TO BENZENE INTOXICATION

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In order to ascertain the primary agency of benzene, firstly the peripheral blood pictures and the histological findings in the subacute benzene poisoning, in an experimental comparison with those pictures and findings in the toluene and xylene intoxication were examined.

The rats, treated with benzene, showed striking and characteristic changes either in case of peripheral blood pictures and of the histological findings. It proves that benzene, immediately giving degenerative or destructive changes, directly affected the hematopoietic organs, but the disorders of mitosis in blood cells must be a more significant effect of the benzene poisoning.

Next, in a further attempt to ascertain the primary agency of benzene on the blood-forming organs and to clarify the mechaism of benzene poisoning, the bone marrow insufficiency and hemolytic phenomena, were observed when compared with those in case of the toluene and xylene poisoning. As for the disorders of cell-division in benzene affected marrow cells, not only the mitotic inhibition at the stage of metaphase and the chromosomal damage but also the deceleration of the mitotic speed in marrow cells and the inhibition of the mitotic motivation at pro- and inter-phases are of possible significance.

For the more substantial clue to the benzene poisoning, further experimental researches were carried out in the hematological findings, especially, in the mitotic disturbances in the marrow of the benzene-affected rats to which were administered some chemical substances which are possibly preventive of the disturbances. The results suggest that the mitotic disturbances of the marrow cells by the action of benzene or its metabolites largely depends on the inhibition of the nucleoprotein metabolism or the nucleic acid synthesis, and that in the benzene poisoning the so-called radiomimetic actions are probably of high significance.

1. Changes in the Peripheral Blood Pictures and Histological Findings Caused by Benzene, in Comparison with the Changes by Toluene and Xylene

It has been deemed patent that after a long spell of exposure benzene affects the bone marrow, producing a great variety of blood pictures accompanied with salient features of aplastic anemia and leukaemia. On the other

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hand, toluene and xylene, the same aromatic hydrocarbons as benzene, are regarded to affect hematopoietic organs less actively[1)2)]. With so many reports on these phenomena and changes of peripheral blood pictures, however, few informations are available concerning the peripheral blood changes caused by benzene in reference to the affected bone marrow[3)], nor are on hand any significant researches in the mechanism of such disturbances of the hematopoietic organs caused by benzene[4)].

As an occupational poisoning, chronic poisoning is quite common. In order to definitely make out the primary agency of benzene upon a living body, the changes of blood-forming organs induced by the administration of large doses for a short spell of time must be taken into consideration.

The present paper is to discuss the changes in the peripheral blood pictures and histological findings under the subacute experimental benzene poisoning, in comparison with those changes caused by the toluene and xylene intoxication.

MATERIALS AND METHODS

Wistar strain male rats, fed on a standard diet of pellets named MF from the Oriental Yeast Co., were divided into four groups when about 3 months old. Twenty rats of the first experimental group were daily treated subcutaneously with 1 ml/kg body weight of benzene [1 ml/kg group or rats, hereinafter] over a period from 1 to 14 days. Five of them to which were administered benzene for 14 days, were examined in the body weight and the peripheral blood findings, that is, erythrocyte counts, hemoglobin content, leucocyte counts, hemogram and appearance rate of Mommsen's toxic granules, for seven times—just before the treatment and 1, 3, 5, 7, 10 and 14 days after that. The Mommsen's toxic granules were stained by the method of Mommsen[5)].

Five of the 20 rats were beat to death 1, 3, 7 and 14 days after the first injection. Of the sacrificed rats, those organs as liver, spleen, kidney, suprarenal gland, testicle and thymus were weighed and the pathologic findings of the principal organs were investigated on the specimens which had been appropriately prepared. To the rats of second, third and fourth experimental groups, toluene, xylene and olive oil were administered, respectively in the same dose level of benzene, and treated in the same way as mentioned above. In an additional experiment, 0.5 ml/kg body weight of benzene, toluene and xylene were given respectively to 10 of the rats, and they were killed 7 and 14 days after the injection [hereinafter, the 0.5 ml/kg benzene toluene or xylene group or rats]. These rats were examined by the same method as adopted in case of the above 1 ml/kg group.

RESULTS

The observations in the 1 ml/kg group, especially of the benzene group,

showed that the animals had apparently lost appetite, and that their growth had been gradually inhibited after the injection.

The inhibition of growth observed in this experiment seemed to be concurrent with the loss of appetite. The fluctuation in the values of the body weight in individual rats, however, did not necessarily go with the peripheral blood findings. In the 0.5 ml/kg groups such an inhibition of growth was also observed, especially definitely in the benzene-injected rats.

Peripheral blood findings—In all the groups but the 1 ml/kg group, the erythrocyte counts showed no remarkable change, and similar was the way with the hemoglobin content. As presented in Table 1, the leucocyte counts in the benzene group decreased rapidly after the injection, but neither in the toluene nor in the xylene groups.

Remarkable changes were observed in the hemogram, especially in the 1 ml/kg group. As is shown in Table 2, the myelocytes and metamyelocytes appeared to be few, the ring, stab and segmented cells increased, the lymphocytes decreased notably, and the ratio of neutrophiles to lymphocytes (N/L) increased in a remarkable degree. The 0.5 ml/kg benzene group also showed much the same phenomena in the hemogram, but in an inferior degree to those of the 1 ml/kg group. In the 1 ml/kg toluene and xylene groups, the segmented cells and the ratio of neutrophiles to lymphocytes increased a little, to the extent not comparable with those of the 0.5 ml/kg benzene group. As is shown in Fig. 1, the appearance rate of Mommsen's toxic granules in the

TABLE 1. Leucocyte Counts (/mm³)

Groups	Benzene		Toluene		Xylene		Control
	1 ml/kg	0.5 ml/kg	1 ml/kg	0.5 ml/kg	1 ml/kg	0.5 ml/kg	1 ml/kg
Pre-exp.	17160 ±2080 (100.0)	16820 ±2020 (100.0)	16860 ±1780 (100.0)	17350 ±2100 (100.0)	17380 ±2060 (100.0)	16840 ±1890 (100.0)	16460 ±1980 (100.0)
1st	16100 ±1890 (93.8)	17100 ±2420 (101.7)	16390 ±2310 (97.2)	17450 ±2040 (100.6)	18140 ±2360 (104.4)	17120 ±1910 (101.7)	16800 ±2010 (102.1)
3rd	12740 ±2410 (74.2)	13700 ±2440 (81.5)	17120 ±2010 (101.5)	18300 ±1940 (105.5)	18590 ±2860 (107.0)	17300 ±1960 (102.7)	16840 ±1880 (102.3)
5th	9720 ±2240 (56.6)	11520 ±2120 (68.5)	19140 ±1880 (113.5)	18860 ±1940 (108.7)	19060 ±2460 (109.7)	18360 ±2190 (109.0)	16840 ±2670 (102.3)
7th	6050 ±1080 (35.3)	9200 ±1940 (54.7)	19140 ±1960 (113.5)	18340 ±1890 (105.7)	18730 ±2140 (107.8)	18100 ±1940 (107.5)	16320 ±2010 (99.1)
10th	4670 ±860 (27.2)	10200 ±1980 (60.6)	18640 ±2560 (110.6)	18040 ±2640 (104.0)	18240 ±1940 (104.9)	18200 ±1860 (108.1)	16720 ±1890 (101.6)
14th	5600 ±960 (32.6)	10840 ±2100 (62.5)	18440 ±1620 (109.4)	18140 ±2140 (104.6)	18850 ±2420 (108.5)	17440 ±2310 (103.6)	16980 ±2040 (103.2)

( ); Percentage to pre-experimental value.

TABLE 2. Hemogram of 1 ml/kg Benzene Group (%)

		Control	Pre-exp.	1st	3rd	5th	7th	10th	10th
Eosinophile		0.6 (0-1.0)	1.0 (0-2.0)	0.8 (0-3.0)	0.6 (0-1.5)	1.2 (0-3.0)	0.6 (0-1.0)	0.3 (0-1.0)	0.2 (0-1.0)
Basophile		0 (0)	0.1 (0-0.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Myelocyte		0 (0)	0 (0)	0.1 (0-0.5)	0.1 (0-0.5)	0 (0)	1.2 (0-3.0)	2.6 (0.5-4.0)	2.9 (2.0-4.0)
Metamyelocyte		0.1 (0-0.5)	0.1 (0-0.5)	0.8 (0-2.5)	0.5 (0-3.0)	1.0 (0-2.0)	2.3 (1.0-4.0)	2.1 (2.0-2.5)	3.9 (2.0-7.0)
Ring		7.1 (5.0 -10.0)	7.9 (5.0 -10.0)	8.8 (3.0 -16.0)	7.9 (5.0 -12.0)	7.9 (4.0 -13.0)	8.2 (6.0 -12.0)	7.0 (5.0 -8.0)	17.0 (13.0 -22.0)
Stab		4.6 (2.5-8.5)	4.5 (3.0-7.0)	6.7 (3.0 -12.5)	7.9 (4.5 -11.5)	9.2 (8.0 -11.0)	10.6 (6.0 -19.0)	11.4 (10.0 -14.0)	10.2 (8.0 -12.0)
Segment	II	1.8 (1.0-2.5)	2.1 (1.0-4.0)	3.1 (1.0-6.0)	5.5 (1.0-8.5)	5.3 (3.0-8.0)	9.1 (3.0-20.0)	8.1 (7.0-9.0)	9.5 (6.0-14.5)
	III	0.3 (0-1.0)	0.4 (0-1.0)	1.1 (0-3.0)	1.6 (1.0-3.0)	2.1 (1.0-4.0)	4.0 (0.5-7.0)	4.5 (2.0-7.0)	4.2 (2.0-7.0)
	IV	0 (0)	0 (0)	0.2 (0-1.5)	0.6 (0-1.5)	0.6 (0-1.0)	1.8 (0-6.0)	1.7 (0.5-4.0)	0.7 (0-1.5)
	V	0 (0)	0 (0)	0.1 (0-0.5)	0.1 (0-0.5)	0.2 (0-1.0)	0.7 (0-4.0)	0.3 (0-1.0)	0.1 (0-0.5)
Lymphocyte		83.1 (79.0 -87.0)	81.9 (78.5 -86.0)	75.8 (65.0 -85.0)	73.8 (67.0 -81.5)	70.8 (62.0 -79.0)	58.9 (43.0 -69.5)	59.3 (54.0 -61.5)	48.2 (43.0 -68.0)
Monocyte		2.2 (1.0-3.0)	2.2 (1.0-3.5)	2.5 (1.0-4.0)	1.8 (1.0-3.0)	1.8 (1.7-3.0)	2.4 (1.0-4.5)	2.6 (1.5-3.5)	3.1 (2.0-5.0)
N/L		0.17	0.18	0.28	0.33	0.37	0.64	0.64	1.01

( ); Variations in each value.

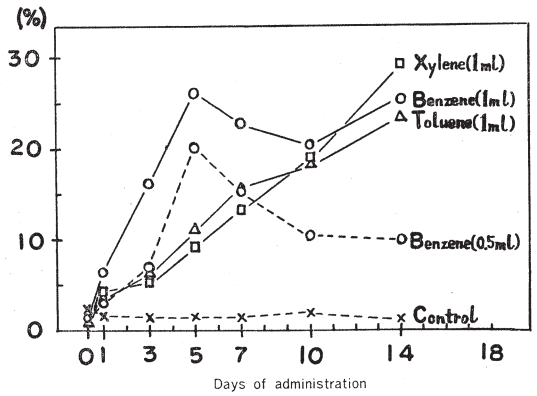

FIG. 1. Average values of appearance rate of Mommsen's toxic granules in 200 neutrophiles.

peripheral neutrophiles rose in either the benzene or the toluene and xylene groups after each administration. These phenomena were more remarkable in the 1 ml/kg group than in the 0.5 ml/kg groups.

In the benzene group, the rate was increasing on the 5th day and decreasing a little on the 7th day and there after. But in the toluene group, it showed a gradual increase after the injection. A similar tendency was also observed in the xylene group.

In general, the toxic granules in the cytoplasma are various in shape and prone to come forth finer in the toluene and xylene groups, while in the benzene group they are fine in the early stage and become larger as the poisoning advances.

Organ weight—The organ weight showed a definite change, especially in the benzene-treated animals. The mean values of the organ weight for the 1 ml/kg benzene group are as presented in Table 3. As is clear in the table, the weights of spleen and thymus had decreased rapidly after the injection, and in 14 days finally reduced to almost one half of the control animals. The testicle and kidney had somewhat reduced in 14 days. On the other hand, in the toluene and xylene administered rats, such changes were not observed except in their thymus and suprarenal gland. The weight of supraenal gland increased a little. In the xylene group, the weight of spleen increased in a certain degree. The significant results are made out in Fig. 2. These findings are also almost identical but less obvious in the 0.5ml/kg groups.

TABLE 3. Mean Values of Organ Weights Treated by 1 ml/kg of Benzene

(g)

Groups		Non-treated	1st day	3rd day	7th day	14th day
Body weight		207.5	186.6	196.3	178.2	201.5
Liver		8.7954 (42.40)	7.8433 (40.03)	8.3400 (42.49)	7.2894 (40.91)	7.9583 (39.50)
Spleen		0.5865 (2.83)	0.5942 (3.18)	0.5311 (2.71)	0.2932 (1.65)	0.3171 (1.57)
Kidney	l	0.9884 (4.76)	0.8753 (4.69)	0.8935 (4.55)	0.8149 (4.57)	0 8150 (4.05)
	r	0.9749 (4.70)	0.8702 (4.66)	0.8853 (4.51)	0.8126 (4.56)	0.8294 (4.12)
Suprarenal gland	l	0.0295 (0.14)	0.0274 (0.15)	0.0267 (0.14)	0.0248 (0.14)	0.0246 (0.12)
	r	0.0235 (0.11)	0.0220 (0.12)	0.0214 (0.11)	0.0198 (0.11)	0.0196 (0.10)
Testicle	l	1.1731 (5.65)	0.9808 (5.26)	1.0340 (5.27)	0.9638 (5.41)	0.9296 (4.61)
	r	1.1581 (5.58)	0.9878 (5.29)	1.0202 (5.20)	0.9058 (5.08)	0.9258 (4.59)
Thymus		0.1832 (0.88)	0.1614 (0.86)	0.1468 (0.75)	0.1146 (0.64)	0.0922 (0.46)

( ); Ratio to body weight ($\times 10^{-3}$).

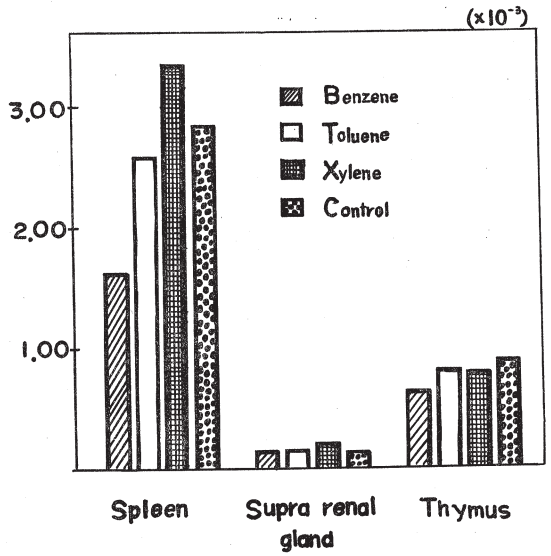

FIG. 2. Ratio of organ weight to body weight treated by dose level of 1 ml/kg on 14th day.

Microscopic findings—Remarkable changes were observed, especially in the 1 ml/kg benzene rats. The veins and capillaries in the liver were distended slightly with blood, but hemosiderosis was almost negative. After 7 days of the injection, a slight vacuolated degeneration was observed in the liver cells of peripheral lobulus, but the structure of liver was not destroyed. The apleen showed such marked changes as hemosiderosis, atrophy of lymph follicles and congestion of sinus lienalis, etc. The hemosiderosis was more obvious in the 3rd day animals than in the other ones. The atrophy of lymph follicles and the diminution of nucleated cells in the spleen had become more striking after the 7th day, and a partial hyaline droplet degeneration was observed either in the wall of small blood vessels or in the reticuloendothelial cells. The cortex of suprarenal gland was slightly hypertrophic and congestive on the 1st and the 3rd days, lots of lipoid pigments were present in the cortex cells, but the cortex proned to be sort of atrophic after the 7th day. In the suprarenal gland, however, scarce hemosiderosis was observed. The testicle was somewhat atrophic on the 14th day, and in the mitotic phase of spermatocytes, pro- and meta- phases seemed to be comparatively numerous.

The lymph nodes in the neck and abdominal cavity tended to be atrophic, and hemosiderosis was partially observed 7 days after the administration. The thymus was atrophic and the diminution of nucleated cells was also observable. Neither kidney nor thyroid gland showed any serious findings.

In general, the pathologic findings were much less striking in the 0.5 ml/kg

rats than in the 1 ml/kg ones. After the 7th day, however, the spleen of the 0.5 ml/kg group had atrophied in almost similar degree to that of the 1 ml/kg ones.

In the toluene and xylene groups, however, scarce pathological findings were obtained. A slight congestion in the liver, spleen and kidney, and a little hemosiderosis in the spleen and hypertrophy in the cortex of suprarenal gland, etc., were partially observed, but no serious atrophy was significantly observed in neither group.

The results from the observations on the bone marrow will be described later.

DISCUSSION

It is generally accepted that the diminution of peripheral blood cell counts should probably be due to noxious agents resulting from the disorder of blood formation, from the transfer disorder of blood cells from hematopoietic organs to blood stream, or from the degeneration or destruction of blood cells. "The reaults of innumerable experiments on animals, mostly by subcutaneous injection of benzol", as Browning[6] reported, "have confirmed Selling's original observation[7] that the striking and characteristic effect of benzol poisoning is a reduction of leucocytes". Under the present experimental condition, the injection of benzene gave rise to a marked diminution in leucocytes number, but no prominent fall was obtained in peripheral red-cell counts and hemoglobin content except in case of the 1 ml/kg benzene group. As can be read in some preceding literatures and will be pointed out later in this paper, the erythrocytes affected with a large quantity of benzene tend to cause stromatolysis[8] and hemolysis[9][10][11]. But taking the facts into consideration that the life span of erythrocytes is short and the decrease in erythrocyte counts is pretty slight, the stromatolysis and hemolysis in this experiment might be never so significant as regards the diminution of peripheral erythrocyte counts. As for the leucocyte counts, the administration of benzene caused a rapid fall in their number, but neither toluene nor xylene did. In the 1 ml/kg benzene rats, the number of leucocytes attained minimum on the 10th day, while in the 0.5 ml/kg rats on the 7th day. The minimum value of leucocytes was about 1/3 to 1/4 of the normal value. But as will be given later, the minimum value of the total nucleated cell counts of the bone marrow in the present case was over 1/3. This difference in number between peripheral leucocyte counts and the nucleated cell counts of bone marrow may have a possible relation to the destruction or the disorders of emigration of blood cells by benzene or its metabolites upon mature leucocytes. As one of the results from the injection of benzene, marked changes in the hemogram were observed, especially obvious in the 1 ml/kg group. The fall of lymphocytes number,

accordingly the increase of ratio of neutrophiles to lymphocytes (N/L) was notable in the benzene-treated animals. Different views and suggestions in literature read on the problem whether a relative lymphocytosis or lymphopenia would be the characteristic feature of benzene intoxication. Batchelor[12] and Engelhardt[13] take with the relative lymphocytosis, while Neumann[14] and Koike *et al.*[3] stand on the relative lymphopenia. Perhaps, the discrepancy has some relation to the difference in experimental conditions, possibly to the species of used animals and the method of drug administration. It is not only of keen interest but of significance, therefore, to make inquiries into the divergency of opinions, and further studies on this problem are highly recommended.

The relative lymphopenia was observed slightly in case of toluene and xylene-treated rats, but an obvious difference from the case of the benzene-treated ones was observed.

The polysegmented leucocyte counts and the appearance rate of Mommsen's toxic granules in the peripheral neutrophiles increased considerably. Generally, it is admitted that the former is a degenerative change of neutrophiles and the latter the disturbance of cytoplasmic maturation of leucocytes. The Mommsen's toxic granules occurred in the benzene-treated group were fine in the early stage and became gross as the poisoning aggravated, and as already pointed out by Yamada[15] who believed that the occurrence of fine granules was an reactive change and had not so high pathologic significance but that those gross granules meaningfully suggested the presence of pathologic regeneration of the bone marrow. It is also of keen interest that the appearance rate of the toxic granules increased also in those toluene and xylene-treated animals, but that the granules in this case were generally so fine in shape that they might be of "reactive change", either.

According to Engelhardt[13], some toxic granules of the leucocytes were observed in case of toluene and xylene poisoning. The occurrence of the toxic granules in the peripheral leucocytes, it is believed, should be accelerated by some chemical substances[16,17] or some infectious diseases[18,19]. It can never be lightly concluded, therefore, whether the increase in the appearance rate of Mommsen's toxic granules in this experiment should be caused by the direct affection of each aromatic hydrocarbon or by the secondary infection resulted from the administration of such substances. Further and intimate studies are required here for the fruitful explanation of this problem.

The characteristic findings of the benzene injured spleen proved the atrophy of lymph follicles and the diminution of nucleated cells and hemosiderosis. These findings are also available in some other papers[20,21]. The hemosiderosis in the spleen is suggesting the destruction of erythrocytes, and the hemolytic effect are aggravated on by benzene affection in the reticuloendothelial system. This problem will be discussed later.

Now, as already pointed out in the paper of Koike *et al.*[3] and others[22)23)], lymph glands showed a rapid degeneration and atrophic changes, and the atrophy of the thymus had also occurred[24]. The results are of keen interest compared with the changes of hemogram, that is, the diminution of lymphocytes in number. Lymphoid cells seem to be more sensitive to benzene than the other cells. The rapid fall in the number of peripheral white-cells accompanied with only a slight degeneration in the other organs than hematopoietic organs can never be explained simply by the direct destructive action of cellular noxious agent to the matured cells.

The spermiogenesis in benzene-injected animals was slightly inhibited, which are affirmatively reported in some papers[25)26)]. The life span of matured leucocytes is very short, and according to Ozogoe[27] and others[28], the turnover time of cell population in the circulating blood seems to be quite brief, especially in lymphocytes and neutrophiles. Those findings suggest that the affection of benzene appears more strikingly in the organs and tissues in which cell-division is very active. The minute consideration over these points will naturally lead to the following conclusions that though benzene produces directly degenerative and destructive effects on the mature hematopoietic cells, the disorder of cell-division is a more important matter on the experimental benzene poisoning. In case of toluene and xylene intoxication, on the other hand, the inhibition of cell-division seems to be much slighter than in case of benzene poisoning.

CONCLUSION

1) Benzene does injury more or less actively to almost all organs, tissues and cells which were examined in the present experiment. But the primary and definite agency by benzene seems to be the disturbances of cell-division, which possibly causes a more desperate injury to the organs and tissues where the cell-division is extremely active.

2) Under the present experimental condition, however, toluene and xylene did not do injuries so seriously as benzene.

2. Bone Marrow Insufficiency and Hemolytic Phenomena in Experimental Benzene Poisoning

As previsionally discussed above, it is noteworthy that benzene has degenerative or destructive effects upon mature hematopoietic cells, but the disorders of cell division, especially in blood forming organs, may be a more significant affection by the benzene poisoning. For the information some reports and papers on this problem are available. An inhibition of mitosis at the stage of metaphase[29)30)31)] and chromosomal damages[3)31)32)] in the benzene-affected marrow cells were observed morphologically. Furthermore, the disturbances of the synthesis of nucleic acids[3)33)34)35)] and of the enzymatic pathway in oxidation and reduction system[36)] were also pointed out. But as regards the disorders of mitosis in the benzene-affected marrow cells, it is not yet convincingly proved what is most important in the explanation of the mechanism of benzene poisoning. In this chapter, therefore, in order to clear up the primary agency of benzene on bone marrow, especially on mitotic marrow cells, and to obtain a clue to the mechanism of benzene poisoning, bone marrow insufficiency and hemolytic phenomena in experimental benzene poisoning were investigated by various ways.

MATERIALS AND METHODS

The bone marrow specimens were readily made from the same animals sacrificed in the preceding experiment. With the benzene-treated rats, the marrow smears were obtained from the rats beaten to death just before the administration, and on the 1st, 3rd, 7th and 14th days after the administration [hereinafter, as 1st, 3rd ··· or 14th day rat, etc.], while with the toluene and xylene groups, from those beaten to death on the 7th and 14th days. The bone marrow used in the present experiment were femoral ones. Right marrows had been smeared and stained appropriately with Giemsa stain, and myelogram and a number of mitotic figures in 2 000 myeloid cells were examined on the stained films. As for the myelogram, a minimum of 500 bone marrow cells were counted according to Stasney's[37)] and Komiya's[38)] classification. On the other hand, nucleated cell counts were examined in other marrows, and pieces of the marrows were fixed with Carney's solution and the sliced specimens were observed microscopically.

After these experiments, in order to compute the proliferation speed of myeloid cells in each group, colchicine-treated marrow specimens* were

* Colchicine was administered subcutaneously to each animal in the dose level of 0.10 mg/100 g body weight[39)], and the specimens were prepared exactly at six hour intervals after the colchicine injection. From the stained films, mitotic rate in 2 000 myeloid cells was examined. In this way, the proliferation speed of myeloid cells in each group is to be checked.

prepared from each 7th day animal, and as for the benzene group, the 14th day specimens were also examined. In this experiment, Wistar strain female rats about three months of age were used. Moreover, crystallization of blood and hemolytic phenomena were tested with other Wistar strain female rats. In these experiments, the adopted blood had been drawn directly from the femoral artery. The crystallization of blood was observed microscopically by adding benzene, toluene and xylene *in vitro* to the whole blood.

To determine the resistance of erythrocytes which had been obtained from benzene-treated animals, the osmotic fragility of the red blood cells affected by benzene for 14 days was examined by Ribière's method[40]. Eighteen female rats were used in the experiment.

RESULTS

Bone marrow insufficiency—Table 1 presents the summary of the principal results of myelogram from the 1 ml/kg benzene, toluene and xylene-administered rats. In the stained films of benzene-treated animals, remarkable changes were notable. Diminutions were recognized in erythroid cells, immature myeloid cells and lymphocytes. On the contrary, there was a marked increase in polysegmented cells, mitotic cells, reticuloendothelial cells and in megacary-

TABLE 1. Principal Results of Myelogram in 1 ml/kg Administered Rats

		Control	Benzene				Toluene		Xylene	
			1st	3rd	7th	14th	7th	14th	7th	14th
Proerythroblasts		1.5	1.2	0.8	1.1	1.0	1.3	1.6	0.9	1.2
Basophilic E.		8.5	9.6	11.2	12.5	12.2	7.9	8.5	6.9	7.3
Polychromatic E.		24.2	21.4	15.0	10.8	9.5	22.4	20.9	19.8	20.1
Orthochromic E.		0.3	0.5	0.8	0.8	1.0	0.3	0.4	0.2	0.3
Myeloblasts		1.9	1.2	1.6	0.7	0.4	2.0	2.1	2.3	2.5
Neutrocytes	Promyelocyte	1.1	1.5	2.2	1.6	0.8	1.2	1.5	1.5	1.7
	Myelocyte	4.8	5.2	4.2	4.4	4.2	4.2	4.8	4.4	3.6
	Metamyelocyte	4.8	5.5	6.9	6.8	4.2	4.5	4.7	5.1	5.7
	Ring and Stab	19.5	21.9	21.6	17.4	18.7	20.3	19.5	18.7	17.9
	Segment *A	10.3	12.0	15.2	17.4	15.3	8.9	7.5	9.8	10.3
	Segment *B	0.6	0.6	1.1	5.0	5.4	1.7	2.6	2.4	3.8
Eosinocytes		3.0	2.3	1.5	1.4	2.4	2.5	2.1	3.1	3.4
Basocytes		0.2	0.2	0.4	0.1	0.2	0.2	0.3	0.4	0.3
Monocytes		4.7	3.3	5.8	3.8	4.1	5.1	5.8	4.8	4.1
Lymphocytes		12.0	7.6	4.1	5.4	4.1	12.5	11.2	13.1	11.4
Plasma cells		0.3	0.4	0.5	0.3	0.2	0.3	0.2	0.6	0.4
Megacaryocytes		0.1	0.2	0.2	0.5	0.5	0.1	0.3	0.2	0.4
Reticuloendothelium		0.3	0.2	1.5	2.5	5.8	0.3	0.8	0.5	0.7
Mitotic cells		0.6	2.0	2.1	3.8	6.5	0.9	1.2	1.2	1.7
Undifferent cells		1.5	3.2	3.3	3.7	3.5	3.4	4.0	4.1	3.2

A minimum of 500 bone marrow cells were classified in each specimens.

*A; below four nuclear segmented cells.
B; four and more ones.

ocytes identified after the benzene administration. In erythroid series, the diminution was notable in polychromatic erythroblasts. The basophilic erythroblasts appeared more numerous than polychromatic erythroblasts. The diminution in the number of the total erythroid cells seemed nearly parallel to the blood destruction in the spleen. Proerythroblasts decreased a little.

The highest value of mitotic indices was observed on the 14th day after the experiment, which was about ten times as high as the value of the control animals. There was a considerable variation both in size and in shape of erythroid and myeloid cells. There were also observed a few vacuoles in the cytoplasma of myeloblasts and promyelocytes, and large azurophilic granules in metamyelocytes. Those findings mentioned above with the 0.5 ml/kg benzene group were almost similar but not so serious as those of the 1 ml/kg group. So far as the toluene and xylene groups were concerned, though a few findings such as the increase in the number of polysegmented cells and of mitotic cells

TABLE 2. Frequencies of Mitotic Figures in the Granulocytic Series at 7th day Injection
Each Values are Ratio to 2 000 Granulocytic Cells

(Mean $\pm\sigma$)

			Prophase	Metaphase	Anaphase	Telophase	Total
Benzene	0.5 ml/kg	7th	38.6±10.3 (56.4)	10.1±4.9 (14.7)	6.4±3.2 (9.3)	13.4±6.8 (19.6)	68.5 (100.0)
		14th	37.4±12.4 (55.7)	9.6±5.2 (14.3)	7.2±4.1 (10.7)	12.9±7.3 (19.2)	67.1 (100 0)
	1 ml/kg	7th	36.6±11.8 (50.9)	15.5±4.7 (21.5)	8.1±4.3 (11.3)	11.7±6.2 (16.2)	71.9 (100.0)
		14th	37.8±11.3 (51.0)	16.1±7.8 (21.7)	7.9±3.9 (10.7)	12.3±8.3 (16.6)	74.2 (100.0)
Toluene	0.5 ml/kg	7th	42.8±10.5 (72.2)	2.0±1.0 (3.4)	2.1±0.7 (3.5)	12.3±6.8 (20.8)	59.2 (100.0)
		14th	43.4±12.7 (73.8)	1.8±0.9 (3.1)	1.9±0.8 (3.2)	11.7±5.6 (19.9)	58.8 (100.0)
	1 ml/kg	7th	43.7±9.1 (71.3)	3.1±0.9 (5.1)	2.7±0.7 (4.4)	11.8±4.7 (19.2)	61.3 (100.0)
		14th	41.6±12.4 (71.8)	2.6±0.8 (4.8)	2.4±1.1 (4.1)	11.5±3.8 (19.8)	58.4 (100.0)
Xylene	0.5 ml/kg	7th	42.8±13.1 (69.8)	2.6±1.3 (4.2)	3.6±1.2 (5.9)	12.3±6.2 (20.1)	61.3 (100.0)
		14th	41.6±12.5 (69.0)	2.7±1.2 (4.5)	3.4±1.5 (5.6)	12.6±5.7 (20.6)	60.3 (100.0)
	1 ml/kg	7th	46.0±10.3 (67.6)	6.3±2.9 (9.2)	5.6±2.3 (8.2)	10.2±6.2 (15.0)	68.1 (100.0)
		14th	44.2±13.6 (66.3)	5.8±3.4 (8.7)	4.9±2.4 (7.3)	11.8±7.2 (17.7)	66.7 (100.0)
Control			39.8±9.2 (71.4)	1.0±0.2 (2.6)	2.8±0.8 (4.3)	11.3±5.4 (21.8)	54.9 (100.0)
Colchicine*			40.2±8.4 (25.7)	100.8±29.6 (64.4)	4.8±0.8 (3.1)	10.7±3.7 (6.8)	156.5 (100.0)

* 0.10 mg/100 g body weight of colchicine group; after six hours incubation (This value is ratio to 1 000 granulocytic cells.)

( ); Percentage to total mitotic cells in myeloid cells.

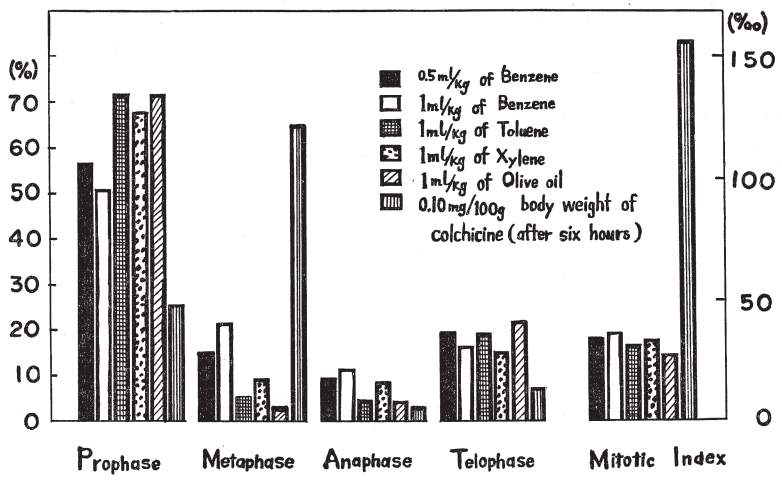

FIG. 1. Frequencies of mitotic figures in the myeloid cells at 7th day administration.

and anisocytosis, were obtained, most of the erythroid and myeloid cells seemed to be left almost intact.

Principal data of the frequencies of the mitotic figures in myeloid cells are presented in Table 2 and Fig. 1. With the benzene-administered rats, remarkable changes were observed in the mitotic figures of meta- and ana-phases. The frequencies of the meta- and ana-phases in the mitotic figures of myeloid series were relatively higher in the benzene group than in the toluene and xylene ones. The appearance rate of meta- and ana-phases in the myeloid cells of the xylene-treated animals was higher than that of the toluene-treated ones. These findings with the benzene-administered specimens showed moderate resemblance to those with the colchicine-treated ones. Moreover, a large number of these mitotic figures showed apparent abnormality, especially in the appearance of their chromosomes. The patterns of the chromosomes injured in the metaphase and in the anaphase were almost irregular and indistinct in their shapes, and also their arrangement appeared to be disorganized. Various pictures of the damaged chromosomal structures were such as bridge formation, partial pycnosis and adhesion, displacement, destruction, tri- and poly-polarization and others. Fig. 2 presents those various pictures of the affected chromosomes. There were pictures of the vacuoled cytoplasma frequently observed in those abnormal mitotic cells. With the 1 ml/kg group, the abnormal mitosis showed about 80% in the metaphase and 65% in the anaphase group on the 14th day, and it showed a tendency of gradual increase in percentage as the poisoning aggravated. Those findings such as mentioned above were not so remarkable in the 0.5 ml/kg benzene-treated bone marrow as in those of the 1 ml/kg group. With the specimens treated with

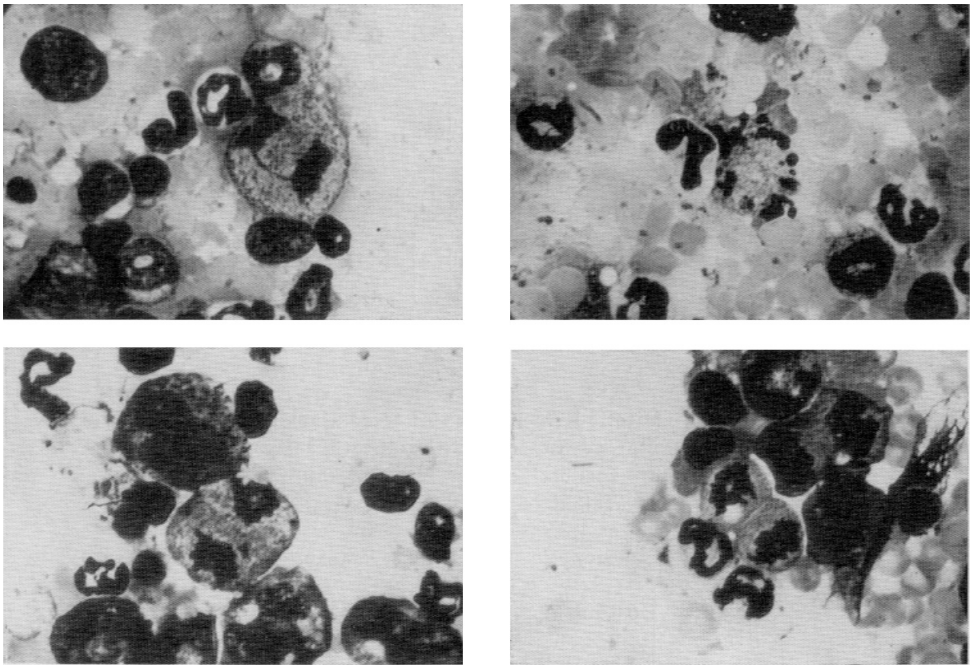

FIG. 2. Various pictures of benzene-affected marrow cells.
(Giemsa stain, oil immersion)

toluene and xylene, mitotic figures were relatively marked in number, but scarce chromosomal abnormality was observable. On the other hand, the mitotic indices on those stained films, that is, the ratio of the mitotic cells to the total granulocytic series in the benzene, toluene and xylene injected specimens, was almost similar to that of the olive oil administered ones.

As is presented in Table 3, the examination of nucleated cell couuts in the benzene-treated marrow showed a marked diminution of them in number. In the 1 ml/kg benzene-treated animals, the nucleated cell counts dropped down below one half of the mean value of the control group on the 7th day, and they showed a more marked decrease on the 14th day. But no significant difierence was observed between the mean value on the 7th day and that on the 14th day. In the 0.5 ml/kg benzene group, the nucleated cell counts decreased to less than 2/3 on the 7th day, but on the 14th day a tendency of gradual recovery was observed. On the contrary, the nucleated cell counts in the toluene and xylene groups showed no remarkable changes. Almost the same tendency was seen in the histological sections. In the 1 ml/kg benzene-treated marrow, hypoplasia, gelatination and slight fatty degeneration were observed. With the 0.5 ml/kg benzene-injected group, a slight regeneration of the affected marrow was observed in the 14th day rats. The toluene-treated

TABLE 3. Nucleated Cell Counts in Each Bone Marrow
(Mean±σ/mm^3)

Groups	Doses	7th day	14th day
Benzene	0.5 ml/kg	91.4±27.3	96.2±20.3($\times 10^3$)
	1 ml/kg	62.1±25.3	59.2±16.5
Toluene	0.5 ml/kg	153.8±18.4	155.4±19.1
	1 ml/kg	149.8±26.5	151.8±19.8
Xylene	0.5 ml/kg	155.5±25.9	155.8±18.4
	1 ml/kg	158.2±26.8	150.5±24.9
Control	1 ml/kg	154.8±21.7	153.3±24.4

TABLE 4. Mitotic Rate in Each Affected Myeloid Series of Bone Marrow Determined by Mitotic Index at Six Hours Incubation after Injecting 0.10 mg/100 g Body Weight of Colchicine Subcutaneously

Groups			(Mean±σ/10^3)
Benzene	0.5 ml/kg	7th day	110.2±20.4
		14th day	115.4±19.3
	1 ml/kg	7th day	86.5±20.4
		14th day	80.6±21.6
Toluene	1 ml/kg	7th day	132.4±19.8
Xylene	1 ml/kg	7th day	128.8±18.6
Control	1 ml/kg	7th day	128.4±17.8

marrow, on the other hand, showed no remarkable degeneration but rather a slight proliferation, especially in the 1 ml/kg group. With the xylene-injected animals, also a slight degeneration occurred on the 14th day in the 1 ml/kg group.

As is presented in Table 4, the mitotic rate of granulocytic series in each group was estimated by determining the mitotic index at six hour intervals after the injection of 0.10 mg of colchicine per 100 g body weight. In the very method, the proliferation speed in each affected marrow may be assessed approximately. In the benzene-treated rats, the mitotic rate was lower than that of the control specimens, especially in the 1 ml/kg group, and it seemed to decrease gradually as the poisoning advanced. In the 0.5 ml/kg benzene group, the proliferation speed in the benzene-treated marrow seemed to be considerably hindered, but not significant. On the other hand, the mitotic rate of the toluene and xylene-treated rats showed approximately the same value as that of the normal ones.

Hemolytic phenomena and stromatolysis—Approximately 0.04 ml of benzene was added, ***in vitro***, to 2.0 ml of oxalated whole blood, and the blood changed into a jelly mass containing numerous red crystals. Thin specimens were prepared from the blood-benzene mixture which had been kept in test tubes at the room temperature and at 37°C for various needed periods of time. After the fifteen minute incubation at 37°C, many yellowish-orange or red crystals, about three to five times as long and with a diameter one time as wide as the red blood cells, were seen in the film, but many erythrocytes were left intact. After thirty minutes, the crystals in the same shape increased remarkably, and there were minimal or no red blood cells. In 60 minutes of incubation, the red blood cells had dissolved completely into many large crystals, in size about fifteen times as long and two to three times as broad as that of erythrocytes. These materials taken out into the room temperature in the procedure of incubation, the crystals seemed to become larger in size. And as shown this experiment, the crystals of erythrocytes mixed with benzene at room temperature seemed to be larger at 37°C. When approximately 0.01 ml of benzene was added to the 2.0 ml of oxalated whole blood, almost the same phenomena were observed either in their shape and in its speed. The crystals of the blood toluene mixture were somewhat smaller in size and less in number than those of benzene at the same intervals during the incubation. For example, by adding approximately 0.04 ml of toluene to the oxalated whole blood at 37°C, the erythrocytes changed into crystals, less than four to ten times as long and one to two times as broad in 60 minutes incubation, and many intact erythrocytes were observed. In case of xylene, the crystallization under the same conditions revealed itself far slighter than in case of toluene, and were observed a few crystals about twice and three times as long and one half as broad as erythrocytes. Nearly the same findings as the above mentioned were obtained in case of the room temperature, and the crystallization was slighter in case of the 0.01 ml of toluene and xylene groups than in the 0.04 ml ones. The blood added heparin as anticoagulant also crystallized in nearly the same way. Still more, in case of the pure erythrocytes prepared by rinsing away the whole blood completely with 0.85% NaCl solution, also occurred the same phenomenon, only the crystal formation somewhat faster than in case of whole blood and the crystals various in shape.

The osmotic fragility of the benzene-affected erythrocytes was examined by Ribière's method after one hour incubation. In the non-treated rats, the values of maximum and minimum resistance of red blood cells were 0.34 ± 0.02 and 0.44 ± 0.02 per cent, respectively. But in case of the 1 ml/kg benzene-administered rats, the values of maximum and minimum resistance on the 7th and 14th days were 0.40 ± 0.02, 0.52 ± 0.02 and 0.36 ± 0.02, 0.48 ± 0.01 per cent, respectively, with statistically significant differences between them. As is evident in these data, the osmotic fragility in the erythrocytes showed an

obvious increase in the benzene-treated rats.

DISCUSSION

There are only a few informations available about the myelogram of benzene-affected bone marrow. Perhaps, it may be due to the difficulty in the classification of bone marrow cells, because the nomenclature of the cells and diseases of blood and blood-forming organs has not yet been standardized internationally[41]. In this experiment, the ways of classification by Stasney and Komiya and others[42,43,44,45] were adopted for reference.

Literatures report that of the bone marrow cells the youngest ones are most seriously injured by benzene exposure[46] and that finally the bone marrow becomes aplastic[47]. In this experiment, a remarkable decrease in erythroid cells, especially in polychromatic erythroblasts, was observed, and myeloblasts showed a tendency of a slight decrease, but there was no remarkable sign that the youngest cells had been most severely injured. There were a notable increase in the number of polysegmented neutrophiles and vacuoled cells, and those findings were regarded to prove degenerative changes. The mitotic cells in the 1 ml/kg benzene-affected specimens on the 14th day reached about ten times the value of the control marrow, which suggested a mitotic disturbance of the bone marrow cells. In order to compare the findings from the marrow cells with those from the peripheral leucocytes, which had shown a remarkable decrease in number after the benzene administration, the changes in myeloid cells were taken into deliberate consideration.

The increase in the frequency of the mitotic figures on smear films has some relation firstly with that of the cell-division in a given time, and secondly with the delay in the time required for mitosis. Accordingly, they were considered to be the sum findings of the contrary phenomena in the mitosis.

With the benzene-administered rats, notable changes were seen in the mitotic figures of meta- and ana-phases, and the frequencies of the meta- and ana-phases in the mitotic figures of myeloid cells were relatively much higher than those of the toluene and xylene groups. Moreover, a large number of those mitotic figures showed apparent abnormality, especially in the appearance of thier chromosomes, and the mitotic rate determined by the colchicine method in the benzene-affected marrow decreased markedly. An obvious increase in the mitotic rats may partly be explained as an expression of the succeeding regeneration, while the frequent occurrence of abnormal mitotic figures, especially in chromosomes was the fact highly suggestive of the injurious effects of benzene and its metabolites upon the mitosis. And the rapid decrease in number of peripheral leucocytes accompanied with a slight cellular degeneration might be regarded as the result of mitotic disturbance rather than as that of direct cellular destruction. The mitotic disturbance due to the action

of benzene and its metabolites, therefore, might be considered to be a most important factor to cause the primary reduction in the number of marrow cells under the experimental benzene poisoning.

As for the mechanism of mitotic inhibition induced by certain chemical substances, as indicated by Dustin[48], "the poisons of the trypaflavine or radiomimetic group disturb nucleoprotein metabolism and the coating of the chromosomes with thymonucleic acid at prophase, and the poisons of the second type, acting principally on the spindle, may perhaps combine with -SH proteins that have been histochemically demostrated in the spindle and also in the nuclear sap". A few papers mention that benzene affects the cell-division at the stage of the metaphase[29) 30) 31)], so that it is called "mitotic poison of colchicine type". As "changes in distribution of protein-bound SH groups which are oxidized during spindle formation forming disulfide (S-S) bridge between adjacent protein molecules are significant in the cell division at the stage of the metaphase and anaphase"[49], benzene and its metabolites might affect this portion. Makino *et al.*[29] observed that "in benzene and phenol treated material the chromosomes could move to the poles, while the mitochondria were inhibited from elongating, and the cleavage furrow was not formed at all". Moreover, Sakai *et al.*[50] observed electron-microscopically the marked vacuoled degeneration in the benzene-affected marrow cells, especially in the mitochondria of promyelocytes, so that it may be asserted that the affection of benzene and its metabolites to the mitochondria might be also of significance.

As to the action of the metabolites of benzene to the mitosis, quinol and catechol are regarded as of trypaflavine type of mitotic poisons, which attack cells immediately before the division to cause nuclear destruction by pycnosis[48]. Furthermore, "hydroquinone seemed to precede the radiomimetic (pycnotic) effect and there was a progressive accumulation of arrested metaphase"[51]. A higher ability to oxidize benzene[52] and a greater activity of the sulphating enzyme system in liver[53], therefore, may play an important role in susceptibility to benzene, and "the failure of toluene and xylene to carry out this attack on bone marrow is due to the fact that their metabolites are not the toxic phenols"[54]. But, as benzene independently affects the mitosis[29], further studies are required for the more profound knowledge abont the role of benzene and its metabolites played in the benzene poisoning.

Benzene destroyed partly marrow cells, especially savagely at the stage of metaphase and anaphase. While, the mitotic rate in the benzene-administered marrow cells which was determined by the colchicine method decreased considerably, so that it means that the findings indicated the inhibition of mitotic motivation in the inter-, pro- and meta-phases or at least the deceleration in the speed of cell division at those stages. These findings will reasonably lead to the conclusion that benzene and its metabolites should have a toxic action on mitosis, not only colchicine-like but also radiomimetic. Which of them is

more significant and primary in the benzene poisoning, however, is a question yet to be determined.

As already pointed out by Gerarde[8], by adding benzene to the oxalated whole blood *in vitro*, the blood changed into a jelly mass containing numerous red crystals, which was also confirmed in the present experiment. Furthermore, almost the same phenomena were also observed in case of the heparin-added blood and in pure erythrocytes. It is so highly significant and interest to clarify the mechanism of this crystallization that further studies of this problem are hopefully required. The osmotic fragility of benzene-affected erythrocytes increased apparently. The notable hemosiderosis in the spleen and the reticuloendothelial system, therefore, might be regarded to have an implication with stromatolysis and with the increase in osmotic fragility of benzene-affected erythrocytes.

CONCLUSION

1) As for the disorders of the mitosis in the benzene-affected marrow cells, the inhibition of cell division in the blood forming cells, especially at the stages of metaphase and anaphase, and the chromosomal damage were regarded as significant as the delay in the mitotic speed of marrow cells and the inhibition of mitotic motivation at the stages of inter-, pro- and meta-phases.

2) The reasonable conclusion, therefore, is that benzene has toxic actions on mitosis, not only colchicine-like but also radiomimetic.

3) Benzene, added *in vitro*, converted erythrocytes into a jelly mass containing numerous crystals, and the increase in osmotic fragility of the affected erythrocytes was also observed. Those findings might be regarded to have an implication with notable hemosiderosis in the reticuloendothelial system treated with benzene.

4) In case of the toluene and xylene intoxication, any disorders of mitosis were not observed, nor were so striking the hemolytic phenomena as in case of the benzene intoxication.

3. Effects of Some Chemical Substances on Hematopoietic Organs in Experimental Benzene Poisoning

In the preceding chapters, it was discussed that benzene had directly degenerative or destructive effects upon hematopoietic organs, and that, as for the disorders of cell-division in the benzene-affected marrow cells, not only the mitotic inhibition in metaphase and the chromosomal damage but the deceleration of the mitotic speed in mitotic cells or the inhibition of the mitotic motivation at pro- and inter-phases were of great significance in the explanation of the mechanism under subacute benzene poisoning. Looking further for a substantial clue to the mechanism of benzene poisoning, our interests were directed toward the hematological findings, especially to cell-division of the marrow cells, which proved influences under the administration of benzene together with some chemical substances. There may be many drugs effective in improving the findings from the benzene administration, yet, in the present case, such substances as possibly play an important role in the normal cell-division were taken up into account. As such substances, glutathione, ATP-salt, adenine, nucleo, namely a compound of some breakdown-product of nucleic acids, and bone marrow extract were chosen. The significant effect of the protein-bound SH groups in the peripheral blood and bone marrow by benzene poisoning was already discussed[36) 55) 56)], and changes in the distribution of protein-bound SH groups which are oxidized during the spindle formation of mitosis were also indicated[49)]. From this point of view, glutathione was adopted. It goes without saying that ATP *in vivo* is of great importance. Next, administered adenine is considered to be utilized in the normal and infected cells[57)] and participate in the nucleic acid synthesis[58) 59) 60)], and its stimulating action for blood formation by benzene intoxication was also proved[61) 62)]. Almost the same effect will be expected in case of the nucleo administration. After Leake[63)] reported that the oral administration of the combined splenic and red bone marrow extracts increased the number of circulating leucocytes, the bone marrow extract has been regarded to have a stimulating effect for blood formation.

The aim of the present discussion is to ascertain the preventive effects of some chemical substances on the benzene affected hemopoietic organs, and to obtain a more substantial clue to the mechanism of benzene poisoning.

MATERIALS AND METHODS

Wistar strain male rats about 2.5 months of age were divided into seven groups of six, A to G. The rats of all the groups but G were treated daily with 0.5 ml/kg of benzene, while, those of G were treated with 0.5 ml/kg of olive oil subcutaneously. At the same time, chemical substances were subcutaneously administered into the rats of A-F groups, as follows:

Group	Substance	Quantity
A	Glutathione (reduced type) dissolved in physiological saline solution	2 mg
B	Adenosine triphosphate disodium salt (ATP) dissolved in physiological saline solution	1 mg
C	Adenine (6-Aminopurine) dissolved in buffer solution of phosphoric acid disodium salt	0.4 mg
D	Nucleo (commericial name)*	0.4 mg
E	Bone marrow extract**	30 mg (as marrow weight)
F	Physiological saline solution	0.5 ml
G	Not treated with any substance	

Nothing but olive oil was given to G group. The doses adopted in the experiment were determined on reference to preceding reports and the therapeutic doses for human beings.

In each group the body weights and the peripheral blood findings, namely, erythrocyte counts, hemoglobin content, leucocyte counts and hemogram, were examined, for six times—just before the treament, 3 days, 1 week, 2 weeks, 3 weeks and 4 weeks after the first administration. All rats were beat to death four weeks after the treatment, and those organs as liver, spleen, kidney, suprarenal gland, testicle and thymus were weighed. The sliced and smeared specimens of the bone marrow were secured from the femoral marrows and prepared appropriately with the same method adopted in the previous experiment.

From the smeared films, the frequencies of mitotic figures in the 5 000 myeloid cells were examined accurately. In order to compare the nucleated cell counts of bone marrow with one another between the groups, the number of the nucleated cells within a sweep of microscope under one thousandfold was calculated, where about twenty spots were set and the cells were counted in each film.

Next, in a further attempt to compute the proliferation speed in each affected myeloid cells, Wistar strain female rats, six in each group, were prepared and after the four-week administration the colchicine method was used in the same way as in the previous experiment.

RESULTS

Body weight and general appearance—The growth of the animals treated

* A breakdown-product of nucleic acid which contain four mononucleotide sodium salt, namely, adenyl-, guanyl-, cytidyl- and uridyl-acids.

** Bone marrow extract was prepared from the red marrow of the rats, and the marrow were homogenated with sterilized water and stored in a freezing room. The supernatant liquid was used after 15 minutes of 3 000 r.p.m. centrifugation.

with benzene was inhibited to some degree after the injection, but no significant differences were observable between benzene-injected groups at that time. The general appearance of benzene-treated groups was affected to a marked degree, and the motive activity of the rats was slightly depressed in about two weeks after the injection. The appearance of hair, especially A, B and F groups, was similarly bad, but in C and D groups comparatively good. Three weeks after the administration, a slight tendency to bleed at the injected spots and a few incrustations and suppurative focuses were observed in the benzene affected rats, but little difference was observable between the groups.

Peripheral blood findings—The mean values of erythrocyte counts of benzene-affected rats were prone to decrease slightly in F group and no effects observable in the other groups; no significant difference, however, was ever proved between the groups. Almost the same phenomena were also observed in hemoglobin content. As shown in Fig. 1, the leucocyte counts decreased markedly in benzene-administered rats. By the injection of some chemical substances, more or less preventable is the diminution of the leucocytes, especially more possible in C, D, and E groups (statistically significant in the values got in the 4th week) than in F group.

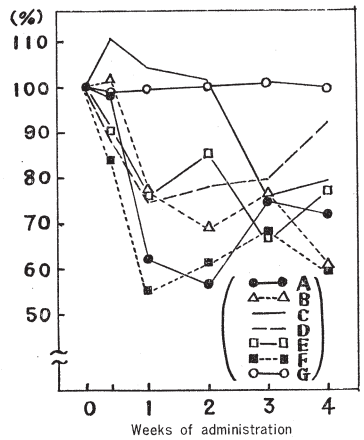

FIG. 1. Leucocyte counts.

In Table 1 are summarized the results of hemogram of each group four weeks

TABLE 1. Hemogram, 4 Weeks after Administration

Groups		A	B	C	D	E	F	G
Eosinophile		1.8	2.3	2.9	1.6	1.8	2.7	2.1
Basophile		0	0	0.2	0	0.1	0.2	0.1
Myelocyte		0.5	0.6	0.7	0.2	0	1.3	0
Metamyelocyte		1.2	1.4	2.3	0.8	0.7	2.4	0
Ring		12.0	13.7	15.9	12.5	8.9	14.7	6.8
Stab		12.9	11.8	11.3	9.3	11.7	11.3	4.2
Segment	II	7.3	6.4	4.8	4.9	7.1	4.7	1.0
	III	2.6	1.4	1.0	1.7	1.6	1.8	0.7
	IV	0.7	0.3	0.2	0.3	0.3	0.3	0.1
	V	0.3	0.2	0	0.3	0	0	0
Lymphocyte		56.3	57.8	54.4	65.5	64.3	55.4	83.0
Monocyte		4.6	4.4	4.5	2.9	3.7	5.3	2.6
N/L		0.67	0.62	0.67	0.46	0.47	0.66	0.15

A minimum of 200 leucocytes were classified in each preparat.

after the injection. In all the benzene-affected rats the relative lymphopenia and the neutrophilia were observed in proportion to the progress of benzene intoxication, therefore, the ratio of neutrophiles to lymphocytes (N/L) increased. Those tendency had been prevented a little in the nucleo and marrow extract treated groups. The immature and polynuclear leucocytes were also found in the blood smears.

Organ weight—The average values of the ratios of organ weight to body weight by group are shown in Fig. 2. There were considerable variations in the values of liver weights and there were no significant differences between groups except D group, but the liver weights of the rats treated with nucleo were prone to increase. The spleen weights showed drastic reduce in the benzene-affected group, but the decrease had been significantly prevented in C, D and E groups. F group lost more the weight of the testicle than the none benzene-treated ones, which proved to be preventable by the injection of some chemical substances (statistically not significant). On the contrary, no kidney showed any influence in its weight. The thymus in F group decreased in its weight remarkably, and its reduction was more or less suppressed by the administration of glutathione, adenine, nucleo and bone marrow extract (statistically not significant).

Frequencies of mitotic figures in the myeloid series—The average values of the frequencies of mitotic figures in the five thousands myeloid cells are shown in Table 2. The mitotic rate of granulocytic cells in the benzene-affected rats increased more obviously, especially in C, D and E groups than the non-treated

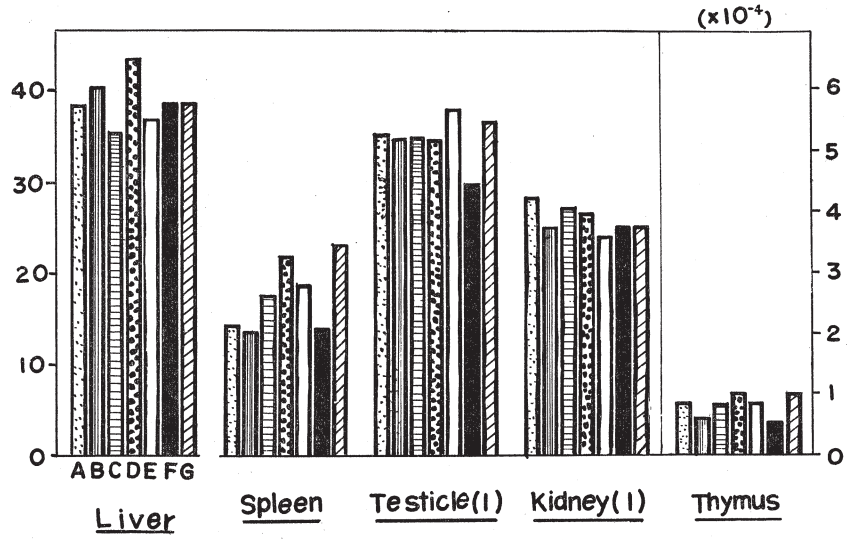

FIG. 2. Ratio of organ weight to body weight in each animals.

TABLE 2. Frequencies of Mitotic Figures in the Myeloid Cells 4 Weeks after Administration
Each values are ratio to 5 000 granulocytic cells.
(Mean±σ)

Groups	Prophase	Metaphase	Anaphase	Telophase	Total
A	255.5±23.2 (61.8)	34.1±5.9 (8.3)	28.2±2.5 (6.8)	95.3±11.2 (23.1)	413.1 (100.0)
B	230.2±20.4 (56.0)	48.4±6.3 (11.8)	25.5±3.6 (6.2)	107.2±9.6 (26.1)	411.3 (100.0)
C	272.5±24.7 (60.6)	41.3±7.2 (9.2)	26.4±4.1 (5.9)	109.4±13.1 (24.3)	449.6 (100.0)
D	274.8±21.9 (60.9)	40.2±5.3 (8.9)	28.5±3.7 (6.3)	107.5±9.6 (23.8)	451.0 (100.0)
E	281.0±23.5 (59.5)	44.9±6.4 (9.5)	34.4±2.7 (7.3)	111.9±10.3 (23.7)	472.2 (100.0)
F	192.5±20.2 (49.2)	66.3±8.2 (16.9)	32.6±4.0 (8.3)	99.8±9.8 (25.5)	391.2 (100.0)
G	186.2±13.6 (71.0)	5.9±0.9 (2.3)	8.8±0.8 (3.4)	61.2±5.6 (23.3)	262.1 (100.0)

( ); Percentage to total mitotic cells in granulocytic series.

ones, but there was no significant difference between the values of the benzene-affected groups. The highest value in the mitotic index of myeloid series was observed in the rats treated with the bone marrow extract, but there were considerable variations in the values of each benzene-affected group. As seen in the previous experiment, the ratio of the mitotic cells in the myeloid cells at the metaphase increased notably in the benzene-treated animals, and in F group, it was more than ten times as much as that of the non benzene-affected ones.

As for the benzene-administered animals, the total number of mitotic figures and the ratio of metaphase in granulocytic series increased statistically significantly, as compared with those of non-treated ones. Those tendencies were most predominant in F group and least in A group, but no significant difference was found statistically in each of the benzene injected groups, except F group. Concerning the mitotic cells of the anaphase, there were marked differences between the benzene-treated specimens and the non-treated ones, but no significant difference was observed between the benzene-affected groups.

The morphological changes, especially the apparent abnormal pattern of the chromosomes in the benzene-affected marrow specimens, were observed more or less obviously in each group in much the same way as in the previous benzene experiment. The chromosomes in the metaphase were comparatively intact in glutathione and marrow extract groups, and the damaged chromosomal

displacement or the bridge formation at the stage of meta- and ana-phases were done in less degree in ATP and marrow extract treated rats. In the adenine and nucleo-injected rats, it showed a tendency to increase the abnormal mitotic cells such as the vacuoled cytoplasma, tri- and poly-polarization, adhesion and partial pycnosis of the chromosomes.

Nucleated cell counts in the bone marrow—The average values of the nucleated cell counts in the marrow specimens under each sweep of a microscope in one-thousand fold are shown in Table 3. The nucleated cell counts were markedly decreased under the four-week benzene administration and reached about 1/2 to 1/3 as many as those of the non-treated group. As for the adenine, nucleo and marrow extract administered rats, however, the decrease of the nucleated cell counts was considerably prevented. There were statistically significant differences between them, especially in C and D groups, and in F group. On the other hand, the nucleated cell counts in A and B groups were almost similar to those of F group.

Mitotic rate of the colchicine-treated marrow cells—Through the assessment of the mitotic rate in a given time, the proliferation speed of the myeloid cells

TABLE 3. Nucleated Cell Counts in Each Group

(Groups)	(Mean$\pm\sigma$)
A (glutathione)	83.2$\pm$10.2
B (ATP)	82.8$\pm$ 9.8
C (adenine)	128.4$\pm$ 9.8
D (nucleo)	122.6$\pm$ 9.6
E (bone marrow extract)	108.2$\pm$10 4
F (saline solution)	75.3$\pm$ 8.8
G (non-treated)	163.2$\pm$ 8.9

These are average values of the nucleated cell counts to each sweep of a microscope in one thousandthfold on the each film.

TABLE 4. Mitotic Rate in Each Myeloid Series of Bone Marrow Determined by Colchicine Method

(Groups)	(Mean$\pm\sigma$) (/10^3)
A (glutathione)	80.3$\pm$16.2
B (ATP)	78.0$\pm$15.6
C (adenine)	105.4$\pm$18.2
D (nucleo)	95.5$\pm$16.4
E (bone marrow extract)	96.6$\pm$18.4
F (saline solution)	75.4$\pm$17.2
G (non-treated)	129.4$\pm$15.8

Each 0.10 mg/kg body weight of colchicine was injected subcutaneously four weeks after the treatment, and marrow specimens were prepared after the six hour incubation.

in each group was determined. As shown in Table 4, the mitotic rate in each of the treated groups was apparently lower than that of control group, especially in A, B and F groups. But the decrease in the mitotic rate in the rats treated with products of nucleic acids and marrow extract was hindered (statistically significant). There were no statistically significant differences between A, B, F groups and G group.

DISCUSSION

As discussed in the preceding chapters, benzene and its metabolites seemed to have a toxic action on mitosis, partly colchicine-like and partly radiomimetic. For a more substantial clue to the mechanism of benzene poisoning, some chemical substances which were expected to prevent such disorders in the hemopoietic organs as induced by the benzene administration, were chosen and injected to the rats together with benzene, and their preventive effects on the hematopoiesis were investigated. The administration of glutathione to the rats treated with benzene proved any protective effect in the peripheral blood findings, organ weights, frequencies of mitotic figures in the myeloid series, nucleated cell counts, and the mitotic rate in bone marrow, except in the diminution of thymus weight and in the chromosomal damage and the increase in number of the frequencies of mitotic figures at the stage of metaphase were prone to be prevented a little. As the protein-bound SH groups, which are oxidized during spindle formation, forming S-S bridges between adjacent protein molecules, are significant for cell-division[49], the administered glutathione may have effects upon the cell division at the metaphase, namely, into the colchicine-like disorder. Though the dose level of glutathione adopted in this experiment would admit some arguments or other, under the present experimental conditions, the administered glutathione would be principally not so important for the improvement of the benezene-affected hematopoietic organs. Now, ATP, namely, high energy phosphate, exists *in vivo* fairly much endogenously, but the exogenous administration of it also has proved some clinical effects. In this case, the mechanism of an effect, induced by the administered ATP, is generally supposed to be related to the hormonal function. But in the present experiment, the ATP group did not show any inclination to improve the hematopoietic organs injured by the benzene intoxication. The disorders of chromosome in cell-division, namely the chromosomal displacement or bridge formation at the stage of meta- and ana-phases, seemed to be less obvious in the ATP treated rats. From these findings, it was concluded that the ATP administration gave some improving effects to the blood forming organs injured by benzene and its metabolites, but it was not so important in the improvement of the affected hemopoiesis.

It is well-known fact that the nuclei, as well as the chromosomes, contain mainly DNA and basic proteins of histone character and other proteins, lipids, ions and several enzymes, namely for the glycolytic pathway and for the synthesis of pyridine nucleotides[64], and that organismus is able to synthesize the purines required for nucleic acid formation. It is noteworthy that some components of nucleic acid, namely adenine and nucleo, were effective for the improvement in the hematopoietic function affected with benzene. There have been few papers reporting about the influence of benzene on the nucleic acid metabolism, which testified the fact that benzene arrested the synthesis of nucleic acid[33][34][35]. The adenine and nucleo show an inclination to prevent the diminution in the peripheral leucocyte counts, spleen weights, nucleated cell counts and mitotic rate in the benzene-treated marrow cells, but not the increase in the abnormal mitotic cells, such as vacuoled cytoplasma, tri- and poly-polarization, adhesion and partial pycnosis of the chromosomes. The stimulating and therapeutic effects of them on blood formation were in rough accordance with those ascertained in the preceding reports on the benzene poisoning[61][62] and on the agranulocytosis caused by the radiation and nitromin[65].

From these findings it was concluded that some nucleic acid components were effective on the improvement of the blood forming organs injured by benzene, and that the effective point of them would be principally in the motivating factors of the mitotic cells at the stage of prophase or of interphase. Furthermore, it is quite likely that benzene and its metabolites would arrest mainly the synthesis of nucleic acids and the metabolism of nucleoprotein and chiefly cause the so-called "radiomimetic action". There were some difference of the changes in the peripheral leucocyte counts between the adenine and the nucleo treated rats, and more researches are yet expected for the further explanation of the phenomena.

Next, bone marrow extract gave certain effect to prevent the disorders induced by the action of benzene[66]. In the experiment, the improvement in the diminution of peripheral leucocyte counts, spleen and thymus weights, nucleated cell counts and the mitotic rate in the myeloid series of bone marrow determined by the colchicine method etc., were observed. These findings suggested that the hematopoietic organs affected by benzene were deprived of some components which would be necessary for blood formation.

As shown in Fig. 1 and Tables 2, 3 and 4, some chemical substances might improve the benzene-affected hematopoietic organs, but the frequencies of mitotic figures in the myeloid cells were nearly twice as many as those of non-benzene-treated group. Consequently, it demonstrated once again that benzene and its metabolites have a serions effect upon the blood forming organs.

Under the present experimental conditions, the inhibition of the nucleic acid synthesis and of the metabolism of nucleoprotein should be most signi-

ficant for the explanation of the mechanism of myelotoxicity caused by the benzene injection.

CONCLUSION

1) By administering some chemical substances, the injuries of the hemopoiesis which occur in benzene intoxication were more or less prevented.

2) In case of the glutathione treated rats, a majority of the experimental findings, except the thymus weight and the chromosomal damage at the stage of metaphase, were not protected.

3) The chromosomal disorders at the stages of meta- and ana-phases were liable to be less in number in the ATP salt administered rats than in the other groups, while the other findings proved it almost similar to that of saline treated ones.

4) Adenine and nucleo, namely breakdown-products of a nucleic acid, kept back the disorders of cell division in the marrow cells, so that the diminution in the peripheral leucocyte counts and the nucleated cell counts in the affected bone marrow was considerably prevented.

The injuring point of benzene was estimated to be mainly in the motivating factors of the mitotic cells at the stage of prophase or interphase.

5) By injecting bone marrow extract, the disorders which occur in benzene intoxication were also fairly prevented.

These findings suggest that the mitotic disturbances caused by the action of benzene or its metabolites may principally depend on the impediment by the metabolism of nucleoprotein and the nucleic acid synthesis, and that in the benzene poisoning the so-called "radiomimetic actions" are possibly of high significance.

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