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EXPERIMENTAL STUDIES ON BENZENE POISONING

1. EFFECT OF BENZENE ON THE BLOOD AND BONE MARROW IN ALBINO RATS

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As is generally known the toxic action of benzene is most prominent in blood and hematopoietic tissue. Numerable studies have so far been made on the change of peripheral blood in benzene poisoning. However, little information is available on the blood changes caused by benzene in refernce to the bone marrow findings. In fact, Gerarde[1] has been the only one who undertook studies on the changes of nucleic acid content and nucleated cell count in bone marrow as well as the changes of the leucocyte numbers in peripherial blood after daily injection of benzene in rats for two weeks and further on the recovery period of three weeks.

As an occupational poisoning, chronic poisoning is common; however, in order to study the primary effect of benzene upon living body, the change of bone marrow due to the administration of large doses for a short time must be taken into consideration. The present paper deals with the change of bone marrow in animals with a special reference to the alteration of peripheral blood in acute poisoning.

Experimental Methods

Growing, male rats of the Wistar strain, weighing from 150 to 200 gm., were maintained with a commercial commpressed food. The animals were subjected to daily subcutaneous injections of a mixture of equal parts of benzene and pure seasame oil. In the first group of experiment, 43 rats were given one ml. of benzene per kg body weight daily over a period of three to thirty five days. In the second group, twenty rats were received two ml. per kilogram daily from three to twenty one days.

As controls in each group, three to seven animals received daily injections of seasame oil. In the first group, three to seven animals were killed 3, 7, 10, 14, 21, 28 and 35 days, in the second group two or three animals were killed 3, 5, 7, 10, 14 and 21 days after treatment.

Blood was taken from the tail for leucocyte, erythrocyte and reticulocyte count. After the animals were sacrificed by heart puncture under ether anesthesia, a complete autopsy was made.

Femoral and tibial marrow were fixed in Zenker's solution and other visceral

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organs were fixed in 10% formalin. Histological observations were made through routine paraffine sections and H. E. and Giemsa stain. Painted and touch preparations were also taken from the bone marrow and some other organs, to observe detailed cytological changes.

Femoral marrow was extruded with 5% acetic acid. A part of cell suspension was diluted with EDTA solution and used to the nucleated cell count.[2] The rest of the suspension was deproteinized with trichlor acetic acid solution. The precipitat was submitted to extract nucleic acid P according to the method of Shmidt and Thannhauser,[3] and extracted DNA-P and RNA-P were determined by the method of Allen.[4]

Experimental Results

Following benzene injection, the animals seemed to lose the appetite and refused food and water. The lower part of the body was coverd with reddish-brown dirty dry crust. The animals began to lose their body weight shortly after the injection, and attained minimum level ten days thereafter and maintained this level to the thirty fifth day (Fig. 1). The loss of weight was greater in animals injected larger dose than in those injected smaller dose. Fig. 1 demonstrates fluctuations of mean body weight. To consider the same data individully, from rat to rat, certain animals showed the gain of body weight, in spite of daily injection of benzene (Table 1). Table 1 shows the change of peripheral blood and bone marrow findings in some rats injected with daily 1.0 ml. of benzene.

A rapid fall occured in the number of white cells in peripheral blood during two weeks after daily administration of one ml. of benzene and a slight tendency to recovery was noted, but in two ml. injected animals any such tendency for recovery was not found (Fig. 2).

Femoral marrow nucleated cell count decreased for consecutive ten days and thereafter tended to increase. There maximum value, however, was lower than those of control group (Fig. 3).

In large dose group, the number of total nucleated cells showed a steady decrease throughout the experimental period and there was no tendency to increase.

As a parallelism is noticed between the alteration of total nucleated cell count and that of body weight, the correlation coefficient between the nucleated cell count and loss of body weight per day is calculated, and obtained the coefficent as 0.71 which is proven highly significant.

The concentration of deoxyribonucleic acid in bone marrow, expressed as μg. DNA-P per mg. dry weight showed the minimum value at the tenth day and thereafter developed a tendency toward recovery (Fig. 4).

An appreciable parallelism was noted between DNA-P content and total nucleated cell count in bone marrow. DNA-P content seemed to be markedly diminished

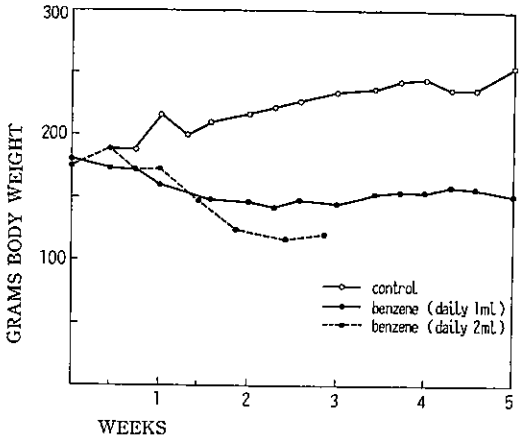

Fig. 1 Effect of benzene on body weight

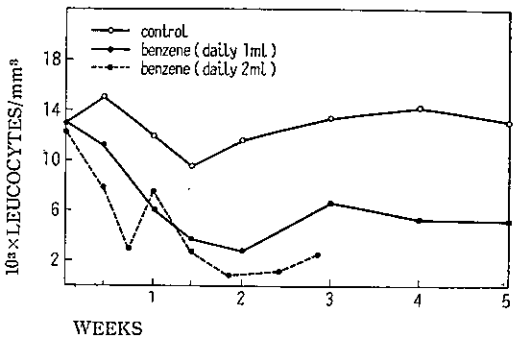

Fig. 2 Effect of benzene on leucocyte count

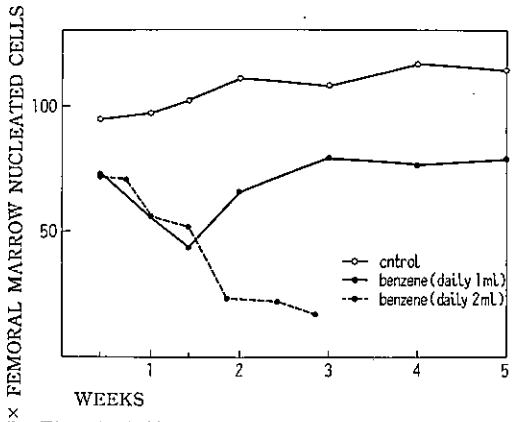

Fig. 3 Effect of benzene on total femoral marrow nucleated cell population in single rat femur

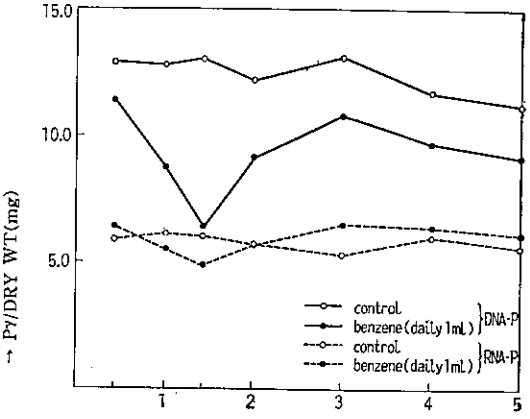

Fig. 4 Effect of benzene on femoral marrow nucleic acid in rat femur

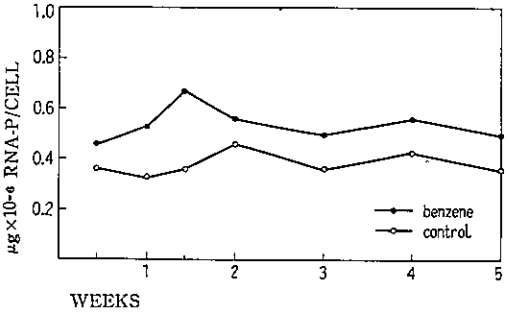

Fig. 5 Effect of benzene on RNA-P content per cell in rat femoral marrow

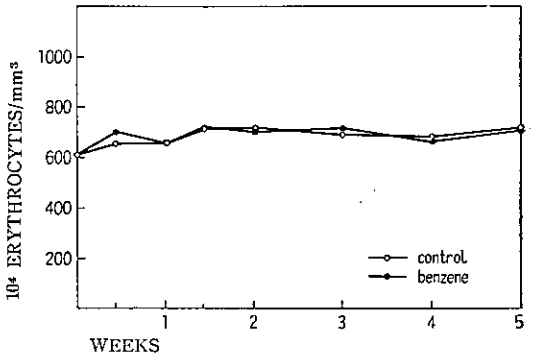

Fig. 6 Effect of daily 1 ml. of benzene on erythrocyte count

Table 1. Change of peripheral blood and bone marrow findings in rats injected with 1, 0 ml per kg body weight of benzene

weeks after treatment	Animals	Exp. no.	Body weight (g) before and after treatment	Gain of weight per day (g)	Peripheral Blood				Bone Marrow			mitotic index
					Red cells $\times 10^4$	White cells $\times 10^2$	Hb g/dl	Retioulocytes	Total nucleated cells $\times 10^6$	Nucleic acid: DNA-P per dry tissue γ/mg	Nucleic acid: RNA-P per dry tissue γ/mg	
1	control	11	203—245	+4.6	576	122	13.7	64	116	12.4	5.6	
		10	210—228	+2.6	650	107	12.6	51	111	10.9	6.2	
	benzene	6	203—174	−4.1	656	27	14.3	21	35	8.1	6.2	
		7	199—152	−6.7	636	14	14.1	40	54	6.3	4.0	
		8	207—169	−5.4	606	20	14.5	13	45	7.5	6.8	
		9	200—151	−7.0	688	28	14.6	8	50	3.8	2.6	
2	control	27	163—244	+5.8	662	80	12.0	43	129	13.8	6.0	
		28	173—213	+2.8	782	100	15.1	44	112	10.8	5.4	
	benzene	29	141—133	−0.5	766	37	14.9	24	87	12.5	6.7	
		30	177—145	−2.3	696	40	15.0	24	49	4.8	4.8	
		31	182—153	−2.0	702	34	15.4	18	58	8.0	5.6	
		32	165—171	+0.4	707	41	14.9	39	103	10.6	5.9	
3	control	25	167—199	+1.5	787	228	13.6	34	115	15.1	6.0	
		26	160—207	+2.2	599	100	13.0	35	92	12.3	6.4	
	benzene	33	175—157	−0.8	766	58	15.1	33	99	12.2	6.6	
		34	172—194	+1.1	742	53	16.4	16	81	10.2	6.8	
		35	149—185	+1.7	673	148	13.7	36	101	12.2	6.3	
		36	139—160	+2.0	701	82	14.5	33	121	12.9	7.7	
4	control	2	137—215	+2.8	724	187	14.6	45	108	9.5	5.3	
		4	180—303	+4.4	678	114	14.5	43	95	12.5	6.7	
	benzene	1	208—224	+0.5	661	58	14.9	36	114	7.9	4.7	
		3	174—194	+0.7	654	60	14.6	26	73	10.2	7.6	
		5	168—128	−1.4	652	77	15.7	26	66	7.7	5.6	
		22	135—164	−1.0	653	59	15.6	49	76	11.2	6.8	
		18	181—181	0	692	30	15.3	10	71	5.3	3.8	
5	control	13	158—263	+3.0	632	101	12.5	42	119	8.9	5.5	
		12	180—166	−0.4	739	100	15.9	64	74			
	benzene	17	182—175	−0.2	560	38	13.8	36		9.1	6.2	
		16	148—143	+0.2	620	102	17.6	40	76			
		20	153—196	+1.2	616	76	15.0	45	121	9.5	5.1	
		21	187—132	−1.6	670	23	16.0	42	41			
		24	207—211	+0.2	741	31	16.5	22	71	10.8	9.1	

in large dose animals in comparison with small dose. The difference between these two groups of animals is not difinite because the number of animals is so small in the latter group to make a conclusion.

The DNA-P content per cell ranged 0.68 to $0.88\times10^{-6}\gamma$ in the exposured animals and showed no significant difference compaired with that of control group. The amount of DNA is calculated with multiplying DNA-P by 10.1. The calculated value of DNA content per cell consisted with the data of some investigators.[5),6)]

The concentration of ribonucleic acid in bone marrow, expressed as μg. RNA-P per mg. dry weight varied within a limited range and no significant difference could be observed between each weekly value (Fig. 4).

The RNA-P content per cell in intoxicated animals showed an increase at the end of the first week, and reached its peak at the tenth day, thereafter it tended to decrease slightly but all of the values remained at relatively high level throughout the experimental period in comparison with the control one (Fig. 5).

As shown in Fig. 6, erythrocytes were not affected.

The alterations of values for the different types of leucocytes in the poisoned and normal animals are given in Table 2.

In this table the relative lymphopenia is noted in animals injected with daily one ml. of benzene.

Table 2. Effect of benzene on relative values for leukocyte counts in rats

weeks	Administration with	Neutrophils	Eosinophils	Basophils	Lymphocytes	Monocytes
Pretreatment	none	32.0	3.0	1.0	61.0	4.0
1	benzene	42.0	1.5	2.0	51.5	3.0
	oil	34.5	3.0	0.5	59.0	3.0
2	benzene	54.5	1.0	0.5	40.0	4.0
	oil	28.0	0	0.5	69.5	2.0
3	benzene	50.5	1.5	0	45.0	3.0
	oil	24.5	3.5	0.5	69.5	2.0
4	benzene	59.0	1.0	0	35.0	5.0
	oil	22.0	1.0	0	72.0	5.0
5	benzene	60.0	1.5	0.5	37.0	1.0
	oil	18.0	3.5	0.5	75.0	3.0

Morphological Observation on the Bone Marrow

In every control animals, the bone marrow of the hind legs showed rather uniform appearance in nacked eye observations, and this fact corresponded with the above described data that the number of total nucleated celles showed comparatively little variation. On histological examination the majority of the femoral marrow

were red cellular. Mixed marrow were rarely found only in a few tibia, and there was no animal with total fat marrow.

It has been considered that, there was no essential difference in cellular composition of the marrow in every bone of rats,[7-8] when their colonies were supplied from a definite source and from a definite age group. Neverthless, inidvidual values in differential count submitted by several authors showed in fact extremely wide variety.[7,9] Reasons for these curious descrepancies seemed to be partly explained from the results obtained by Harris et al.,[10] who pointed out that the cellular composition of the rat bone marrow undergoes two significant changes during the first year of their life.

Taking these points into consideration, our observations on differential count were made chiefly on painted preparations from the femoral marrow, and the results obtained on the control animals showed considerable similarity to those of 8 to 10 weeks of age found in the description of Harris et al.. A only little difference was comparatively low value in percentage of the so-called undifferentiated reticulum cells in our experimental animals.

During the first week of daily administration of benzene, there was difinite decrease in number of the nucleated cells in the femoral marrow as described above, and histological observations in this stage revealed that cellular density became somewhat loose, especially in the area around the blood space. Slight hemorrhage was sometimes associated; however, essential structure of the bone marrow appeared to be preserved, even in the animals of higher dose group.

Definite decrease in number of normal erythroblasts, especially of orthochromatic ones, was one of characteristic feature in this stage, and about one half of the cells of erythroblastic series showed apparent abnormalities. They were somewhat larger in size than the usual ones and were predominated by more abundant basophilic cytoplasm and by hyperchromatic nuclei with densely arranged network of chromatin. On the other hand, proerythroblasts showed definite increase even in calculated actual number, and more or less apparent abnormalities were evident almost in majority of these cells.

Concerning the leucopoetic series, there was slight relative increase in differential count, though calculated actual number diminished to almost one half of the control value. This relative increase in differential count of total leucocytes was chiefly due to the relative rise in count of myelocytes and in those of mature polymorphnuclear leucoytes including segmented and as well as ring shaped nuclei. These polymorphnuclear leucocytes frequently showed their winkled or pycnotic nuclei, and sometimes contained basophilic granules resembling toxic granulation in their cytoplasm. Relative percentages of the myeloblasts and of the stab cells were not yet significantly altered in this stage. Eosinophilic leucocytes and megakaryocytes appeared to show no essential abnormalities in their distribution and in mor-

phological features.

Another prominent change in this stage was a significant increase in number of nuclear division in variable stage of mitosis. The most high value in mitotic index was observed at the end of the first week and it reached almost ten times or more to the average value of 0.35 per cents of all nucleated cells in the control animals. Moreover, a large number of these mitotic figures showed apparent abnormalities, especially in the appearance of their chromosomes. The pattern of the chromosomes in the metaphase were frequently irregular and indistinct in their shape, and their arrangement also appeared to be disorganized. There were rather frequent pictures of delayed or disturbed refusion of the chromosomes in two adjuscent daughter cells, in which their cytoplasmic structure obviously recovered from cellular division. These abnormalities in mitotic division were noted more frequently in the cells of erythroblastic series, and they were also evident in the leucopoetic cells especially in the proliferated myelocytes. Occasional karyolexis or framentation of the nuclei were appeared to be drived rather from the cells in damaged mitosis than from the cells in resting cells in maturation. And comparatively slight signe of degeneration in hematopoetic cells seemed to be noteworthy.

During a week after the onset of the treatment, morphological changes in the bone marrow of the rats treated with daily administration of 2 ml. of benzene, were almost similar to those received 1 ml. daily. And so far as this stage was concerned, the differences in severity of the lesions were not so remarkable between these two series.

From the second week, signs of regeneration took place evidently in the damaged bone marrow in most of the animals of 1 ml. group. Generally said, just from this stage, corresponding to gradual recovery of total nucleated cell number of the bone marrow, a gradual tendency to return to usual level was observed also in differential count. And some of the animals sacrificed after four or five weeks revealed nearly normal values in their differential count. In these animals, however, slight predominace in myelocytes and still somewhat reduced value in mature neutrophilic leucocytes might suggest that, these bone marrow received preceeded damage. Moreover, abnormal erythroblasts and somewhat higher mitotic rate were also definitely recongnized. It seemed to be noteworthy that, changes in erythroblastic series and in mitosis persisted even after five weeks, though nearly complete recovery was already established.

On the other hand, the bone marrow after the second week showed considerably wide variety in their morphological changes, which was possibly determined by severity of the initial damage and by the degree and tendency of the succeeded regeneration. Some animals sacrificed after three or four weeks showed rather predominated value in differential count of the erythroblastic cells which were composed principally of usual erythroblasts and several per cents of abnormal ones.

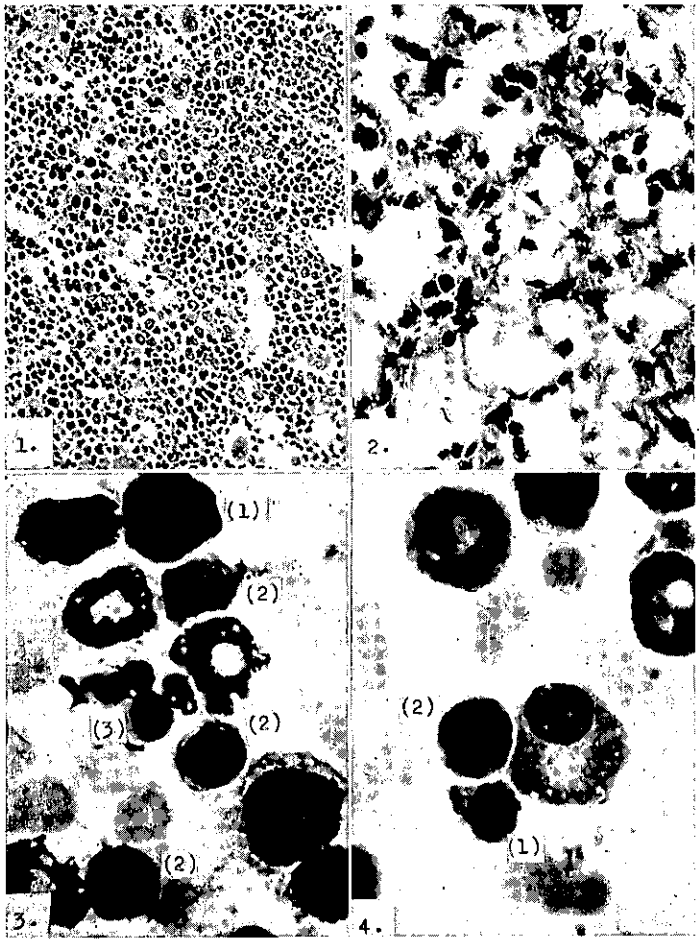

Fig. 1. General view of the femoral bone marrow taken from a rat received daily one ml. of benzene for a week. (paraffin section, H.E. stain, lower magnification)

Fig. 2. Hypoplasia of the femoral marrow produced by three weeks duration of daily injection of two ml. of benzene. (paraffin section, Giemsa stain, higher magnification)

Fig. 3. A large erythroblatic cell (1) with abundant basophilic cytoplasm. The other three (2) are also larger in size as compared to usual normoblast (3). From the painted preparation of the femoral marrow after a week. (Giemsa stain, oil immersion)

Fig. 4. A small undifferentiated mesenchymal cell (1) and a plasma cell in the painted preparation from a femoral marrow after three week. A larger erythroblast (2) is also present. (Giemsa stain, oil immersion)

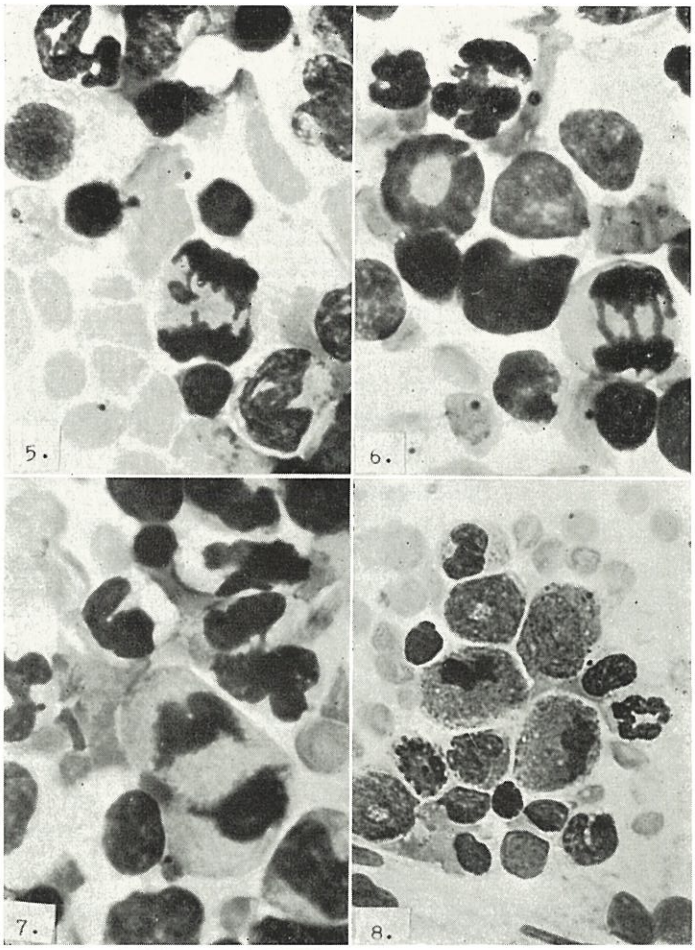

Fig. 5. to Fig. 8. Variable pictures of mitotic divisions with apparently damaged chromosomal structures. Note the bridge formation and chromsomal abberation in later metaphase. From the painted preparations in variable stages. (Giemsa stain, oil immersion)

Some animals revealed considerable predominance in leucopoetic series especially in myeloblasts and in myelocytes. Moreover, a few animals after three weeks showed considerably advanced reduction in number of erythroblastic cells with relatively preserved leucopoetic series; however, the damaged picture as a whole showed some resemblance to those which usually found in the 2 ml. group described later.

These varieties in the response of bone marrow to the initial damage and the following regeneration, might be regarded as an expression of so-called "individuality" in each animal, but as discussed later, actual basis of such "individuality" might not necesarily be considered only in relation with the difference in sensitivity of the marrow cells to any noxious agent. At any rate, it seemed to be noteworthy that, these varieties in tissue response in the 1 ml. group became more apparent from the second week, when reactive regeneration took place more evidently just under the influence of still continued administration of benzene.

And just from this stage, from the second week, difference in the features of damage between two groups treated with different dose of the benzene became definitely apparent. When daily administration of 2 ml. of benzene continued over two weeks, total nucleated cell count of the bone marrow showed more or less significant reduction in all of the animals, and finally almost complete destruction of the hematopoetic cells were resulted in some of the animals at the end of the third week. In such instances, total nucleated cells decreased to nearly one twentieth of those of control animals, and the hematopoetic cells were extremely scanty also in histological sections. Small islets of remained hematopoetic cells were sparsely distributed in the edematous and gelatinous stroma with only minimal signs of fibrotic tendency. Any remarkable regeneration was hardly recongnized. Rather scanty cellualr elements in painted preparations were chiefly composed of unusual erythroblastic cells and of remaining leucopoetic cells in small number. Some smaller cells resembling to undifferentiated reticulum cells were also found. Plasma cells were also present in small number. Significant decrease in number of myelocytes and as well as that of more matured neutrophilic leucocytes were evident. Eosinophilic leucocytes and megakaryoctes were also severely damaged, but the latter appeared to be still preserved to some degree. Mitotic rate was still higher than that of control and all of mitotic figures appeared to be affected.

From the above described morphological findings, it is concluded that daily administration of the benzene in sufficient amount may cause progressive destruction of the haematopoetic tissue in the bone marrow, as seen in several foregoing descriptions[11)-13)] on experimental benzene poisoning. The result of this destruction was most clearly revealed as the significant decrease in number of total nucleated cells especially in the animals of 2 ml. series. In this series, however, the severity of affection made the difference in features of damage among the different cell series hardly discernible. On the other hand, it seemed highly interesting to note

that, in the group treated with somewhat lower dose of the benzene, some differences in the features of the damge were observed between two main series of hematopoesis, in the bone marrow. In the erythropoetic cells, the most predominant change in early stage was the appearance of numerous abnormal erythroblasts and of more immature "proerythroblasts". And it seemed to be noteworthy that these abnormalities in erythroblastic cells remained definitely at least to the end of the fifth week though the grade was not so marked as seen in the first week. On the the other hand, in the leucopoetic series, evidence of any destructive or alterative changes were not so prominent in the immature cells, and the course of their maturity appearred not to be so severely affected so far as morphological observations concerned. Only in the matured polymorphnuclear leucocytes, some signs of nuclear damege were discernible from the early stage of the experiment. From these results, it might be suggested that, if administered dose of benzene was not so high, its destructive effects found in resting cells were not so severe except for in mature neutrophilic leucocytes. This suspicion seemed to be supported by the fact that necrosis or degeneration of the haematopoetic cells were only rare findings and a few pictures of karyolexis were appeared to be drived from the destruction in mitotic stage. Thus, if these direct injury in the haematopoetic cells were not severe enough to explain completely the definite and rapid fall in number of total nucleated cells during the initial stage, it might be assumed more likely that significant increase in mitotic rate associated with a numbers of abnormal mitotic figures might be an important finding. The benzene and its derivatives have been recongnized as mitotic poisons,[13] and some authors pointed out an increased mitotic rate in some visceral organs in experimental benzene poisoning. In our experimental study, significant increase in mitotic rate in the bone marrow may be partly explained as an expression of the just succeeding regeneration; however, frequent occurrence of abnormal mitotic figures were highly suggestive of injurous effect of benzene or its derivatives upon the mitotic division. Possibilities were not excluded that some latent injury endured in resting stage became apparent at the time of mitosis. From these considerations, it seemed more likely to assume that the rapid initial destruction of hematopotic cells in the bone marrow with only slight signs of cellular degeneration might be chiefly explained by the mitotic disturbance rather than direct cellular injury.

Results from the observations on the bone marrow other than the femur and on the systemic visceral organs will be given in succeeding report in preparation.

Discussions

A rapid fall occured in body weight in almost all animals treated with benzene. The decrease of body weight stopped at a certain level seven to ten days after the beginning of the treatment in animals with daily one ml. of benzene and two weeks

after in those with two ml. of benzene. The level of former remained higher than the latter. The fluctuation of body weight corresponded with that of total nucleated cell count in bone marrow and of peripheral leucocytes number. When observed separately, the fluctuations of body weight differed according to animals, but, when animals are observed as a whole the body weight showed above mentioned tendency. In animals injected with one ml. of benzene, a few of them showed little decrease or even slight gain in body weight irrespective of the administration continued. Any decrease in total nucleated cell count in bone marrow was scarcely noticed in these animals. Some of these animals showed a normal level in peripheral white cell number, while in others, sharp decrease was noted in leucocytes number. The individuality inherent in each animal may be responsible for these unusual cases.

As the sensitivity of the marrow cells to the noxious agent may be regarded to be insufficient to elucidate individual variety in bone marrow, the capacity of detoxication in liver may be taken into consideration.

In animals given one ml. of benzene, the number of leucocytes attained to the minimum on 14 th day, while the nucleated cell count on 10 th day. The discrepancy observed in the measurement to attain the minimum values may be explained from the brief span of neutrophilic leucocytes and the disturbance of supplement of the mature cells of myeloid series from the bone marrow into blood. The minimum value of leucocytes number were one-third or one-fourth of normal value, while that of nucleated cell count in bone marrow were only about a half of normal value.

This numerical difference may be related to the destructive action of benzene or its derivatives upon matured polymorphonuclear leucocytes in bone marrow and presumably also in the circulating blood.

Suspected loss of capacity of these damaged neutrophiles to emigrate into the blood stream, might be also one of probable explanations for such too rapid initial fall and for relative predominance in differential count in bone marrow of these neutrophilic leucocytes.

It seems to be odd that no evident decrease was observed in peripheral red blood cell count throughout the experimental period. The stationary erythrocyte count may be surmised to be concerned with the life span of rat red blood cell, which has been estimated[14] in a range of 49-55 days, and with the duration of the experiment. The present experiment continued through only 35 days. Therefore, the long life span of erythrocyte may be responsible for little fluctuation of erythrocytes number. On the other hand, abnormal erythroblasts and proerythroblasts were clearly shown in erythroblastic series in bone marrow throughout the experimental period. It may be noteworthy that the appearance and persistency of such morphological abnormalities in erythroblastic series corresponded rather well with those of abnormal pattern in erythrophoresis of hemoglobin described in the third report in this bulletin.[15]

In the animals administered daily one ml. of benzene, a rapid fall was observed in the total nucleated cell count in bone marrow and leucocytes number in peripherial blood, but they tended to recover shortly thereafter, and myelogram showed that the reactive regeneration took place evidently from the third week. On the other hand, in animals administered daily two ml. of benzene, any tendency toward recovery was hardly recognized in nucleated cell count, leucocyte number as well as in morphological observations.

The difference between two recovery process may by explained from the presumption that daily one ml. of benzene administration was insufficient to interfer completely the reactive regeneration following the primary damage in the bone marrow.

A definite and rapid fall in numbers of total nucleated cell of the bone marrow during first week was not completely explained only from the morphological changes in the hematopoietic cells.

In this connexion one must pay attention to the definite increase in mitotic rate associated with numbers of abnormal mitotic figures. A significant increase in mitotic rate may be partly explained as an expression of the succeeding regeneration; however, frequent occurence of abnormal mitotic figures were highly suggestive of injurous effect of benzene or its derivatives upon the mitotic division.

From these considerations, it seemed more likely to assume that the rapid initial decrease in number of hematopoietic cells in the bone marrow, accompanied with only slight of cellular degeneration might be explained from the action of benzene as the mitotic inhibitor rather than as a direct cellular noxious agent.

The fluctuation of DNA-P content per dry weight of tissue in bone marrow run parallel with that of total nucleated cell count, while DNA-P content per bone marrow cell underwent a slight change following administration of benzene. From these facts fluctuation of DNA content may be presumed partly from the change of total nucleated cell count in bone marrow.

In regard to relative proportions for leucocyte count in peripheral blood, it was proven that a relative lymphopenia occured following benzene administration, and this fact consisted with the result of Latta et al.[16] From a consideration of this result, it may be reasonable to give attention to the changes of lymphatic tissue as well as that of myeloid tissue in acute benzene poisoning.

Summary and Conclusion

Male, albino rats weighing 150-200 gm. were administered daily with one ml. per kilogram of body weight of benzene for five weeks and two ml. for three weeks respctively. A rapid fall was observed in body weight, in leucocytes count in peripheral blood, in femoral marrow nucleated cell count and in DNA-P content per dry weight of bone marrow during the first ten days of daily injection of benzene.

In the one ml. series, thereafter, these values tended to recover, neverthless, they maintained low level in comparison with those of control animals. Morphological observations revealed that the reduction in number of the bone marrow cells was chiefly due to that of erythroblastic cells and that of the cells of neutrophilic leucocytes, while eosinophilic leucocytes and megakaryocytes appeared to be relatively preserved in this dose of benzene. Abnormalities in the features of erythroblastic cells and significantly increased mitotic rate were noticed, and persistency of these changes even after proceeded regeneration were pointed out.

Rats given daily with two ml. of benzene underwent more marked changes and any signs towards recovery was hardly recognized throughout the whole course of the observation. And after three weeks, some animals showed nearly aplastic bone marrow.

Erythrocytes count in peripheral blood and RNA-P content per dry weight of the bone marrow were not significantly affected.

It was also suggested that mitotic disturbance due to the action of benzene or its derivatives might be a most important factor to cause primary reduction in number of marrow cells especially in early stage of experimental poisoning.

ACKNOWLEDGMENTS

We wish to express our thanks to Miss Michiko Yoshiike for the determination of nucleic acid, to Miss Minako Takada for hematological investigation and to Miss Satoko Shimizu for preparing specimens for morphological observations.

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要　　旨

ベンゾール中毒の実験的研究

第 1 報　ベンゾールの骨髄及び血液に及ぼす影響

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産業中毒としてのベンゾール中毒の多くは慢性の経過をとり，末梢血液の異常を来す。従つて，ベンゾール中毒の研究には，少量のベンゾールが長期に亘つて作用した場合に，造血器官にあらわれる変化を末梢血液の変動と関連して観察する要がある。然し，ベンゾールの生体に対する一次的な作用機序を知る為には，多量のベンゾールを短期間作用させて生ずる造血器官の急性変化を知る事も必要である。こういう観点から，白鼠に急性，ベンゾール中毒を起させ，その経過を末梢血液の変化，骨髄の病理学的所見，骨髄の有核細胞数及び核酸の変動を通じて追究した。

即ち，雄の白鼠（体重 150～200 瓦）にベンゾールを体重 1kg 当り 1cc 或は 2cc の割合で毎日背部皮下に注射して夫々 5 週間，3 週間に及んだ。対照白鼠には胡麻油を注射した。その間，毎週 1 回（初期には 3，10 日目にも）殺して，下肢骨を採り，大腿骨の骨髄は 5%の醋酸で洗い出して有核細胞数を測定すると共に，Schmidt-Thannhauser の方法によつて核酸を抽出分離した後，Allen の方法により DNA-P と RNA-P を定量した。尚殺す前に尾部より採血して赤血球数，白血球数，白血球の百分比，Hb，網状赤血球等を測定した。下肢骨の骨髄は，組織学的に観察すると共に塗沫標本を作成しギームザ染色によつて観察した。
以下，本文の図表を中心にして簡単に説明する。

Fig. 1. ベンゾールを注射してゆくと白鼠の体重は 1 週後に既に急激に減少しその後，この低下した水準を実験期間中続ける。2cc 注射の場合は 1cc 注射に比べて，体重の減少の程度が大きい。

Fig. 2. 末梢血液中の白血球数は注射後 2 週までは急速に減少し，その最低値は対照群の⅓から¼に当る。1cc 注射群ではその後，稍〻恢復する傾向が見られた。2cc 群の最低値は 1cc 群の最低値よりも更に低く，しかもその値は，実験終了の 3 週まで恢復しなかつた。

Table 2. 白血球像の百分率ではベンゾールの注射に伴い，相対的淋巴球減少が見られた。

Fig. 3. 大腿骨の骨髄の全有核細胞数は 1cc 群では，1 週目から 10 日目にかけて，最も減少し，その値は対照群の½に達した。併し，3 週目から恢復に転じ，実験終了までその値を続けた。2cc の注射群では，一路下降を続け，3 週後には対照群の⅓或はそれ以下まで低下した。

Fig. 4. 大腿骨の骨髄細胞の核酸を DNA-P と RNA-P に分けて 1cc 注射群について観察すると，乾燥重量当りの RNA-P 量には対照群に比べて顕著な変動が見られない。乾燥重量当りの DNA-P は 1 週から 10 日後にかけて最も減少し，その後は恢復に向うが，対照群の水準までは恢復しなかつた。DNA-P の曲線の走行は Fig. 3 の骨髄中の全有核細胞数の走行と平行

している。然し細胞 1 個当りの DNA 量にはベンゾール群と対照群との間には差が見られなかつた。

Fig. 5. 細胞 1 個当りの RNA 量の変動では連続 1cc の注射後 10 日目に山が見られた。又，ベンゾール群は対照群に比べて常に高い値を示した。

Fig. 6. 末梢血液の赤血球数ではベンゾール群と対照群の間に，殆んど差が見られなかつた。

Table 1. ベンゾール 1cc 注射群の代表例についての体重，末梢血液，骨髄の逐週的変動の個々の数値を掲げた。

骨髄の病理学的検索では，注射後 1 週で赤芽球の減少と，その約半数の異常像が見られた。白血球系では未熟の細胞はベンゾールによる障害が少く，成熟好中球に異常像が見られた。又，種々の段階の有糸分裂像を持つた細胞が増加し，対照群に比べて約 10 倍に達した。又，これ等有糸分裂像の多くのものは染色質の形態配列に異常を示した。注射後 2 週には 1cc 注射群では既に再生像があらわれて，3 週後からは益々顕著となり，骨髄は全般に多彩な像を示した。5 週後には骨髄像はかなりの恢復を示すが，赤血球生成系の異常と，有糸分裂像異常は，この時期でも軽度ながら存続した。

2cc 注射群では初期の像は 1cc 注射群と本質的には同じであるが，その後骨髄細胞は全面的に破壊され，2 週以後に於ける再生の徴は全然見られなかつた。

Bull. Nat. Inst. Indust., Health 2, 17~28, (1959)

EXPERIMENTAL STUDIES ON BENZENE POISONING

2. EFFECT OF BENZENE ON THE CATALASE ACTIVITY IN BLOOD

Hiromichi HASEGAWA and Mitsuo SATO

Many reports have been issued on the changes of blood and blood forming organs in benzene poisoning, but none of which has refered to the interaction between a certain substance such as a protein or an enzyme and toxic substance in blood caused by benzene injection. It has been assumed that the metabolites of benzene such as phenol, catechol or hydroquinone might cause benzene poisoning, but no experimental fact to prove this assumption has been yet observed except the reports that phenols were very effective as mitotic poison[1].

We took up the problem of catalase as a part of studies of benzene poisoning by the following reasons:

a) Many investigators, especially Williams et al[2,3], have clarified the metabolic pathway of benzene in which benzene was oxidized into catechol, hydroquinone and hydroxyhydroquinone through phenol.

b) Catalase reaction is inhibited with phenols which combine with imidazol radicals of catalase-protein[3,4,5,6,7].

c) Hirokawa[8] reported that sexual difference exerted a serious influence upon the appearance of benzene poisoning.

d) Adams[9,10] and Begg[11,12] have found the possibility of an interrelationship between catalase and sexual hormones.

e) The measurement of catalase reaction is very easy.

But regretfully the physiological action of catalase is still obscure[13,14,15]. The authors have undertaken an experiment expecting the phenolic substance was responsible for the inhibition of blood catalase activity, but in studying benzene poisoning in rat a new non-phenolic substance was found and this substance was just an agent which could induce the suppression of blood catalase action. The present paper deals with the change of blood catalase activity as well as the extraction of poisonous substance due to benzene poisoning.

Method

Enzyme solution was prepared by adding 2 ml. of distilled water to 0.1 ml. of heparinized blood drawn from heart of rat. In general, the action of various poisons upon the catalase reaction has two distinct phases which are called "initial" and "final" state of the inhibition.

In the present experiment, the catalase activity was measured in the "final"

state abolishing totally the phenomena of initial state by previous treatment of catalase with a small quantity of H_2O_2. And the following mixture was used; 28 ml. of distilled water, 2 ml. of pH 6.8 phosphate buffer solution $\left(\frac{1}{100}\text{ M in final experimental solution}\right)$, 2 ml. of enzyme solution, 2 ml. of H_2O_2 solution $\left(\frac{1}{2000}\text{ M in final}\right)$ and 4 ml. of H_2O_2 $\left(\frac{1}{100}\text{ M in final}\right)$. To 32 ml. of bufferd catalase solution was added 2.0 ml. of H_2O_2 solution (pretreatment). After 5 min., 4.0 ml. of H_2O_2 was introduced to it and the course of H_2O_2 decomposition was followed by titration pipeting out 5 ml. from the reaction mixture into conc. H_2SO_4 solution.

The log $[H_2O_2]$-t-curve became linear showing the constant inclination which was regarded as corespoding to the "final" state. We prescribed expediently that the enzyme solution had the activity of 100% when it decomposed 1.3×10^{-4} M of H_2O_2 per second. All experiments were carried out at 0° to avoid the denaturation of catalase by H_2O_2.

Results

The activity of blood catalase in rat injected with benzene.

Following daily injection of 1 ml. or 2 ml. of benzene per kg of body weight blood catalase showed approximately 60—80% activity of that in control rat at the seventh day (Fig. 1), but it recovered to the normal level, 100%, at the tenth day regardless of the amount of benzene and maintained the same level until the fourteenth day.

After three weeks, the sharp decline of catalase activity was noted, particularly in the case of 2 ml. of benzene injection. The cause of the recovery in the second week and rapid decline of activity thereafter remained still obscure.

The decrease of body weight had little influence on catalase activity, because 100% activity was obtained in rat of which body weight was decreased by food reduction as same extent as that of rat injected with benzene.

The effect of pretreatment of a small quantity of H_2O_2 upon catalase activity.

In above experiments the authors pretreated the catalase solution with a small quantity of H_2O_2 $\left(\frac{1}{2000}\text{M in final concentration}\right)$, and the log $[H_2O_2]$-t-curve became linear in both normal and benzene injected rats.

To clarify the nature of the catalase reaction in the absence of pretreatment, further experiments were carried out. To a buffered catalase solution (30 ml. of distilled water, 4 ml. of buffer solution and 2 ml. of enzyme solution) 4 ml. of H_2O_2 $\left(\frac{1}{100}\text{ M in final}\right)$ was added to start the catalase reaction and the results were shown in Fig. 2.

In a normal rat, the log $[H_2O_2]$-t-curve became linear, but on the contrary in

CATALASE ACTIVITY IN BLOOD

Table 1. Relative catalase activity in blood of rat injected with benzene

a) Daily injection of 1 m*l*. of benzene per 1 kg of body weight

Duration of treatment (days)	Animal No.	Catalase activity (%)
4. Normal	31	100
	1	100
7, Normal	16	100
	43	100
	47	95
10, Normal	19	110
	18	104
14, Normal	5	100
	9	92
	22	102
	28	100
21, Normal	33	100
	8	100
	25	100
	26	100
28, Normal	14	115
	35	100
	29	109
	2	101
	4	103
35, Normal	2	100
	7	100
	38	100
	12	96
38, Normal	23	100
	36	100
	37	100
42, Normal	15	110

Duration of treatment (days)	Animal No.	Catalase activity (%)
4, Benzene	4	100
	34	74
	24	100
7, Benzene	17	87
	26	67
	42	79
10, Benzene	46	102
	49	90
	10	90
14, Benzene	6	99
	13	110
	20	99
	29	100
	31	100
	32	100
21, Benzene	12	60
	32	55
	21	65
	33	55
	34	70
	35	44
	36	56
28, Benzene	27	64
	44	68
	30	50
	5	70
	1	65
	3	46
35, Benzene	11	70
	15	80
	16	40
	17	42
	20	37
	21	42
38, Benzene	45	47
	50	61

Tabel 1. b) Daily injection of 2 m*l*. of benzene per 1 kg of body weight

Duration of treatment (days)	Animal No.	Catalase activity %
3, Benzene	24	100
	25	107
	26	101
	28	80
5, Benzene	38	66
	39	73
	40	80
	41	71
7, Benzene	33	74
	34	64
	35	57
	43	77

Duration of treatment (days)	Animal No.	Catalase activity %
10, Benzene	17	100
	18	91
13, Benzene	21	100
	22	100
17, Benzene	29	65
	32	60
20, Benzene	20	27
	46	37

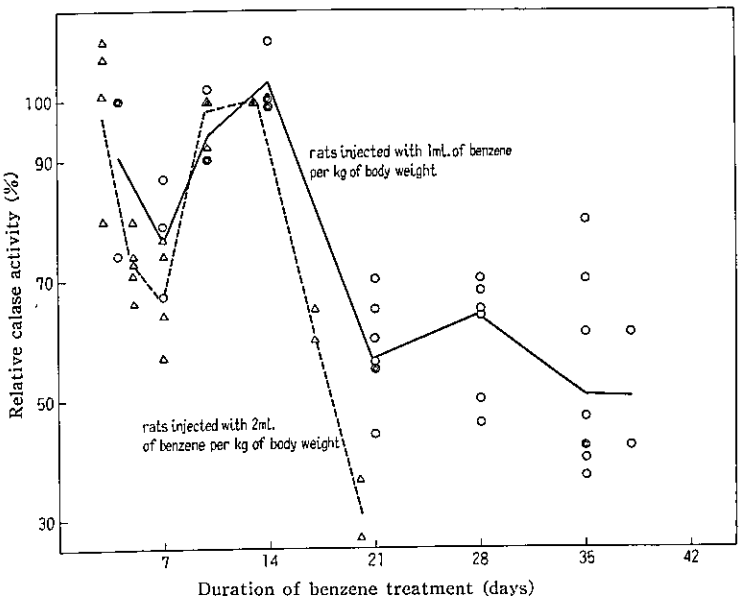

Fig. 1 Changes of catalase activity in blood of rat caused by the injection of benzene.

Enzyme solution was prepared by adding 2 ml. of water to 0.1 ml. of blood. Following reaction mixture was used: 28 ml. of water, 2 ml. of pH 6.8 phosphate buffer($\frac{1}{100}$ M in final experimental solution), 2 ml. of enzyme solution, 2 ml. of H_2O_2($\frac{1}{2000}$M), and 4 ml. of H_2O_2($\frac{1}{100}$ M). When an enzyme solution decomposed 1.3×10^{-4} M of H_2O_2 per second in the reaction system described above, it was defined that this enzyme solution had the activity of 100%. (pH 6.8, 0°)

the rat injected with benzene, the reaction curve bended with time and finally run parallel with that of the solution pretreated with a small quantity of H_2O_2.

Ogura et al[4,5,6] explained the phenomena observed with or without pretreatment that the mechanism of catalase action was composed of following three consecutive reactions

$$E+S\rightleftarrows ES$$
$$ES+S\rightleftarrows SES$$
$$SES\rightarrow E+H_2O+O_2$$

where E was the free catalase molecule, S hydrogen peroxide, E intermediate complex and SES a complex in which another molecule of H_2O_2 is bound reversibly to ES. If a certain poison (G) is added to a catalase solution in the absence of H_2O_2, the following reversible reaction occurs

$$E+G\rightleftarrows EG \qquad (1)$$

When H_2O_2 is added to a system in which the equillibrium between E and G had been attained, all existing E will be changed into ES, because the reaction $E+S\rightleftarrows$ ES can take place almost instantaneously and the equillibrium attained is shifted

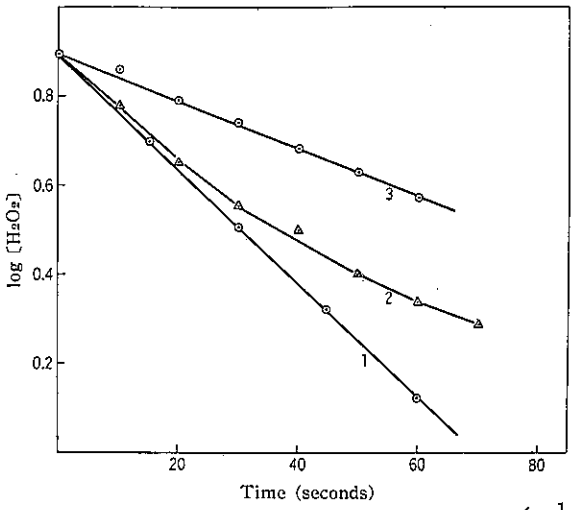

Fig. 2 Effect of pretreatment with a small quantity of H_2O_2 $\left(\frac{1}{2000}\text{M in final}\right)$

Curve 1: for a normal rat with and without pretreatment. In normal rat, the effect of pretreatment was not observed.

Curve 2: for a rat (No. 17) injected with benzene for 5 weeks without pretreatment.

Curve 3: for a rat (No. 17) with pretreatment. After 5 minutes of the pretreatment, catalase reaction was started with the addition of much quantity of H_2O_2 $\left(\frac{1}{100}\text{M in final}\right)$ (pH 6.8, 0°)

extremely toward the right hand side. The poison G will then commence to react with ES in the following manner:

$$ES+G \rightleftarrows ESG \qquad (2)$$

In many experiments[4,5,6,] it has been shown that the dissociation constant of EG was much smaller, about $\frac{1}{100}\sim\frac{1}{1000}$, than that of ESG. From the result of the experiment, it was assumed that a certain poisonous substance which could inhibit catalase action appeared in blood of poisoned rat. In the absence of H_2O_2, the reaction (1) was established in equilibrium. When the reaction was started with the addition of H_2O_2, however, free catalase molecule E was changed instantaneously into ES entailing the destruction of the equillibrium (1), so the reaction (2) would take place gradually.

Accordingly, the log $[H_2O_2]$-t-curve would be bended with the lapse of reaction time. As for the reaction system pretreated with a small amount of H_2O_2, the equillibrium (2) could take place before the catalase reaction had started, so the log $[H_2O_2]$-t-curve became linear showing the constant inclination which may be regarded as corresponding to the "final" state.

The catalase activity in blood of poisoned animals showed approximately 40—60 % of that of control animals, however the activity was recovered as far as nearly 100% by dialysis at 0° against streaming water for 60 hours. Therefore, the log $[H_2O_2]$-t-curve became linear even if the pretreatment of a small quantity of H_2O_2

was omitted. From these facts, it might be assumed that catalase molecule reacted reversibly with the poisonous substance in blood which got through out a small hole of cellophane dialysing bag into the dialysed solution.

The reversibility of the reaction between catalase molecule and this poisonous substance was clarified by the following experiment, too; that is, with the dilution of enzyme solution which was prepared according to the predescribed method some decrease of the molar concentration of the poisonous substance was expected in the total reaction system. In fact, as shown in Fig. 3 the degree of inhibition of catalase reaction was proved to be more smaller in the poisoned animals than that of control animals.

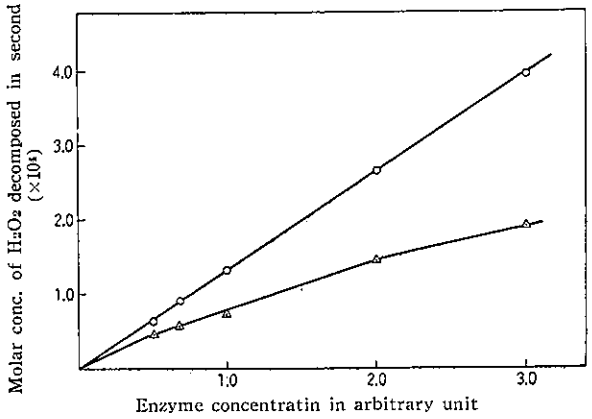

Fig. 3 Relation between enzyme concentration and reaction velocity. When 2 ml of water was added into 0.1 ml of blood, enzyme concentration was defined as unit 1.0. The reaction velocity was measured according to the catalase reaction system described in the term of method.
○·····normal rat (No. 16)
△·····abnormal rat (No. 32) injected with benzene for 3 weeks daily.
In a normal rat the catalse activity corresponds to enzyme concentration in a linear relationship, but in a rat injected with benzene it is not linear and bends as enzyme concentration increases. This bending shows that the interaction between catalase and the poisonous substance is reversible. (pH 6.8 0°)

Extraction of the poisonous substance from blood of rat injected with benzene.

To ascertain in which fraction the poisonous substance existed, blood of benzene injected animals, which had merely 30—40% of the catalase activity, was fractionated into the fraction of plasma and erythrocytes and each fraction was added to the reaction system of the normal rat as follows:

a) Addition of the plasma moiety.

b) Addition of the moiety of erythrocytes which was kept for 5 hours at 60° to inactivate catalase contained in erythrocytes.

c) Addition of the moiety of erythrocytes which was boiled in the bath for 2 min. at 100° and was filtered off the precipitation.

The inhibition of catalase reaction was not observed in either case of (a), (b), (c).

Therefore, the butanol method[16], which had been employed as an effective method in preparing many enzymes combined with lipid or lipoprotein, was adopted to extract the poisonous substance from blood, but this attempt did not succeed.

Only with the following treatment, in which acetone was employed, a satisfactory result was obtained in extracting the poisonous substance from blood. To 10 ml. of blood drawn from the poisoned rat was added 100 ml. of distilled water to destroy erythrocytes. To the mixture, 110 ml. of acetone was added at 0° and it was kept at 0° overnight. The precipitation, in which catalase was contained, was centrifuged off. Acetone contained in yellow supernatant was distilled off at 60°~80°. Addition of this supernatant, in which the poisonous substance might be contained, to the reaction system led to a marked inhibition of catalase reaction as shown in Fig. 4.

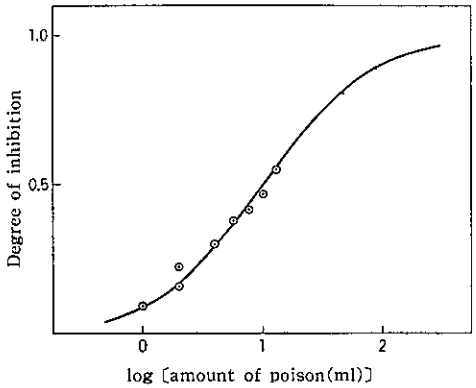

Fig. 4 Inhibitiory effect of the poisonous substance extracted with acetone from blood of rat injected with benzene.
Catalase solution was prepared from blood of a normal rat and experiments were carried out in final state. Total volume of reaction system was 20 ml: 2 ml of phosphate buffer, 1 ml of catalase solution, 14 ml of water and poisonous substance, 1 ml of H_2O_2 $\left(\frac{1}{2000}\mathrm{M}\right)$, and 2 ml of H_2O_2 $\left(\frac{1}{100}\mathrm{M}\right)$ (pH 6.8 0°)

The degree of inhibition H showed the sigmoid curve of the first order which was defined by

$$H=1-\frac{V_G}{V}=\frac{[G]}{\phi+[G]}$$

where V and V_G denoted the apparent velocity constant of H_2O_2 decomposition in the presence and absence of the supernatant contained the poison described above, respectively, and $[G]$ the concentration of the poison and ϕ a constant corresponding to the concentration of poisonous substance which would cause 50% inhibition. In Fig. 4, $[G]$ or ϕ was shown in ml., because we could not as yet determined

the property of the poison. Moreover, treating with acetone, the poison was verified both in plasma and in erythrocytes. After adding three times of ethylether to the acetone free supernatant and vibrating mechanically for 10 hours, the poisonous substance was removed completely into etheral layer. A small quantity of water was added to etheral layer and after ether was distilled off, the extremely concentrated poisonous substance was remained in water.

The effects of some phenols upon crystalline catalase of equine liver.

If phenols, such as phenol, catechol or hydroquinone, which were verified by many workers[2,3] as metabolites of benzene, exist in blood as free form from the conjugation with glucuronic or sulfuric acid, it may be presumed that the catalase action is inhibited with phenols. Using crystalline catalase extracted from equine liver according to the method of Shirakawa[17], the inhibitory effects of phenols upon catalase action were examined in the following reaction system: 13 ml. of H_2O, 2 ml. of pH 6.8 phosphate buffer $\left(\frac{1}{100}\text{-M in final concentration}\right)$, 2 ml. of catalase solution (about 10^{-9} M), 1 ml. of H_2O_2 for pretreatment $\left(\frac{1}{2000}\text{M}\right)$, and 2 ml. of H_2O_2 $\left(\frac{1}{100}\text{M}\right)$ in 20 ml. of total volume. The decomposition of H_2O_2 at 0° was followed by titration at different intervals (15, 30 and 45 seconds). The ϕ-value corresponding to the concentration of poisonous substance caused 50% inhibition was $10^{-5.0}$ M for catechol, $10^{-4.6}$ for hydroqinone, $10^{-4.2}$ for resorcine and $10^{-3.0}$ M for phenol as shown in Fig. 5, that is, the inhibitory effect of these phenols was in

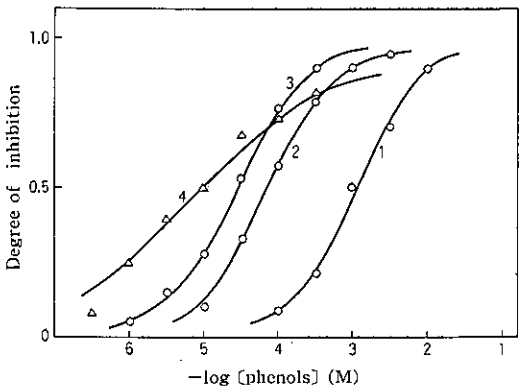

Fig. 5 Inhibitory effects of phenols upon crystalline catalase of equine liver.
Experiment was carried out in final state at pH 6.8 and 0°
Curve 1: for phenol
2: for resorcine
3: for hydroquinone
4: for catechol

the order

catechol > hydroquinone > resorcine > phenol

As shown in Fig. 5, only catechol showed a characteristic curve comparing with phenol or hydroqinone and the order n which was defined by the equation

$$H=1-\frac{V_G}{V}=\frac{[G]^n}{\phi^n+[G]^n}$$

was $n=\frac{1}{2}$ for catechol

but $n=1$ for phenol, resorcine and hydroquinone.

Discussion

This report forms a part of the investigation on experimental benzene poisoning in rat, and takes up a change of blood ingredients, which is regarded as important subjects for the understanding of the nature of benzene poisoning. In spite of no notable change could be observed in the erythrocytes number and the concentration of hemoglobin in the poisoned animals, the level of catalase activity in blood dropped till 30−50% of that of control animals.

This result may afford a better understanding of a fact that the catalase activity in blood reflect sensitively the severity of benzene poisoning and submit a sure method to diagnose benzene poisoning in an initial stage. The authors succeeded in extraction of the poisonous substance from blood in rat suffering benzene poisoning, but the nature of poison is still obscure.

In 1945, Baernstein[19] reported that after the injection of 2 ml. of benzene daily into rabbit, phenols such as phenol, catechol or hydroquinone were excreated into urine as conjugated form with glucuronic or sulfuric acid and the maximum level was attained at the ninth day. It may be possible to assume that if free phenols, namely in an unconjugated form, appear in blood, it will cause the decrease of catalase activity. On this assumption, the inhibitory effect of phenols upon equine liver catalase was examined as described above. When the catalase activity of rat injected with benzene show 50%, the amount of poisonous substance contained in 1 ml. of blood must be calculated 37.6 mg as phenol exclusive of other phenols, because the ϕ-value is $10^{-3.0}$ M for phenol, and 1.21 mg as hydroquinone and 0.44 mg as catechol, respectively. However, the existence of so much phenols in free form in living body is not conceivable.

Moreover, adding 20 ml. of poisonous substance extracted with acetone to the reaction system (total volume 40 ml.) and if the catalase reaction is inhibited 50%, the water solution concentrated into 1 ml. by the treatment of ether has to contain 1.8 mg as phenol, 0.6 mg as hydroquinone and 0.22 mg as catechol. If so much amount of phenols exist in the solution, the search of phenols may be easy by the method of diazo reaction or absorption spectrum in ultra-violet region or paper chromatography, but phenols could not be proved in any method.

On the other hand, it was defined enzymatically that the poisonous substance was not phenols. Acting crude peroxidase extracted from garden radish by Morita

and Kondos'[18]method upon the poisonous substance in the presence of 10^{-2} M of H_2O_2 for 30 minutes at 20° and the solution was boiled for 2 minutes at 100°. After centrifuging off the precipitations, the supernatant solution was added to the catalase reaction system. If phenols exist in the supernatant, it will be oxidized to the other by peroxidase. Therefore, the inhibitory effect of the poison upon catalase reaction will be changed by the treatment of peroxidase. But their effect was the same.

In consideration of these facts, it will be regarded as appropriate that the poisonous substance is not phenols. Finally, we must call the attention to the point that all measurements of catalase activity being shown in Fig. 1 are carried out at 0° in the reaction system (40 ml. of total volume) in which 0.1 ml. of blood is contained. In living body, the catalase reaction in blood is carried out at 37° and the concentration of the poisonous substance in blood is 400 times of that in the above reaction system. In general the ϕ-value increases as temperature increases. Ogura et al showed that in phenols the ϕ-value at 37° was greater about $10^{0,5}$ M than that at 0°. Supposing that the effect of temperature on the combination of catalase and poisonous substance is similar to that of catalase and phenols, and catalase activity in above reaction system drops to 50%, the catalase action in blood of living body will be inhibited entirely. The catalase activity in living body calculated on the assumption described above is shown in Fig. 6.

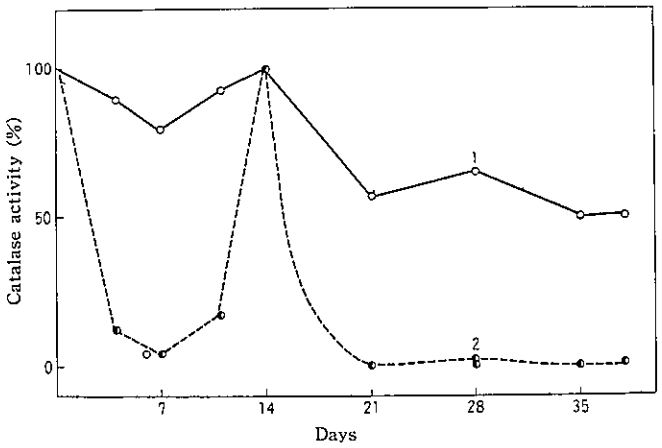

Fig. 6 Catalase activity in blood of living body.
Curve 1 was reprinted from Fig. 1.
Curve 2 shows the catalase activity in blood of living body which is calculated from Curve 1. The bases of the calculation are as follows.
1) The inhibition curve of the poison upon catalase action was the sigmoid of the first order. 2) The combination of catalase and the poison was reversible. 3) In Curve 1, 0.1 ml. of blood is contained in 40 ml. of reaction mixture. Therfore the concentration of poison in blood of living body must be 400 times of that in the above reaction mixture.

SUMMARY

1) Injecting 1 or 2ml. of benzene per 1kg of body weight daily for 4-5 weeks, catalse activity in rat blood lowered considerably, but the actvity in normal rat remained at normal level, 100±5%.
2) The decrease of catalase activity ascribed to the poisonous substance appeared in blood and the total quantity of catalase in blood was equal to that of a normal rat.
3) This poisonous subtance was not extracted with boiling or butanol, but with acetone. This poison was concentrated into a small quantity of water by the treatment of ether.
4) The inhibitory effect of phenols upon catalase prepared from equine liver was examined, and the order of inhibitory effect was as follows

   catechol>hydroquinone>resorcine>phenol

5) In consideration of many facts it was defined that the poisonous substance did not belong to phenols.

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要　旨

ベンゼン中毒の実験的研究

(2)　血液中のカタラーゼ活性の変化について

長谷川弘道　佐藤　光男

カタラーゼは血液及肝臓中に多量に存在する酵素であるが，此の活性がベンゼンの注射によ

つてどの様に変化するかを調べてみた。第一図に活性の変化と注射日数の関係を示した。ベンゼンをラッテの体重 1 kg 当り 1 ml の割合で毎日注射すると活性は一週間目には早くも 60% まで減少するが，10 日目には 100% まで恢復し，二週間目から再び活性の低下がみられる。体重 1 kg 当り 2 ml を注射した時には活性の減少は更に顕著である。但し正常ラッテには個体差は殆んど認められず其の活性値は 100±5% であつた。

この様なカタラーゼ活性の低下という現象はベンゼンの注射によつて血液中に出現して来る或る種の毒物によつて惹き起される。すなわち反応溶液中にあつて最初

$$E+G \rightleftarrows EG \qquad (1)$$

E: カタラーゼ，G: 毒物

の平衡が成立している時，H_2O_2 が導入されると，カタラーゼと H_2O_2 は非常にすみやかに反応してカタラーゼと H_2O_2 の複合物が生ずる為

$$ES+G \rightleftarrows ESG \qquad (2)$$

S : H_2O_2，ES: カタラーゼ-H_2O_2 複合物

が (1) の反応にかわつておこる。一般に ESG の解離恒数は EG の解離恒数の約 $\frac{1}{100}\sim\frac{1}{1000}$ 程度の大きさしか持たない。(1) の平衡が成立している反応系に H_2O_2 を加えてカタラーゼ反応を出発せしめると，反応時間の経過と共に (1) に代つて (2) の平衡が成立して来る為反応曲線は時間と共に折れ曲つて来ることが予想される。これを実証したのが第二図である。

尚第一図は (2) の平衡系を極く少量の H_2O_2 で予め処理することによつて成立せしめた状態に於て行われた。

この毒物の存在を更に確定的にしたのが第 4 図である。ここではベンゼンを注射してカタラーゼ活性の低下した血液に 10 倍量の水を加えて，血球を破壊，ついで等量のアセトンを加えて一夜冷蔵庫中に放置するとカタラーゼ反応を阻害する物質が上澄中に出て来ることを示した。この上澄からアセトンを除去したものを正常ラッテの血液に加えてカタラーゼ反応の阻害度が毒物の量とどの様な関係にあるかをまとめたのが第 4 図である。

尚この毒物は血漿中にも又赤血球中にも存在することが確められた。

私共は最初はこの毒物が或はフェノール類ではないかと考えたので馬の肝臓から抽出した結晶カタラーゼに対するフェノール，カテコール，ハイドロキノン（之れ等はいづれも体内に入つたベンゾールが代謝されて生ずるものである）等の阻害効果をしらべた。其の結果が第 5 図である。其の阻害効果は

カテコール＞ハイドロキノン＞レゾルシン＞フェノール

の順序であつた。

この結果から考えて血液中のカタラーゼ活性が 50% である時の血液 1 ml 中には，若しこの毒物が

フェノールならば	37.6 mg
ハイドロキノンならば	1.21 mg
カテコールならば	0.44 mg

含まれていることになり，これだけ多くの量のフェノール類が生体内で遊離の形で存在するとは考えられないし，又吾々はフェノール類を検出することは出来なかつた。従つてこの毒物はベンゾールの代謝産物そのものでなく何か他のものであると考えられる。

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STUDIES ON THE PATHOGENESIS OF SILICOSIS

EFFECT OF SILICA DUSTS ON THE PHAGOCYTIC CELLS IN VITRO

Kimiko KOSHI, Kiyoyuki KAWAI and Hiroyuki SAKABE

In the previous paper it was found that the endogenous respiration of the liver homogenate of rat and succinoxidase activity of liver homogenate or mitochondria was not inhibited by any kind of silicic acid such as quartz solution containing monosilicic acid and colloidal silicic acid of various proportion released from quartz particles,[1] amorphous colloidal silicic acid[2] and quartz powder[1),2)] specificically.

It is still obscure what property of quartz plays an important role in the process of silicotic nodule formation, but, dust laden histiocytes are seen frequently in histogical sections of the lung or peritoneum exposed to dust.

Therefore, in the present paper we have studied whether the injury of cell was caused by phagocytosis of dust. Marks[3),4)] examined already the influence of various silica particles on the intraperitoneal monocyte of guinea pigs in vitro and demonstrated the toxicity of silica particles and silicic acid[5)] on it.

It is well known that nodules are found on the omentum when the free silica particles are introduced into the peritoneal cavity of rats or guinea pigs. But, the type of tissue reaction against silica particles is different according to the species of the experimental animal.[6)] Namely, the nodule in rats is composed of collagen fiber, but in guinea pigs fibers are seen only in the peripheral zone of the nodule in the very early stage.

We have studied the toxicity of silica particles on the intraperitoneal monocytes of rats by endogenous respiration, counting of cell number and morphological change.

Experimental Method

Male rats of Wister strain, weighing 150 to 350 gr. were used as experimental animals.

CELLS:

Exudates were induced in rats by intraperitoneal injection of 4 ml. of sterile Tyrode's solution containing 0.01 per cent glycogen. After injection the exudate cells were collected by glass capillaly stricked into peritoneal cavity and differential cell counts were made daily. The counting results were shown in table 1. In this study the exudates obtained 2 days after injection were used, because the proportion of monocytes was usually over 70 per cent and the cells were fresh.

The exudates were washed out with sterile Tyrode's solution containing 12 units of heparin per ml., and the exudates from several animals were pooled for an

experiment. The cells were freed from this heparinized solution by centrifugation at 1,000 r. p. m. for 5 minuites and washed twice with sterile Tyrode's solution and then were suspended in the culture medium. The cells were maintained in the cold (0-4 °C.) after removal from the animals.

Table 1. Number of intraperitoneal monocyte after injection on Tyrode's solution containing 0.01 per cent glycogen.

Days after injection	Mean number of monocyte $\bar{x}$	Root square of unbiased variance u
0	79.1	1.04
0.25	44.8	6.23
1	67.1	3.07
2	71.5	2.27
3	73.1	3.11
4	76.1	2.50
5	76.2	11.79

Cell component of 2 days after injection

	$\bar{x}$	u	u/x
Monocyte	71.5	2.27	0.032
Polymorph Neucleus	20.3	2.41	0.118
Mast cell	2.4	0.48	0.200
Neucleus Rest	5.9	0.72	0.122

CULTURE CHAMBERS:

As culture chambers Warburg flasks were used for measuring oxygen uptake and for counting cell number, and the square glass tube with 10 ml. of capacity were used for examination of morphological change.

MEDIUM:

Culture medium was prepared aseptically from Tyrode's solution of 70 per cent and rat serum of 30 per cent. Streptomycin and penicillin were added to the medium at final concentration of 50 μg. and 50 units per ml. respectively.

DUSTS:

Quartz; Glass sand having 99.8 per cent purity, and showing the quartz pattern by X-ray diffraction was used. The quartz dust was prepared in the size range from 0.5 to 1.5 μ by repeated sedimentation and centrifugation in distilled water. Particle size was measured by electron microscopie.

Tridymite; Quartz powder was mixed with same amounts of sodium tungstate and the mixture was calcined for 2 hrs. at 1200°C. After calcination, sodium tungstate was removed by Soxlet apparatus. The formation of tridymite was confirmed by X-ray diffraction. Tridymite dust was prepared under the size of 3 μ.

Titanium dioxide; TiO_2 was purchased from Takeda Co. in Japan. TiO_2 particles of 0.5 to 1.5 μ size were prepared by the above mentioned method.

Each dust placed in the Elenmyer flasks with 5 ml. of capacity was sterilized in the dry oven at 160°C for one hour and then sterile Tyrode's solution was added. Aggregates of dust particles in this suspension were dispersed by sterilized magnetic stirrer.

CULTURE PROCEDURE AND ESTIMATION OF CELL ACTIVITY:

Measurement of oxygen consumption:

Cultures of 5 million cells in one ml. of medium were made in Warburg flasks covered with rubber cap and incubated for 24, 48 and 72 hrs. in the incubator at 37°C. Culture medium was not renewed through an experimental period. After incubation, the centre wells were charged with 0.1 ml. of 20 per cent KOH and then flasks were fitted to manometers. Oxgen uptake at 37°C. was recorded for 2 hrs., and in the several cases for 6 or 10 hrs. The flasks used in assay were 5.85 to 6.0 ml. in capacity. Vessel constants were about 0.4 in the case of KO_2.

Counting of cell number:

The culture procedure was the same as the above mentioned. In order to free the cells adhered to the surface of glass, the cultures were mixed by pipetting. The cells in the suspension were counted in a hemocytometer.

Morphological examination:

A small pieces of cover glass was placed in the square tube and then one ml. of medium containing 5 million cells introduced into the tube. After incubation at 37°C for 24, 48 and 72 hrs., cover glass on which cells adhered was taken out from each culture tube. And then the cells on the glass plate were fixed with methanol and stained with Giemsa solution.

EXPERIMENTAL RESULTS

The toxicities of various silica and TiO_2 were estimated by measurement of oxgen uptake, counting of cell number and observation of morphological change.

Change of cell number:

The cell number of each culture tube to which each dust of 50, 200, 1,000 and 3,000 γ was added was estimated after incubation of 24, 48 and 72 hrs. The data were summerized in fig. 1.

The cell number in cultures added each dust was compared with 95 per cent confidence limit of cell number in control cultures. As seen in fig. 1, the cell number of culture incubated with 1 mg. of tridymite or quartz for 24, 48 and 72 hrs. were outside the extent of 95 per cent confidence limit of control cultures. However, the cell numbers of cultures which incubated with 50 and 200 γ of quartz, tridymite or TiO_2 for 24, 48 and 72 hrs. were inside the extent of 95 per cent confidence limit of control cultures.

1 mg. and 3 mg. of TiO_2 added into the cultures had no effect on the cell numbers of cultures.

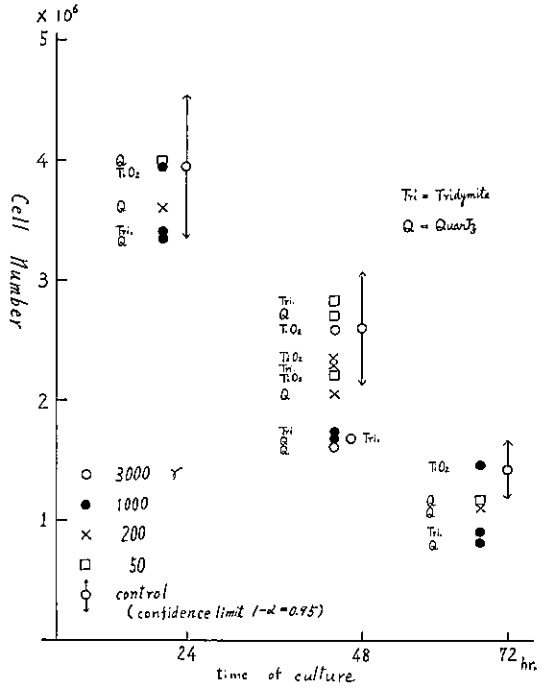

Fig. 1. Mean Cell Number after Phagocytosis of Various Dust.

In the cultures. incubated with 50 γ of tridymite, quartz or TiO_2 for 48 hrs., about 75 per cent of the cells ingested each dust respectively. The half of these dust laden cells phagocytosed less than ten particles and 3 per cent of cells had been satiated with dust particles.

In the cultures added 200 γ of test dust, dust laden cells were found in about 85 per cent and 9 per cent of these cells had been satiated with dust.

By addition of 1 mg of each dust, about 90 per cent of the cells phagocytosed the dust and 30 per cent of these cells were satiated with dust.

In the group added 3 mg of dust, about 95 per cent of the cells phagocytosed

Tabl. 2 Number of phagocytic cells after culture for 48 hr.

Number of phagocytic dust	50 γ			200 γ			1 mg			3 mg		
	Quartz	Tridy-mite	TiO_2	Quartz	Tridy-mite	TiO_2	Quartz	Tridy-mite	TiO_2	Quartz	Tridy-mite	TiO_2
>100	0	1	3	5	5	9	28	26	26	69	—	62
<100	17	17	20	29	23	27	34	37	35	11	—	19
<10	58	57	40	51	52	40	21	29	29	13	—	11
0	25	25	27	15	20	24	7	8	10	7	—	8
Per cent of phagocytic cells	75	75	73	85	80	76	93	92	90	93	—	92

the dust particles and about 70 per cent of these dust laden cells was full up with dust.

The number of phagocytosed cells were shown in Tab. 2.

Photomicrograph of the cells phagocytosed each dust were shown in Figs. 2, 3 and 4.

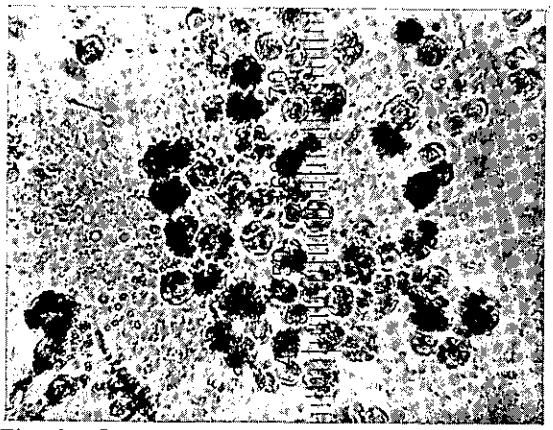

Fig. 2. Intraperitoneal monocyte after cultivation with 1 mg. of quartz for 48 hrs.

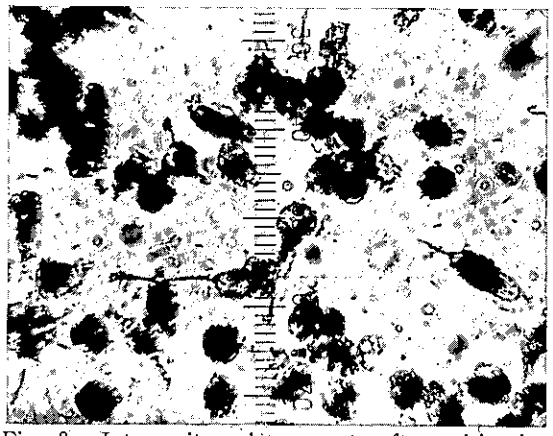

Fig. 3. Intraperitoneal monocyte after cultivation with 1 mg. of tridymine for 48 hrs.

The abnormal morpholgical changes of dust laden cells were not observed.

Endogenous respiration of cultured cells:

The endogenous respiration of the cultures incubated with 50 γ and 200 γ of each dust for 24, 48 and 72 hrs. were compared with that of control group respectively.

No significant differences were found between amounts of oxygen uptake of dust added group and control group.

1 mg of tridymite or quartz had inhibitory effect on the endogenous respiration

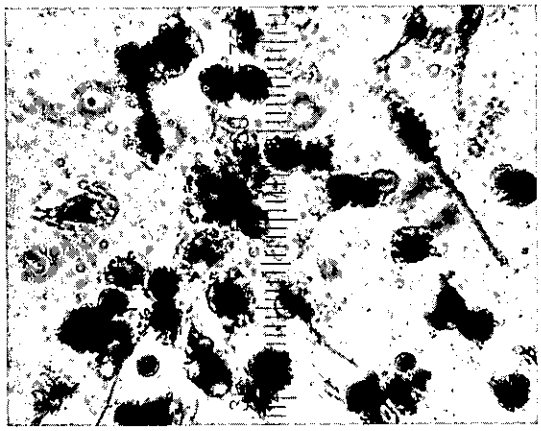

Fig. 4. Intraperitoneal monocyte after cultivation with 1 mg. of TiO_2 for 48 hrs.

of culture which had been incubated with these dusts, but 1 mg of TiO_2 had no effect.

In the cultures which were added 3 mg of tridymite, quartz and TiO_2, the former two inhibited the respiration, and the latter did not.

These were shown in Figs. 5, 6 and 7.

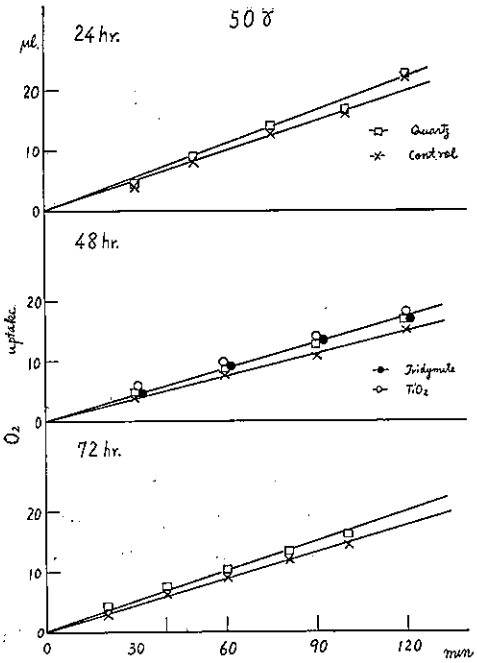

Fig. 5. Time-rate Curves of Endogenous Respiration of Intraperitoneal Monocyte.

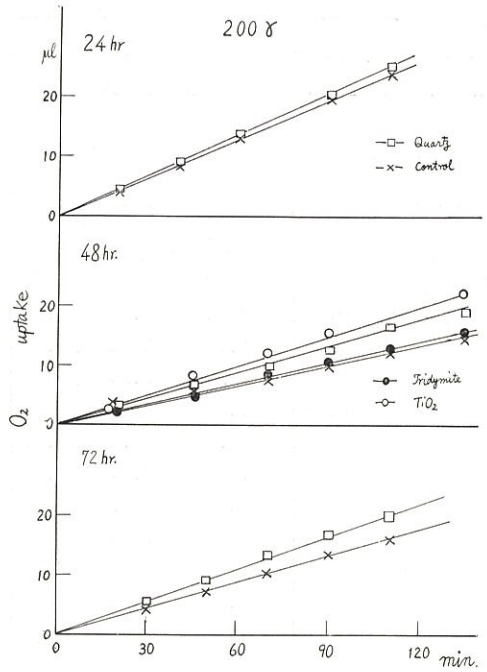

Fig. 6. Time-rate Curves of Endogenous Respiration of Intraperitoneal Monocyte.

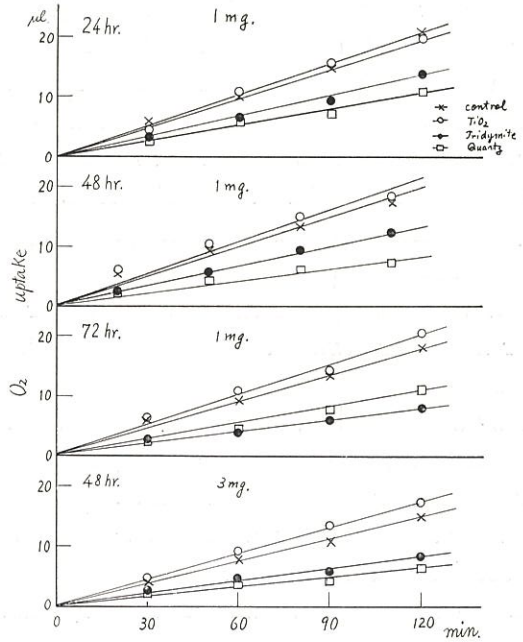

Fig. 7 Time-rate Curves of Endogenous Respiration of Intraperitoneal Monocyte.

From these results, oxygen uptake per cell per hr. were calculated and shown in Table 3. As seen in table 3, drop of oxygen uptake in cultures treated with silica dust was not caused by decrease of cell number.

Table. 3 Amounts of oxygen uptake per cell per hr.

culture hr.	24 hrs.			48 hrs.			72 hrs.		
	dust added group	control	difference	dust added group	control	difference	dust added group	control	difference
50 γ Quartz	3.80	3.10	+0.70	4.20	3.29	+0.91	13.35	10.91	+2.44
〃 Tridymite	—	—	—	3.76	3.34	+0.42	—	—	—
〃 TiO_2	—	—	—	4.21	3.29	+0.92	—	—	—
200 γ Quartz	5.68	4.76	+0.92	5.36	4.74	+0.62	8.78	7.91	+0.87
〃 Tridymite	—	—	—	4.70	4.74	−0.04	—	—	—
〃 TiO_2	—	—	—	5.40	4.74	+0.66	—	—	—
1 mg Quartz	3.07	4.03	−0.96	3.86	6.27	−2.41	4.43	6.55	−2.12
〃 Tridymite	2.89	4.03	−1.14	4.99	6.90	−1.91	7.70	6.55	+1.15
〃 TiO_2	4.15	4.03	+0.12	7.00	6.90	+0.10	7.80	6.55	+1.25
3 mg Quartz	—	—	—	3.91	4.15	−0.25	—	—	—
〃 Tridymite	—	—	—	3.60	4.15	−0.50	—	—	—
〃 TiO_2	—	—	—	4.44	4.25	−0.19	—	—	—

Discussion

1) In the above experiment the most important problem was that 50 γ or 200 γ of quartz and tridymite had no effect on the cultured intraperitoneal monocyte of rat. Namely, in spite of the dust laden cells were clearly observed in the cultures, these cell activities were not different from that of control group so far as cell activity mesured by endogenous respiration and number of cells.

Recently Marks[7] demonstrated that the endogenous respiration of cultures incubated with 1.25 μg. of tridymite per 10^6 monocytes of guinea pigs dropped to the one-half of that of control culures.

Tridymite showed a significant difference of between our results and his. As this reason, the difference of preparation method of tridymite and of the species of animal might be assumed.

Futhermore, Marks[8] showed that there are differences between the toxic dosis of Madagascar quartz and that of Belgian quartz, and he supposed that this phenomena were caused by impurity of Belgian quartz. However, it is assumed also that the difference of toxicity of quartz may be produced by some unknown physico-chemical nature of quartz other than impurity.

On the differences of toxicity due to animal species, rats showed a stronger fibrogenic potency to quartz than guinea pigs, as shown in previous papers. It was observed that oxygen uptake of the phagocytic cells slightly increased except

the cases of adding silica over 1 mg. Sbarra et al.[9] and Mudd et al.[10] showed that increased oxgen uptake during phagocytic activity has been demonstrated with innert particles.

2) By addition of 1 mg. of tridymite or quartz to the cultures, the endogenous respiration of these cultures showed one -half of that of control culture.

1 mg. of dust per 5 million cell corresponds to about 500 particles per one cell if quartz particle has mean size of 1 μ. In the proceess of formation or silicotic nodule it is not clear whether the cells were exposed in such high concentration, but it seems unreasonable to suppose such a high deposition.

3) The endogenous respirations of the cultures incubated with 1 mg. and 3 mg. of TiO_2 were not disturbed but rather slightly increased as compared with that of control culture, in spite of satiated cells were found in about 30 and 70 per cent of phagocytosed cells respectively. The over leaded dust did not depress the respiration of the cells despite that the large amount of dust phagocytosed in the cells was assummed to depress the cell function mechanically.

Summary

Intraperitoneal monocytes of rats were incubated with quartz, tridymite and titanium dioxide in the medium of Tyrode's solution containing rat serum in vitro for 24, 48 and 72 hrs. at 37°C. And the cell activity was estimated by endogenous respiration, enumaration of cell number and morphological change.

The cell activities cultured with 50 γ or 200 γ of tridymite, quartz and titanium dioxide showed no significant differences as compared with that of control group.

In the group of the addition of 1 mg. of tridymite or quartz, endogenous respiration and cell numbers were decreased respectively, whereas in the group of titanium dioxide no inhibitory effect was seen by same dose.

The cultured cells incubated with 3 mg. of each dust showed the same result as seen in the former dosis.

Addendum

Recently, tridymite No. 5691 was kindly supplied to us from Dr. G. Nagelshmidt. The size of its tridymite was 0.5 μ to 2 μ.

200 μg. of this tridymite was added to cultures of 5 million intraperitoneal monocyte of rat. After incubation for 72 hrs., cells were almost completely damaged and oxgen uptake was remarkably depressed.

From this result, it is assumed that the significant differences between results of our experiment and that of Marks were caused by difference of the sort of tridymite.

ACKNOWLEGEMENT

The authors are indebeted to Dr. G. Nagelschmidt of the Safety in Mine Research Establishment, Sheffield for gift of tridymite no. 5691. We wish to thank Miss M. Yasukawa for skilled technical assistance and Mr. I. Naito for keeping animals.

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要　旨

珪症病因に関する研究

腹腔内単核細胞に対するシリカの影響

興　貴美子　河合　清之　坂部　弘之

珪症結節の生成過程において，シリカのどのような性質が関与するかという問題は，尚不明である。

そこで著者らはさきに，珪症結節形成に作用するシリカの形を種々想定し，石英から溶出したコロイド状及び単分子状珪酸，無定型コロイド状珪酸，石英粉末等をラッテ肝の呼吸酵素系に作用させ検討したが呼吸酵素系に対する阻害は見出す事が出来なかつた。

吸入されたシリカがどのような経路で肺内にとり入れられるかについても，種々の説があるが，少くとも珪症結節成立過程にシリカ食喰細胞は必ず見出されるものであるので，シリカは食喰細胞の中で，どのような形をとるにしろ，細胞に何らかの変化を与え，その後，線維増殖をおこすものではないかと考えた。このため著者らは，食喰細胞の組織培養により，細胞を出来るだけ長く生存させ，この培養細胞に対し石英，トリヂマイト及び TiO_2 を加え，これらの物質の細胞に対する影響を検討した。

実験方法として，ウィスター種純系雄ラッテの腹腔内に 0.01 % グリコーゲン加タイロード

氏溶液を注入し，注入48時間後，腹腔内滲出細胞を洗い出した。この滲出細胞は単核細胞を70％以上含んでいる。

この細胞をラッテ血清30％を含むタイロード氏液を培養液として，5×10^6 個の細胞を1ccの培養液に懸濁し，37°C，24，48，72時間培養した。

添加したdustの粒子の大きさは，石英 TiO_2 では0.5～1.0 μ 位で，トリヂマイトでは3 μ 以下である。

添加dust量は，1 μ 粒子の石英として計算した場合，100 γ の石英を 5×10^6 個細胞に入れると，細胞1個に対してdust約50個となるところから，出来るだけ珪症結節生成過程に実際に相当する濃度を用いたいと考え，50 γ，200 γ，1 mg. 3 mg. の量を用いた。

測定方法として，ワールブルグマノメーターによるendogenous respiration，細胞数の算定，短冊培養法による形態学的観察を行つた。

結果は，石英，トリヂマイト，酸化チタンの50 γ，200 γ 添加群では，単核細胞中にdustの食喰像ははつきり認められるが，形態学的にはdust食喰以外に核その他に変化なく，細胞数もまた，変化を示さない。又endogeneus respirationはdust添加群にむしろ促進の傾向があり，阻害の事実は見出せなかつた。

更に，1 mg. のdustを添加したところ，トリヂマイト，石英群では，細胞数の減少，endogenous respirationの低下が見られた。しかし TiO_2 添加群では対照との差が見られなかつた。

そこで更に過剰の量として3 mg. の各dustを添加したが，やはり阻害はトリヂマイト，石英にのみみられ，TiO_2 には見られなかつた。

以上の実験で，endogenous respirationの低下は，石英，トリヂマイトの1 mgを 5×10^6 個の細胞に加えた時に始めておこるが，この量は最近Marksがモルモットの腹腔内培養細胞においてトリヂマイト1.25 μg./10^6個細胞で阻害を示した実験とは，かなりかけはなれた量である。

最近Nagelschmidtによつてつくられた，トリヂマイトNo. 5691を分与され，これによつて実験を行つたところ，200 μg./5×10^6個 細胞で72時間培養したところ，殆ど完全な細胞破壊像がみとめられた。

Marksはベルギー産石英とマダカスカル産石英に毒性の相異を見出しているが，トリヂマイトにおいても，その製成方法なり，原料の相異なりにより，結晶構造又は不純物等何等かの原因で，毒性に差を示すものと考えられる。又，Marksらがモルモット腹腔内細胞を用い，著者らはラッテを用いているので，動物種の違いも一つの因子となるかも知れない。

Bull. Nat. Inst. Indust. Health., 2, 40~47, (1959)

STUDY ON THE MICRODETERMINATION OF CORTICOSTEROIDS BY USE OF BLUE TETRAZOLIUM

Hiroshi YOSHIKAWA

Several methods based upon the reducing capacity of the corticosteroids have been proposed for their quantitative analysis. Silber and Porter[1] used the reduction of phenylhydrazine sulfuric acid ; Chen et al[2], Meyer and Lindberg[3], Weichselbaum and Margraf[4] applied the blue tetrazolium ; and recently Mazarella[5] discribed the use of bathophenanthroline. In general, their methods are lacking of sensitivity and owing to relatively small concentration of corticosteroids in normal urine, large amounts of urine are needed for their methods. The Silber-Porter method has been most widely used. But, in this method, corticoids which have 17, 21-dihydroxy-20-keto group in the side chain C-17 only produced color change, and that of 21-hydroxy-20-keto group in C-17 was not determined by this method. On the other hand, it had been proven a number of Δ^4-3-ketosteroids with a hydroxy or a keto grouping in various position of the molecule reacted with blue tetrazolium reagent, and Chen et al[2] and Mader and Buck[6] found extensive application of the reagent for the quantitative estimation of corticosteroids. Mader and Buck studied with 17 kinds of steroids, and found the following five cortical hormones could develop color: Cortisone, cortisol, desoxycorticosterone, corticosterone, and dehydrocorticosterone. The remaining compounds did not reduce the reagent.

As all known biologically active corticosteroids give the color development by use of blue tetrazolium reagent, the author studied the estimation of corticosteroids by use of blue tetrazolium based upon the procedures of Weichselbaum et al[4] and Mader et al.[6]

Experiments

1) Specificity

The absorption curves for the formazan obtained form blue tetrazolium are given in Fig. 1. The specificity of this method depends upon the reaction of blue tetrazolium with the 21-hydroxy-20-keto configuration to produce compounds, whose absorption is in maximum at a wave-length of 520 mμ. Steroids which bear the 17-keto and 21-methyl-20-keto function (e. g., 17-hydroxyprogesterone and dehydro-epi-androsterone) reacted with the reagent, but absorption was very little. Therefore, this colorimetric procedure can be used for the estimation of corticosteroids. However, it has a weak point to indicate more or less high value for blank test.

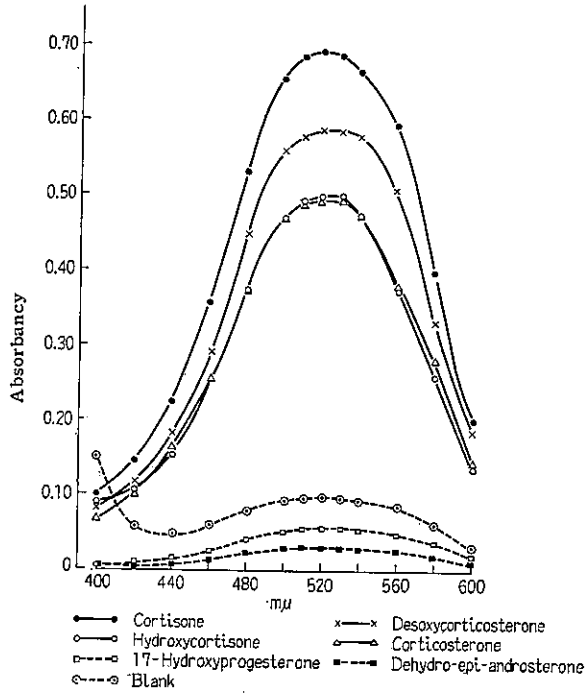

Fig. 1. Absorption Curves

Although a wave length of maximum absorption was recognized at 520 mμ, there was no great difference in the absorbancy from 510 to 530 mμ. In the previous reports, the wave length of 510 mμ was used measurement. This absorption maximum may be influenced with the purity of blue tetrazolium used. Therefore, Weichselbaum et al[4] refined the commercial blue tetrazolium before treatment.

2) Rates of color formation

The rate of formazan formation for various corticosteroids is illustrated in Fig.

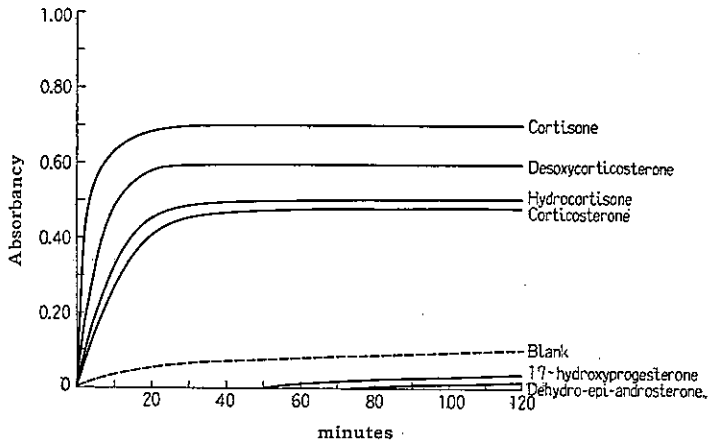

Fig. 2. Rate of Color Formation by Times

2. All of the corticosteroids reached their maximum color in 30 minutes at 37.5°C, and remain stable for at least 2 hours. On the contrary, 17-hydroxyprogesterone and dehydro-epi-androsterone scarcely reduced the reagent. However, in the blank test the color developed progressively with the prolongation of time.

There are a gradual development of color reaction after the incubation in water bath, and Chen et al [2] reported that the color is stable for a few hours with the addition of methanol-acid-pyridine buffer. Nowazynski et al[7] found that the acidification by glacial acetic acid arrests the color reaction. The author examined the change of absorbancy with time after the addition of glacial acetic acid. As summarized in Table 1, the color developments are remained stable for at least 2 hours and pH of this solution was approximately 3.7.

Table 1. The Change of Absorbancy by Time after the Addition of Glacial Acetic Acid

	Blank	Cortisone	Hydrocortisone	Desoxy-corticosterone
immediately	0.055	0.685	0.450	0.585
after 1 hr.	0.055	0.685	0.450	0.585
after 2 hrs.	0.055	0.684	0.449	0.586

3) The influence of the order of addition of reagents

Studying the critical condition of color development of corticosteroids by blue tetrazolium, Weichselbaum et al[4] recognized that it has a bearing on the order of addition of blue tetrazolium and alkali, and when a blue tetrazolium is added to the steroid before the alkali, the rate of reduction is distinctly more rapid than when the order of addition is reversed.

In relation to the order of addition of blue tetrazolium and alkali, the following two procedures were adopted, one is the addition of the tetramethyl ammonium hydroxide followed by blue tetrazolium, the others is after the addition of the blue tetrazolium, the tube is incubated in a water bath, and then the tetramethyl ammonium hydroxide is added to it, and it is again incubated. The author investigated the comparison of the following two procedures: (1) To 5 ml. of ethanol containing 20 γ of cortisone, 0.1 ml. of blue tetrazolium solution and 0.2 ml. of tetramethyl ammonium hydroxide solution were added. After this sample and blank sample were incubated for 30 minutes at 37.5°C. 1.0 ml. of glacial acetic acid was added to each of them, and their measured at 520 mμ. (2) To 5 ml. of ethanol containing 20 γ of cortisone, 0.1 ml. of blue tetrazolium solution was added and the tubes were incubated for 20 minutes at 37.5°C. At the end of this incubation periods, 0.2 ml. of tetramethyl ammonium hydroxide solution was added and again the tubes were incubated for 20 minutes at 37.5°C. After 1.0 ml. of glacial acetic acid was added, the absorbancy was read at 520 mμ. Mean value of absorbancy about five samples treated with the former procedure was 0.312 ± 0.007 and about five samples with the latter procedure

was 0.317±0.008. No significant difference could be recognized between these two values. Therefore, the former procedure may be adopted because of its simplicity compared with the latter.

To determine the most suitable doses about blue tetrazolium and tetramethyl ammonium hydroxide, the absorbancy was measured with samples containing 1, 10, and 50 γ of cortisol and the data was illustrated in Table 2. Nowazynski et al[7] had

Table 2. The Effects of Doses of Reagents

Reagent dose	Sample No.	Blank	Hydrocortisone		
			1 μg	10 μg	50 μg
B. T. 0.1 ml. T. M. A. H. 0.2 ml.	4	0.084	0.006	0.106	0.538
B. T. 0.2 ml. T. M. A. H. 0.4 ml.	4	0.146	0	0.099	0.528
B. T. 0.5 ml. T. M. A. H. 0.5 ml.	4	0.310	0	0.075	0.477

B. T. : Blue tetrazolium solution.
T. M. A. H.: Tetramethyl ammonium hydroxide solution.

used the different doses of the reagents for the determination of aldosterone which is not more than and not less than 2 γ respectively. The values of absorbancy obtained from different doses of reagents are shown in Table 2. The colors obeyed Beer's law on either of those doses within those limits. However, with the increase of reagent doses, it was proven that sensitivity fell off, and the value of blank rised remarkably. From these results, it may be considered that the doses of 0.1 ml. of blue tetrazolium solution and 0.2 ml. of tetramethyl ammonium hydroxide are most suitable for the determination of corticosteroids, at least in the range from 1 γ to 50 γ.

4) Standard Curve

Standard curves of optical density with various corticosteroids are presented in Fig. 3. The colored formazane produced by the reduction of blue tetrazolium by corticosteroids are photosensitive. The curves obtained with the optical densities versus concentration (from 1 to 50 γ) of corticosteroids follows Beer's law. But it was not proven to obey Beer's law with corticosteroids which is less than 1 γ. As illustrated in Fig. 3, 17-hydroxyprogesterone and dehydro-epi-androsterone showed very little absorption and color formation by these two steroids for the determination of corticoids were too weak for interfere.

The color development between these four corticosteroids by same doses differed in some degree. This fact was recognized by Chen et al[2] and Izzo et al[8], and it is possibly because of the difference in the reduction of blue tetrazolium caused by the various radicals of Δ^4-3-ketosteroids. To apply the method for practical use,

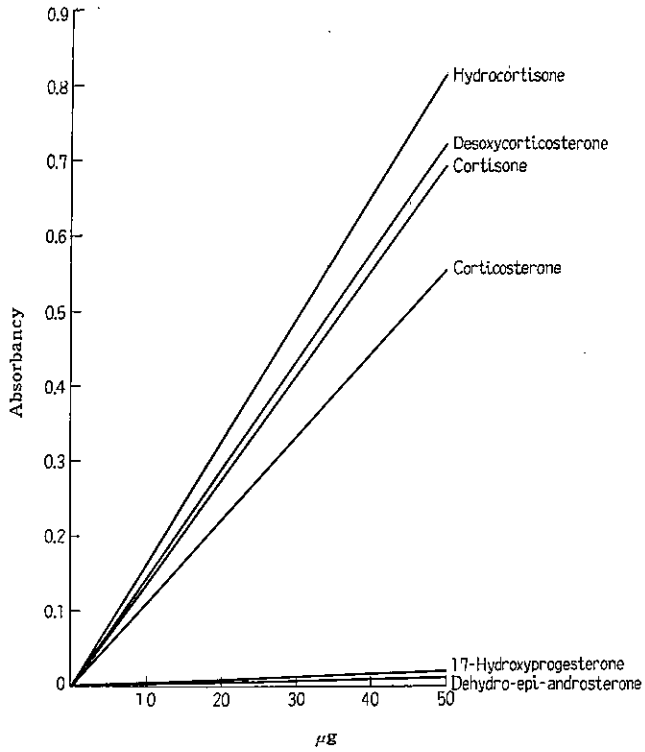

Fig. 3. Standard Curves

i. e., in case of the determination of corticosteroids in urine or plasma, it comes into question what corticosteroids should be selected as the standard. The author maintains the opinion that values should be expressed in terms of cortisone or desoxycorticosterone, which is employed as the standard. However, if a sample to be analyzed is known to contain a large proportion of a corticosteroid such as hydrocortisone, this hydrocortisone should be employed as the standard if we desire to get value which is nearer to absolute value.

PROCEDURE

I. Reagents.

(1) Ethyl alcohol: absolute, redistilled.

(2) Blue tetrazolium (3, 3′-dianiole-bis-4, 4′-(3, 5-diphenyl) tetrazolium chloride): This solution is prepared by dissolving 10 mg. of blue tetrazolium in 2 ml. of 90% ethyl alcohol.

(3) Tetramethyl ammonium hydroxide: This solution is prepared by diluting 0.5 ml. of the 10% aqueous solution of tetramethyl ammonium hydroxide with 9.5 ml. of 90% ethyl alcohol.

(4) Glacial acetic acid: absolute.

(5) Steroids investigated: Cortisone, cortisol, desoxycorticosterone, corticos-

terone, 17-hydroxyprogesterone, and dehydro-epi-androsterone was used. These standard solutions of steroids were prepared by dissolving 1.0 mg. of the steroids with 100 ml. of ethyl alcohol.

Reagents (2) and (3) should be prepared immediately before use.

II. Color development.

The sample containing desired micrograms of corticosteroids in 5 ml. of ethanol is poured into one of the two glass-stoppered test tubes. To the second tube, 5 ml. ethanol is poured. To each tubes 0.1 ml. of the blue tetrazolium solution and by 0.2 ml. of the tetramethyl ammonium hydroxide solution are added, and the resulting solutions are well mixed. The tubes are incubated for thirty minutes at 37.5°C. in a constant-temperature water bath. These solutions are acidified by the addition of 1.0 ml. of glacial acetic acid. The final solution, pink in color, are again well mixed and transferred to 4 ml. Beckman quartz cuvettes. The color intensities are read at 520 mμ in the Beckman Spectrophotometer. The acidification arrests the color reaction, which remains stable for at least 2 hours. The blank sample is treated in the same manner.

Discussion

There are various reports on the methods of estimation of adrenal cortical hormone by using blue tetrazolium as reagent. Izzo et al[8] reported the investigation of the reducing capacity of 40 steroid compounds by using this reagent. An oxygen at C-3, C-11, C-12, C-17, or C-20 had no appreciable effect on the reduction of blue tetrazolium unless an unsaturated 3-keto group was also present, and the 20, 21-diols and 17, 20, 21-tiols did not reduce blue tetrazolium even if Δ^4-3-keto group was present. The author recognized that 21-methyl-20-keto group could not develop color as well. It would indicate that the reduction of blue tetrazolium is dependent upon the primary α-keto group ($-\overset{\overset{O}{\|}}{C}-CH_2OH$) in the side chain attacked to C-17 of the Δ^4-3-ketosteroids.

Studies were undertaken to investigate the optical conditions for color development with blue tetrazolium in the presence of corticosteroids. By the above-stated procedure, a series of determination on solutions of corticosteroids in concentration varying from 1 to 50 γ, evidenced that the intensity of the color varied directly with the quantity of reducing substance present, and agreed with Beer's law.

Furthermore, the author have studied the following procedures in an attempt to improve the sensitivity of this reaction: the maximal absorbancy, the concentration of reagents, and length of time of heating.

Summary

For the simple microchemical method for the determination of corticosteroids, the method by use of blue tetrazolium as reagent was studied. And it was esta-

blished that this procedure is to be applied to the estimation of the adrenal cortical hormones. The colorimetric reactions described are remarkably evident by Δ^4-3-ketosteroids posessing the primary α-ketol grouping ($-\overset{O}{\overset{\|}{C}}-CH_2OH$) at the C-20 and C-21 positions. Steroids such as progesterone and androsterone produced very little color, it was too weak to interfere the determination of adrenal cortical hormones.

Procedures which are used for the quantitative determination of corticosteroids by the reduction of blue tetrazolium have been modified in order to increase the sensitivity of the reaction and the stability of the color produced.

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要　　旨

Blue tetrazolium による Corticosteroids の微量定量についての検討

吉　川　　博

副腎皮質ホルモンの生理学，並びに生化学の最近の急速な進歩に伴い，これらホルモンの分泌の，病態生理の立場からみた動態の把握が重要となつて来た。

このためにはクロマトグラフィーを用い，各種 corticosteroids を分別定量することが最も確実な方法であるが，労働衛生の分野で集団を対象とした研究には殆んど用い得ない。従つて簡単な定量法の確立が必要である。現在一般に広く採用されている方法は Silber-Porter 法であるが，この方法では 17, 21-dihydroxy-20-ketosteroid のみしか定量出来なく，21-hydroxy-20-ketosteroid は定量出来ない。副腎皮質からの各種ホルモンの分泌が，如何なる状態下でも同一比率で起るならば Silber-Porter 法による定量法で，副腎皮質ホルモンの分泌態度を類推しう

るが，stressor の差異によつて各種ホルモンの分泌割合に差異があるとすれば 21-hydroxy-20-ketosteroid の定量も亦必要となつて来る。

以上の点から，著者は Blue tetrazolium がすべての生物学的活性を持つた副腎皮質ホルモンの存在下で Formazan を形成し，呈色反応を呈し，亦この試案を用いた定量方法も 2，3 報告されているので，Blue tetrazolium を用いた定量方法の精度と可能性について検討した。

その結果，本文中に記載した如き定量方法を確立した。即ち，C-17 位の側鎖に $-\overset{O}{\overset{\|}{C}}-CH_2OH-$ を持つた Δ^4-3-ketosteroids は Blue tetrazolium と鋭敏に反応し，且つ 1～50 γ の corticosteroids では Beer の法則に従い，定量可能なことを確認した。併し，1 γ 以下の濃度では定量出来なく，又盲験値が多少高値を示す欠点を持つている。

上述の如く，Blue tetrazolium 法は不純物を含まない溶液では鋭敏な方法であるが，併し，本試薬は庶糖，脂質等にも反応を示す。従つて尿中，血中には此等の多くの干渉物質が含まれるので，此等が corticosteroids の定量に影響することも考えられるので，今後実際の試料について検討したい。

Bull. Nat. Inst. Indust. Health., 2, 48~56, (1959)

PREPARATION OF CARBOXYMETHYLCELLULOSE PAPER AND DIETHYLAMINOETHYLCELLULOSE PAPER

Masami KIMURA

Cellulosic materials with ion exchange functions have been prepared in sheet form by chemical treatment of filter paper. The utility of these materials has been generally shown in the resolution of ternary inorganic mixtures. Wieland et al.[1] reported that the mixture of amino acids could be separated with carboxyl-paper, and Micheel et al.[2] published the report that the succinyl-paper was sufficient to effect an adequate separation of the mixture of basic amino acids. In recent years, the ion exchange paper has been prepared by incorporation of resin with a slurry of paper fibers before the sheet is formed. This paper was found to have the similar separable effectiveness for most of amino acids as ion exchange resin.[3),4)]

On the other hand, certain derivatives of cellulose possessing ion exchange groups have been studied in regard to fractionation of proteins. Sober and Peterson have published reports on the use of carboxymethyl- and diethylaminoethyl-cellulose in the fractionation of plasma protein[5),6)] and enzyme.[7)] Since then their column chromatography has been utilized for fractionation of biological substances, for example: histone[8)], hormonal protein[9)] and nucleic acid.[10)]

The present author has prepared the carboxymethyl*- and diethylaminoethyl*-cellulose paper, and has been examining their applications for rapid and simple fractionations of biological substances. In the following pages the preparation procedure and properties of new ion exchange cellulose papers will be discribed.

Experimental and Results

1. Preparation of Carboxymethylcellulose Paper

Monochloroacetic acid and NaOH were used for carboxymethylation of cellulose paper. Sober and Peterson showed in their original paper[5)] that the powder was treated with NaOH at the begining of the reaction. Ellis and Simpson[9)] reported that the powder was suspended in monochloroacetic acid solution for 1 hour in order to obtain a CM-cellulose powder of the desired degree of substitution. The comparison of both pretreatments of cellulose paper is presented in Table 1. As the result it was shown that ion exchange groups could be introduced more efficiently in cellulose paper by pretreatment of monochloroacetic acid than NaOH.

* Abbreviated as CM- and DEAE-.

Table 1. Pretreatment in the process of carboxymethylation

Reagent of pretreatment	Period of pretreatment hr.	Carboxymethylation		Reaction period hr.	Ionizing group $\frac{\text{m mole}}{\text{gm.}}$
		Reagent I	Reagent II		
4.2 MCA	1	4.2 M MCA, 50 ml.	10 N NaOH, 150 ml.	1	0.12
,,	1	,, 50 ml.	,, 150 ml.	3	0.23
10 N NaOH	1	10 N NaOH, 100 ml.	4.2 M MCA, 30 ml.	1	0.09
,,	1	,, 100 ml.	,, 30 ml.	3	0.14

Cellulose paper: Toyo Roshi No. 51, 5 sheets, respectively.
Pretreatment and carboxymethylation: cooled with ice.

Monochloroacetic acid may be converted to sodium-salt in the reaction. Therefore, when monochloroacetic acid is used as the carboxymethylating reagent, the concentration of NaOH must be high enough to neutralize it and also to carboxymethylate the hydroxyl groups of cellulose. The NaOH with high concentration (10 N) was added to the monochloroacetic acid solution in which filter paper was immersed. The prepared CM-cellulose paper did not keep a smooth surface and did not maintain the original form. Sodium monochloroacetate was applied as the carboxymetylating reagent in place of monochloroacetic acid.

Such deformation may mainly depend upon the concentration of alkali. The carboxymethylation of filter paper, pretreated with 4.2 M sodium monochloroacetate was carried out under various alkaline conditions: 1 N, 2 N, 5 N and 10 N NaOH. The reaction mixture was allowed to cool in an ice bath for 4 hours (Table 2). The higher the concentration of alkali, the more the extent of substitution, but the prepared paper was more deformed. On that account the 4 N NaOH was selected for the reagent in this experiment.

The results of carboxymethylation of filter paper under various conditions are shown in Table 3. The reaction at 60°C for 75 minutes was the most suitable condition of carboxymethylating cellulose paper. The precedure is as follows.

Table 2. Effect of concentration of alkali in carboxymethylation

Reagent 4.2 M MCANa ml.	NaOH Conc. (N)	NaOH ml.	CM-cellulose paper			Carboxy-methylated ratio
			Width cm.	Weight. 10 g/cm	Form	
50	1	50	2.0	8.2	−	1
50	2	50	2.0	8.3	−	1
50	5	50	1.9	10.0	±	4
50	10	50	1.7	12.6	+	8

Form: Plus sign presents that the prepared paper is bent and is not kept a smooth surface.
Carboxymethylated ratio: Messuring with B. P. B. method in chromatographic method.
Pretreatment and carboxymethylation: cooled with ice.
Reaction period of carboxymethylation: 4 hours.

Table 3. Carboxymethylation of Cellulose Paper

Cellulose paper			Reaction reagent* 4.2 M MCA** or MCANa and 4 N NaOH, respectively ml.	Reaction condition		I. G.*** m mole / g.	I. E. C.*** %
Kind	Width and length cm. × cm.	Sheet		°C	min.		
No. 51	2×40	30	100**	20	120	0.26	0.44
50	3×40	26	300	30	30	0.24	0.52
51	8×18	30	250	65	30	0.56	0.74
51	2×19	20	100	60	60	0.54	0.75
51	2×40	30	150	60	75	0.68	0.79
51	3×40	13	150	60	120	0.52	0.72
50	4×40	30	250	60	120	0.45	0.68
50	4×40	30	375	60	150	0.39	0.64
50	4×40	30	375	70	120	0.44	0.72
51	2×40	5	70	80			0.72
Cellulose powder		20 g	100**	65	30	0.56	

* Monochloroacetic acid and sodium monochloroacetate abbreviated as MCA and MCANa.

** Concentration of NaOH is 10 N and its volume is three times of volume of MCA.

*** Ionizing group and ion exchange capacity are abbreviated as I. G. and I. E. C.

Thirty sheets of filter paper (Toyo Roshi No. 51: 2×40 cm.) were immersed in 150 ml. of 4.2 M sodium monochloroacetate for 3 hours at room temperature, after which 150 ml. of 4 N NaOH was added in 3 or 4 portions and mixed thoroughly after addition. The reaction mixture was allowed to stand at 60°C for 75 minutes, with careful stirring by using a magnetic stirrer. It was acidified by adding 1 N HCl to give a pH 1 to 2. After decantated the solution, the CM-cellulose paper was washed with 0.1 N HCl repeatedly, followed by water until the solution became acid-free. Then it was dried on a filter paper at room temperature.

2. Preparation of Diethylaminoethylcellulose Paper

Almost none of ionizing groups could be introduced in diethylaminoethylation according to the same procedure as employed in preparing DEAE-cellulose powder (Table 4, No. 1). By using a reagent of 2 N NaOH in place of the 6 N NaOH, which was employed by Sober et al.[5] in diethylaminoethylating cellulose powder, ionizing groups could be introduced to a certain extent, but the prepared paper was easily torn into small pieces (Table 4, No. 2). When filter paper pretreated with 2 N NaOH containing 10% NaCl was diethylaminoethylated at 80°C for several reaction periods, the ionizing groups were substituted in good extent and the original form of paper was kept well. But by prolonging the reaction period, the paper became partially light brown (Table 4, No. 3, 4, and 5). Being pretreated with 6 N NaOH, which was the most adequate concentration among 1 N, 2 N, 4 N, 6 N and 8 N NaOH, and reacting at 80°C for 90 minutes, the prepared paper had the most ionizing groups but became partially light brown. (Table 4, No. 7). Similarly at this temperature,

Table 4. Diethylaminoethylation of Cellulose Paper

Exp. No.**	Pretreatment	Reaction condition cooled 30°C	80°C min.	State		No. 51 I. G. I. E. C. m mole/g.		No. 51 I. G. I. E. C. m mole/g.	
				deformed	colored				
1	non		120	+	+	0.05			
2	non	120	95	+	−	0.30			
3	2 N NaOH, 10% NaCl		180* 90	−	−	0.48	0.43	0.49	0.43
4	,,		180* 120	−	−	0.46	0.35	0.46	0.43
5	,,		180* 300	−	+	0.13	0.27	0.41	0.40
6	6 N NaOH		55	−	−	0.44			
7	,,		180 90	−	+	0.73	0.58	0.72	0.56
8	,,		180 120	−	+		0.50		0.34
9	,,		480	−	−		0.50		0.34
10	,,	30	480	−	−	0.68	0.49		0.54
11	,,	40	480	−	−	0.69	0.50		0.40

* at room temperature.
** Exp. No. 1 Toyo Roshi No. 50, 10 sheets.
Exp. No. 2–11 Toyo Roshi No. 50, 5 sheets and No. 51, 5 sheets.

the reaction period effected the substitution (Table 4, No. 6 and 8). The DEAE-cellulose paper, which kept the original form of the paper and did not colored and moreover had enough ionizing groups, was obtained under the reaction condition of low temperature and long period (Table 4, No. 9, 10 and 11). The procedure under the most adequate condition is as follows.

Ten sheets of filter paper Toyo Roshi No. 50 and No. 51: (4×40 cm.) (5 sheets, respectively) were immersed in 6 N NaOH for 30 minutes. The container used was an oblong shaped one. After decantation of alkali, they were washed with water until the solution was neutral. The pretreated paper was dried on a filter paper. The alkaline-treated paper was immersed in solution of 12 gm. of 2-chloro-N, N'-diethylaminoethyl chloride* in 14 ml. of water, and was added with 120 ml. of cooled 2 N NaOH. The reaction mixture was allowed to stand at 4°C for 30 minutes, and then at 30°C for 8 hours, with careful stirring by using a magnetic stirrer. When the reaction was finished, 150 ml. of 2 M NaCl was added to the mixture and the solution was mixed carefully and completely. The DEAE-cellulose paper was allowed to stand in 1 N NaOH for 15 minutes, then after decantation, 1 N HCl was added to it and it was allowed to stand for 15 minutes. After discarding HCl again, the paper was immersed in 0.1 N NaOH overnight. It was washed with water until the solution became neutral, and then washed with alcohol and dried on a glass

* The solution which was obtained thionylchloride (200 gm.) and chloroform (250 ml.) was added titratedly to a solution of 2-diethylaminoethanol (100 gm.) in chloroform (100 ml.) under cooling and stirring by using a magnetic stirrer. Thereafter the stirring was continued for one hour. The reaction mixture was distilled at reduced pressure. Adding alcohol to the residue, it was distilled again at reduced pressure. The residue was recrystallized from hot alcohol.

plate at room temperature.

4. Mesurement of Introduced Ion Exchange Groups

Titration Method

After a CM-cellulose paper was dried at 105°C for 15 minutes, it was weighed. The paper was placed in a small beaker and 30 ml. or 40 ml. of 0.5 M NaCl was added to it. The solution was allowed to stand overnight. The miliequivalent of acidic groups per gm. of CM-cellulose paper was determined by titrating the ion exchanged cation with standard alkali (0.01 N NaOH) by using a glass electrode. The solution was thoroughly stirred after each addition of standard alkali until the solution approached to equilibrium. The titration curve of both CM-cellulose paper and powder are presented in Fig. 1.

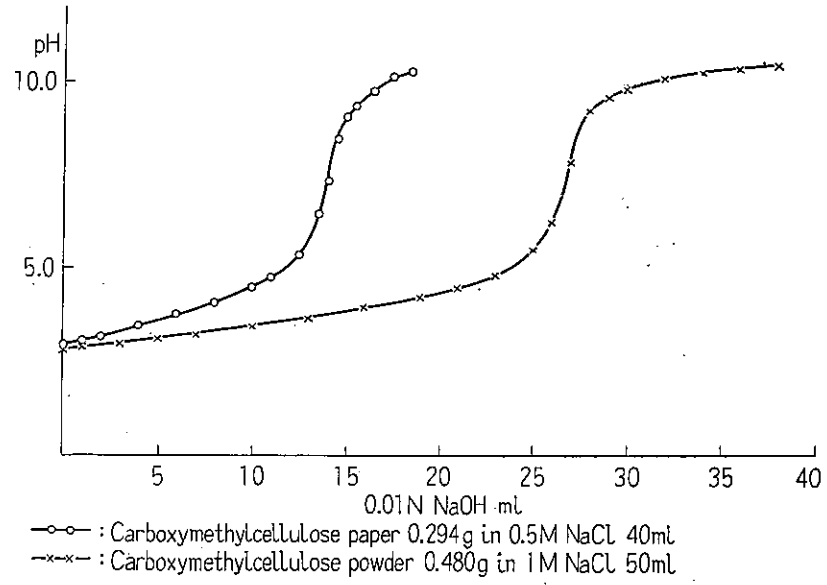

Fig. 1. Titration Curve of Corboxymethyl Cellulose Paper and Carboxymethyl Cellulose Powder

The air-dried DEAE-cellulose paper was weighed. The paper was treated with 30 ml. of 0.5 M NaCl for 30 minutes. The miliequivalent of basic groups per gm. of DEAE-cellulose paper was determined by titrating the ion exchanged anion with standard acid (0.01 N HCl) at the similar manner of CM-cellulose paper. Fig. 2 shows the titration curve of the DEAE-cellulose paper.

Chromatographic Method

The chromatographic method which could estimate simply and rapidly the ion exchange capacity was invented by the author.

As a chromatographic developer ammonium acetate and or buffer containing sodium chloride was used for CM-cellulose paper and used sodium glutamate for DEAE-cellulose paper. A paper for testing was placed side by side with paper which

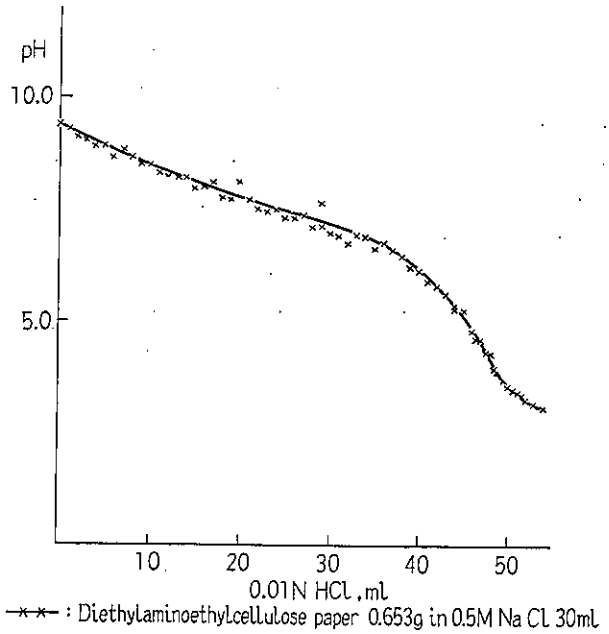

Fig. 2. Titration Curve of Diethylaminoethyl Cellulose Paper

posessed the known capacity with titrating method and was chromatographed ascendingly. After the papers were developed to the equal level, they were dried. The color was developed with an adequate method. The experiments are as followes:

CM-cellulose paper 2×18 cm.

Developing distance 15 cm.

Developing period 50–80 minutes

Developer A 1/10 M ammonium acetate

,, B 1/5 M sodium borate buffer containing 0.1 M NaCl

Developing color A Ninhydrin method:

Sprayed by 0.1% ninhydrin (gm./ml.) solution in saturated n-buthanol containing 2% glacial acetic acid (ml./ml.) anp heated at 105°C for 5 minutes. The color of ion exchanged part was violet.

,, B B. P. B. method:

Immersed in solution of bromphenol blue (0.05 gm.) and, glacial acetic acid (2 ml.) and mercury chloride (1.0 gm.) in 100 ml. of water. Ion exchanged part was stained blue.

DEAE-cellulose paper 1.0×11 cm.

Developing distance 10 cm.

Developing time 30-60 minutes
Developer 0.05 M sodium glutamate
The color was developed with Ninhydrin method.

Ratio was calculated comparing the area of total developed part with the colored part, in which the ionizing groups was ion exchanged with cation and anion. The ion exchange capacity (I. E. C.) presented is represented in Table 3 and 4 as the value which is reduced from 1.

5. Ion Exchange Ability of Carboxymethylcellulose Paper

The chromatography of basic amino acids was carried out with CM-cellulose paper. The following borate buffers were used as the developer.

A 1	1/5 M sodium borate buffer,	pH 7.1	
A 2	,,	pH 7.6	
A 3	,,	pH 7.8	
A 4	,,	pH 8.1	
A 5	,,	pH 9.0	
A 41	1/5 M sodium borate buffer (pH 8.1)	containing	0.005 M NaCl
A 42	,,		0.05 M ,,
A 43	,,		0.1 M ,,
A 44	,,		0.2 M ,,

CM-cellulose paper was equilibrated with each buffer respectively. The paper was half-dried. The sample solution (0.005 ml.) containing arginine, histidine and lysine (0.1%, respectively) was spotted on the paper and was chromatographed. The chromatogram is shown in Fig. 3.

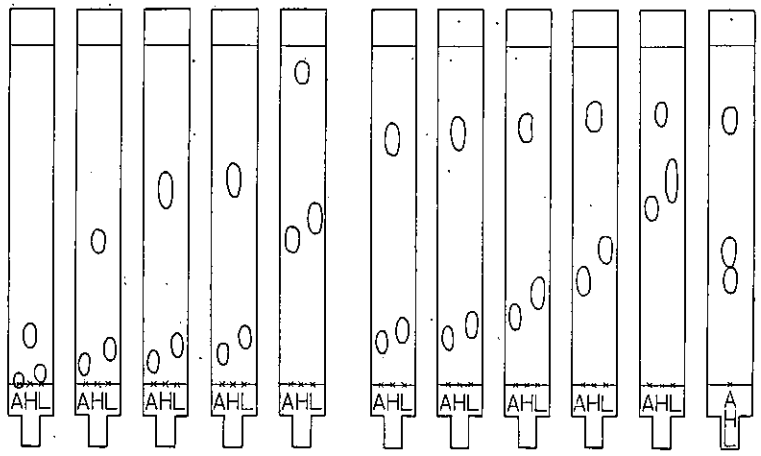

Fig. 3. Chromatogram of Basic Amino Acids with Carboxymethylcellulose Paper
A: Argine, H: Histidine, L: Lysine (each 0.005 ml of 0.1% solution)
Developing temp. 22°C
Developing Time 30~60 min.
Carboxymethylcellulose paper 2×18 cm (I. G.=0.68 mmole/g.)

Conclusion

Monochloroacetic acid has been applied for the carboxymethylation of cellulose powder. In the carboxymethylatieg cellulose paper sodium monochloroacetate was used in place of monochloroacetic acid. The carboxymethylation with NaOH above 5 N could not keep a smooth surface and could not maintain an original form of the paper. Therefore, 4 N NaOH was used as the most adequate reagen. Examining to find out the suitable condition of reaction with the object of obtaining the most degree of substitution, the reaction at 60°C for 75 minutes was proven as the most suitable one. In this condition, the ionizing groups were introduced more in the cellulose paper than in the cellulose powder. With regard to the titration curve, the ionizing group may be similar in the cellulose paper and powder. The ion exchange ability of CM-cellulose paper was proved by chromatography of basic amino acid.

The cellulose paper could not be diethylaminoethylated in the same condition as the powder. Accordingly for the diethylaminoethylation of the paper 6 N NaOH was chosen and proved to be most adequate. The alkaline-treated paper was further treated with 2-chloro-N, N′-diethylaminoethyl chloride and 2 N NaOH at 4°C for 8 hours. Thus prepared DEAE-cellulose paper was found to introduce enough ionizing groups and was not deformed nor colored. The titration curve of the paper showed a similar tendency to the curve of DEAE-cellulose powder prepared by Sober et al.

Acknowledgements

The author wishes to express his gratitude to Prof. T. Ando and Dr. S. Ishii for valuable discussion, and to Miss M. Oka for skilled technical assistance.

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要　　旨

カルボキシメチルセルローズペーパーとヂエチルアミノエチルセルローズペーパーの調製

木　村　正　己

沪紙（セルローズペーパー）を化学的に処理して性質を変え，それを用いたペーパークロマトグラフ法で物質をよりよく分離する試みがなされている。しかもこの方法は簡易かつ迅速であることが望ましい。

労働衛生の分野に於ては職業病の診断方法に普断の改良を加えてゆくことが要請される。近時ペーパークロマトグラフの進展に伴い，労働衛生の分野に於ても生体内物質の確認定量にこれが広般に使用されるに至つた。しかもこの方法は簡易迅速に実施されれば有用性が倍加される。著者はペーパークロマトグラフに使用される沪紙に化学的処理を加えることによつて物質の分離を容易にした。即ち，セルローズパーパーにカルボキシメチル基（カチオン交換性）或はヂエチルアミノエチル基(アニオン交換性)を導入することによつてこの目的を達し得ることを見出した。これらのイオン交換セルローズペーパーはその導入基のイオン交換性とセルローズペーパーの吸着性の両性により，イオン交換樹脂によるクロマトグラフと同様の効果を示し，かつ迅速に分離定量の操作が行いうる利点がある。

交換基のセルローズペーパーへの導入における反応条件を検討した結果，以下述べる方法が最良であつた。

カルボキシメチルセルローズペーパー：　沪紙（東洋沪紙 No. 51）を 4.2 M モノクロル醋酸ソーダに室温，3時間浸した。その溶液に同量の 4 N 苛性ソーダを加え，60°C にて 75 分間処理した。調製されたペーパーのイオン交換基量は 0.68 mmole/g を示し，他の反応条件によつて調製したものより大きな値であつた。

ヂエチルアミノエチルセルローズペーパー：　沪紙（東洋沪紙 No. 50, No. 51）を 6 N 苛性ソーダに 30 分間氷冷下で浸し，水洗後風乾した。この前処理に際しては沪紙の原形を変えないように注意しなければならない。かくて前処理したペーパーに 2-クロロ-N, N′-ヂエチルアミノエチル塩酸塩水溶液と 2 N 苛性ソーダを加え，4°C にて 30 分間処置してから 30°C で 8 時間処理を行つた。このペーパーは 0.68 mmole/g のイオン交換基が導入され，しかもペーパーの変形も着色もなかつた。

カルボキシメチルおよびヂエチルアミノエチルセルローズペーパーの滴定曲線からそのイオン交換性が証明できた。さらに前者を用いた塩基性アミノ酸のクロマトグラフによりそのイオン交換能が実証され，かつ迅速な生体物質の分離定量に利用できることが示された。

Bull. Nat. Inst. Indust. Health., 2, 57~62, (1959)

STUDY ON THE PREPARATION METHODS OF SUBMICRON PARTICLES

Katsunori HOMMA, Shigeji KOSHI and Hiroyuki SAKABE

From the viewpoint of industrial hygiene, preparation of aerosol composed of the optionally sized particles less than 1 micron, should be necessary to investigate their retention to repiratory truct, to test the efficiency of the dust collector against very fine particle and to study their physico-chemical properties.

Many reports on the preparating method of particulate clouds have been issued. These methods may be classified as follows.

Condensation method of volatile matter.[1~3]

Generation of aerosol by chemical interaction.[4) 5)]

Aerosol generation by combustion.[6) 7)]

Formation of aerosol by photolysis.[8) 9)]

Generation of smoke by electric arc.

Dispersion method of air blast atomization.[10) 11)]

Spinning disk atomization method.[12)]

Atomization method by ultrasonic[13) 14)] etc.

However, reports for study on the production of submicron particles are very few.[3) 15)]

We have tried to obtain the aerosol of submicron particles by electric arc method and condensation method.

Experiment and Result

a) Metal fume by electric arc method.

When high electric potential charges on the electrode, electric spark can be

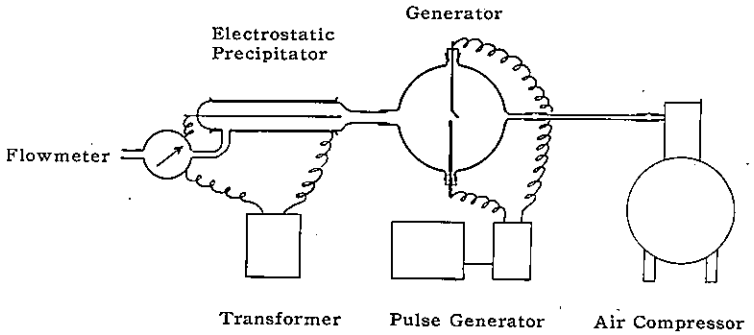

Fig. 1. Diagram of metal fume generating System

seen, and the metal of an electrode evaporates as metallic ions. Then, these metallic ions condense with one another and very fine particles can be produced.

In this experiment, we made the metal fume generator shown in Fig. 1.

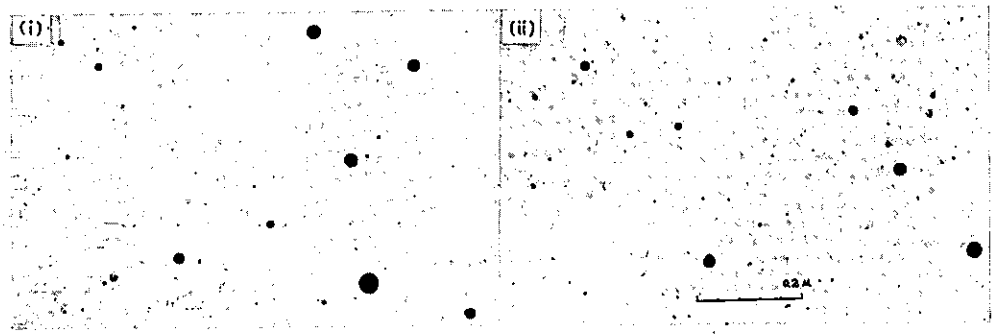

Fig. 2. Electron micrograph of metal fumes
(i) Iron oxide (ii) Lead oxide
Magnification: ×14,000

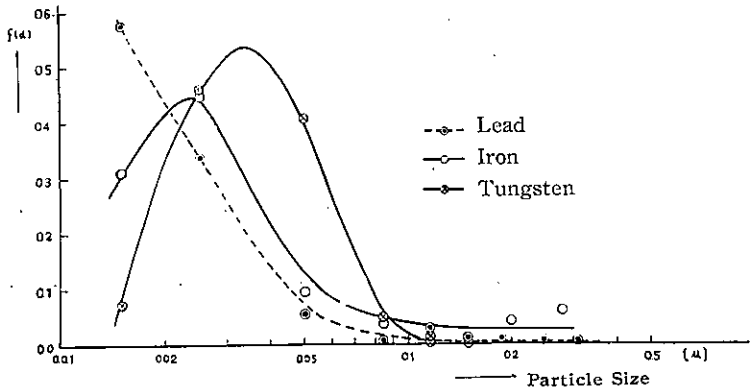

Fig. 3. Particle size distribution curve of three different kinds of metal fume

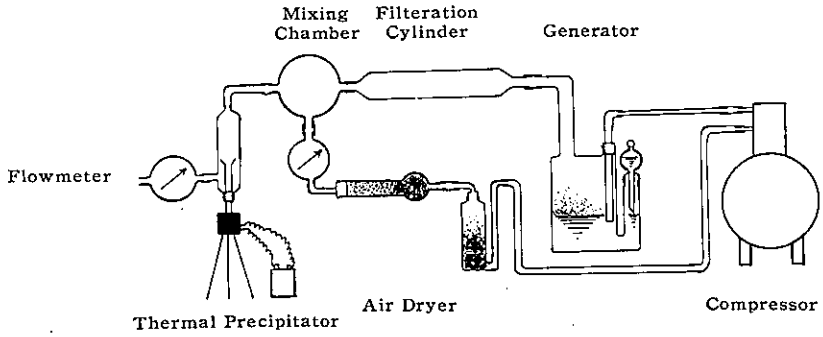

Fig. 4. Diagram of NaCl particle generating system

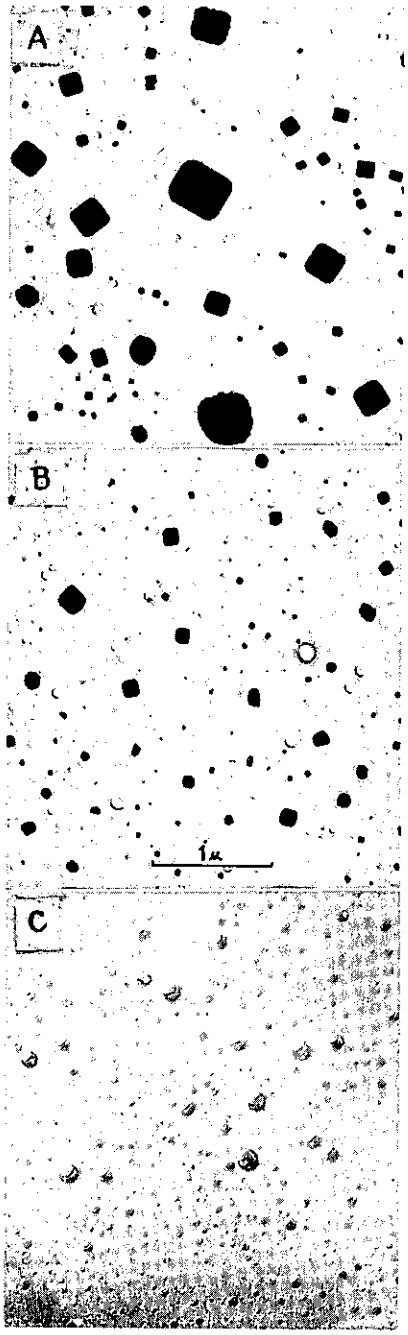

Fig. 5. Electron micrograph of NaCl particles
A: NaCl concentration 10^{-1}%
B: ,, 10^{-2}%
C: ,, 10^{-3}%
at 420 m/sec. of air velocity
Magnification: ×16,000

As metals of the electrode, iron, lead and tungsten were used in the form of wire. 9,000 volt of A, C, was discharged intermittently at the rate of 300 times per minute between a pair of electrode whose distance was 7 mm. Electric discharge was controlled with pulse generator. In this way, metallic ions of the electrode evaporated by discharge were condensed to fine fumes in the generating chamber. The fume laden air was passed through the electrostatic precipitator at the rate of 30 l/min, with air compressor. Fumes collected in the precipitator were examined electron microscopically in this method, about ten thousands patricles could be generated per one spark. Electron micrographs of the fumes of iron oxide and lead oxide are shown in Fig. 2.

As shown in Fig. 2, the particles of iron oxide and lead oxide obtained by this method showed a spherical shape, and the aggregation of particles was not seen. Particle size distribution of each metal fume is shown in Fig. 2.

The rich frequent particle size was observed to be about 0.04 μ in tungsten oxide and 0.02 μ in iron oxide, and could not be determined in lead oxide as some of these particles were too small to investigate by our electron-microscope.

b) NaCl particle formation by air blast atomization.

The apparatus of NaCl particle generating system is shown in Fig. 4. By this method, at first, NaCl mists were formed by the atomizer, which blowed air against the surface of the aqueous solution of NaCl. The generated mists were conducted into the filteration cylinder, and during passing through the cylinder set in horizontal, large droplets fell on its bottom. Length and radius of the cylinder and velocity of air passing through the cylinder were effective factors to the upper limit of particle diameter

passable through it. We selected these conditions to be not passable for the particles larger than 15 microns. Mists carried into the mixing chamber through the filteration cylinder were dried up by dried air from the other inlet. Then, completedly dried, solid particles of NaCl were collected by the thermal precipitator. Their particle sizes were examined by the electron microscope.

Then, we have studied the effects of concentration of NaCl solution and the air velocity at the nozzle of atomizer to the particle size. The experiments were carried on the following conditions.

(1) Concentration of NaCl solution: 10^{-1}, 10^{-2}, and 10^{-3}% (in weight)

(2) Blasting air velocity at the nozzle: 310 and 420 m/sec.
(Air pressure at the nozzle: 20 and 30 lb/in^2)

Experimental results are shown in Figs. 5 and 6.

As seen in Fig. 5, NaCl particles produced from the solution of the concentration of 10^{-1} and 10^{-2}% took a cubic crystal form but typical crystal form was not obtained in the particles from the solution of 10^{-3}% concentration.

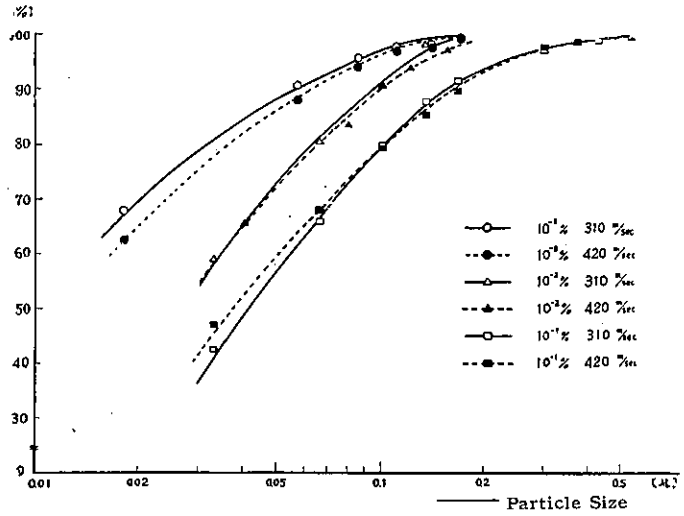

Fig. 6. Particle size distribution curve of NaCl aerosols

As seen in Fig. 6, the particle sizes were different with the concentration of NaCl solution, and the sizes decreased with the lowering of concentration. Air velocity at the nozzle of atomizer had no effect on the size of particles in these NaCl concentration of solution.

Discussion

By these method we could produce the very fine and uniformly dispersed particulate clouds of the size smaller than 0.5 μ.

In the metal fume generating method by electric arc, we obtained the metal

fumes of different sizes with the kinds of metal. We could not clarify why these differences were produced, as the mechanism of fume formation was not yet clear.

In the NaCl particle formation by air blast atomization, we could obtain the particulate clouds of various different size distribution with various NaCl concentration of solution in our experimental condition. Air velocity at the nozzle through which the air blow against the surface of the solution was not the determining factor to the size in the narrow range of our experiment. It was assumed that the particulate clouds formation from the mist is very convenient method, as we can obtain the aerosol of desirable upper limit of the size distribution. It may be said that the clouds formation of submicron particles of iron or NaCl will contribute to industrial hygiene concerning with these small sized particles, as isotopically labelled iron or NaCl will be examined simply and accurately without any serious harmful effect.

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要　　旨

Submicron 粒子の生成方法について

本間克典　輿　重治　坂部弘之

労働衛生に於て，Submicron 粒子の人体に及ぼす影響とか，或は，かかる微細粒子に対する集塵器の集塵効率の決定，又それら粒子の物理化学的性質を調べる為に，任意の大きさのそろつた粒子を作る必要がある。

Aerosol の生成については，極めて多くの報告があるが，1 μ 以下の粒子の生成については，LaMer 一派のステアリン酸等の凝縮法，Dautrebande 等による食塩水の分散濃縮法及び Walkenhorst による NaCl の凝縮法等，二，三の報告があるにすぎない。

微小粒子の生成の際には，多くの困難な問題がある。即ち，一次粒子は極めて微小でも，凝集を起し，大きな団粒を生じてしまうと云う事である。粒子の凝集は，物質の物理化学的性質，気体中の粒子の濃度，気体の種類，粒子の生成方法等，多くの因子により引き起こされる。

我々は，金属アーク法及びアトマイザーによる食塩水の分散濃縮法により，よく分散された Submicron 粒子を生成した。

a) 金属アーク法

電極として，鉄，鉛，タングステンを用い，電極間距離を 7 mm に保ち，交流 9,000 V をかけ，1 分間 300 回の割合で断続放電させた。生成した金属フユウム (fume) は電気集塵器にて捕集し，電子顕微鏡により調べた。三者のいずれの金属の場合に於ても，粒子は球状で，殆んど凝集は認められなかつた。平均粒子経は，鉄：0.03 μ，タングステン：0.04 μ，鉛は電子顕微鏡でも測定出来ないような微細粒子がかなり多い為，平均粒径は求められなかつた。

b) 食塩水の分散濃縮法

食塩の濃度を 10^{-1}，10^{-2} 及び 10^{-3}% とし，アトマイザーのノズルを通過する際の空気の流速を 310 及び 420 m/sec と変化し，食塩水の表面に於て，ミストを発生し，そのミストを乾燥空気と混合し，塩の粒子を生成せしめた。生成粒子は，Thermal Precipitator で捕集し，電子顕微鏡で粒径を測定した。

この方法により作られた塩の粒径分布は，ミスト発生時の空気の流速には，殆んど影響されず，溶液の濃度により粒度が調節出来るようである。

食塩水溶液の濃度と空気の流速とによる生成粒子の平均粒径は次のようである。

濃度 (%) / 流速 (m/sec)	10^{-1}	10^{-2}	10^{-3}
310	0.10_5	0.07_8	0.04_0
420	0.11_3	0.09_1	0.04_2

労 衛 研 報 第2号, 63～69, (1959)

ゴム糊溶剤中のベンゼンの定量

坂 部 弘 之　左右田礼典　木 村 正 己　松 村 芳 美

DETERMINATION OF BENZENE IN THE SOLVENT OF RUBBER PASTE

Hiroyuki SAKABE, Reisuke SODA, Masami KIMURA and Yoshimi MATSUMURA

昭和34年，東京都に於て，ヘップサンダルの家内工業に従事する人々に多数のベンゼン中毒患者が発生したが，其の原因が接着剤のゴム糊に含まれるベンゼンにある為，労働省からの依頼に基き，これらの市販ゴム糊製品中のベンゼン含量を測定した。

分 析 法

ゴム糊を溶剤そのまま分析することは，その性状から困難である。又揮発性成分を蒸気で測定することは分析精度の点及び蒸気成分濃度が必ずしも溶剤それ自体の成分濃度に等しくないという点で不充分な結果しか得られない。そこで今回の分析に際しては以下述べる如く，先ずその溶剤を単蒸溜で出来る限り生ゴムその他不揮発性成分と分離して集め，この溜出液を分溜塔で分溜し，各溜分について分析を行つた。分析は主としてガスクロマトグラフ法に依つたが，尚結果が不明確な場合は赤外スペクトル，又は紫外スペクトルを測定して確定した。従つて操作途中に起る試料の損失以外には大きい誤差を生ずる原因が考えられず，又ベンゼンと他成分を誤つて分析するおそれがない。

以下操作順序に従つて詳述する。

(1) 単 蒸 溜

容器は残渣除去が楽であるように 1 l のセパラブルフラスコを用い，これにゴム糊を大体 500 グラム程入れ，全共通擂合せガラス蒸溜セットで蒸溜した。加熱は湯浴によつて行い，先ず 90°C 位迄の溜分を軽く吸引しつつ蒸溜する。通常の沸石やギャピラリではすぐにつまつたりして気泡が出なくなるおそれがある。又此の際吸引が強すぎると低沸点成分の損失が多くなる事が考えられたので注意して僅かに気泡の出る程度で且出来る限り減圧にならぬように努めた。更に高沸点の成分はやや減圧にして蒸溜し，残渣が乾涸した所で終了した。一試料のこの操作に要する時間は大体一日であつた。溜出液はまとめて分溜塔で分溜を行つた。

(2) 分　　溜

分溜蒸溜塔は東京科学精機製作所製の手動式のものを用いた。前記単蒸溜溜出液を 1 l のフラスコに入れ電熱器で加熱して蒸溜を行つた。塔頂部と塔内の温度が常に等しく保たれるよう分溜塔ヒーターの電流を加減した。温度は銅コンスタンタン熱電対の起電力を電圧計で読んで求めた。還流比は大体 1 : 10 で，溜分の溜出温度が一定で可成り長くそのまま持続している場合は 1 : 5 位に溜出を早くした。溜分は一定温度範囲内のものを一つにまとめた。流出速度は溶剤の

種類，条件によつていろいろであるが大体一分間に 0.5 cc 乃至 2 cc であつた。塔内残溜分 (Hold up) は約 10 cc であつた。これ以上速く蒸溜を行うと分離が極めて不完全になる。200 cc の試料の場合この操作に要する時間は 4 時間乃至 7 時間であつた。

(3) ガスクロマトグラフ

ガスクロマトグラフ装置への試料の注入は注射器で行つた。使用した注射器はツベルクリン用の 1 cc のもので同一の注射器を常に用いた。試料の注入量は 0.05 cc とした。検出器は熱伝導度セルで抵抗体は 20 Ω の白金線コイルである。記録計は 60 mV フルスケールの範囲で記録を行つた。充塡剤は 30 乃至 65 メッシュのセライトに 10 : 3 の割合でディオクチル・フタレートを滲ませたもので，これを内径 4.5 mm，長さ 2 m の銅管につめ恒温槽内に入れる。操作時の温度は室温その他の条件によつて，変化したが，一日の分析中は恒温になるようにした。He をキャリアーガスとして使用し，流量は石鹸膜法で測定，大体 35 乃至 40 cc/分 で測定を行つた。クロマトグラム中のベンゼンの位置は，各溜分のクロマトグラムにベンゼンと思われる山が出現した時，その試料に 5 : 1 乃至 10 : 1 位の容量比で小量の純ベンゼンを加えて，再びクロマトグラムを書かせ，その山がもとの形を失わずに高くなることによつて決定された。この際，非常に近くに山を持つ成分があつた場合は決定が困難になるし，又山の形も変る事がある。僅かにベンゼンと異る位置に山をもつ物質がある時は純ベンゼンを加えることにより，山の形は非対称に変形する。この様な場合には後述する如く分光学的にも検討してベンゼンの有無及び量を確定してある。ベンゼン含有量の計算はクロマトグラムの山の面積によらず，山の高さが，成分の重量に比例するものとした。面積法によらない為の誤差は他の種々の誤差よりは遙かに小さい。混合試料の場合は各成分の示す位置は溶剤の種類，濃度比によつて多少ずれるし，又測定条件の影響も受けるので注意を要する。

(4) スペクトル分析

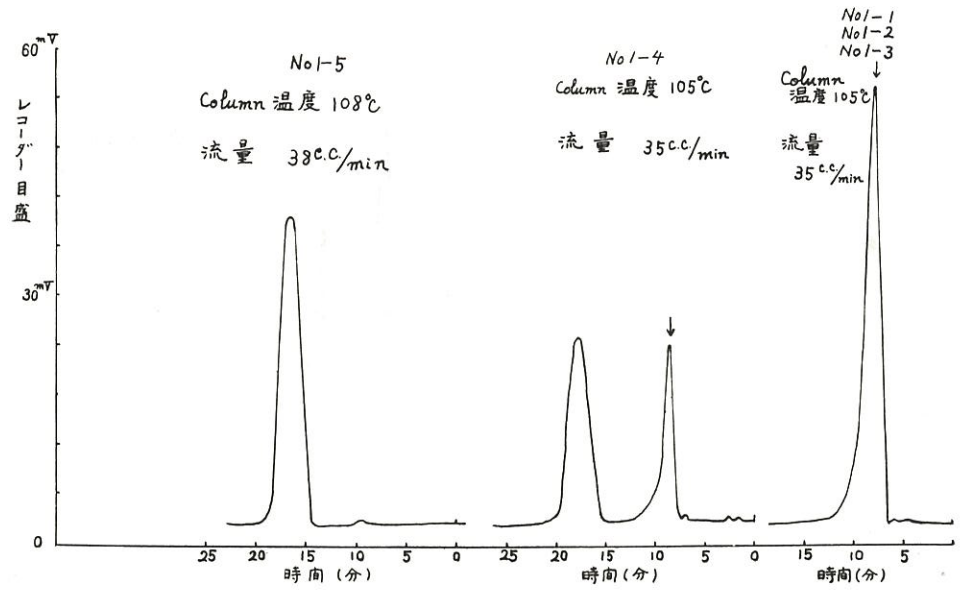
ガスクロマトグラフのみの測定で定量が不確実な場合，主に紫外吸収スペクトル分析によつて測定，定量値を確定した。分光器は東京工業試験所第二部の厚意により Cary Model 14 分光光度計を使用した。赤外吸収スペクトルは同試験所に於て島津製作所製の簡易型分光器 IR-2 型を使用して一，二の試料につき測定したが，今回の場合はベンゼンに注目しているため紫外吸収スペクトルを利用することが有効であつた。従つて測定結果は紫外吸収スペクトルによるものを利用した。その測定に於ては，試料をエタノールで 1/121 乃至 1/2662 に稀釈，その数 cc をセルに入れてスペクトルを取つた。トルエン，キシレン及びその他共軛不飽和結合を有する化合物を除けばベンゼンのスペクトルの領域 300 乃至 200 mμ に吸収を示して妨害するものはない。又高分解能の分光器であるため混合物の場合でも最大吸収波長及び山の数が化合物によつて皆異るため連立方程式をつくつて成分量を決定することが可能である。但し一成分が特に多量の場合や含まれている成分の定性が不完全な場合には誤差が当然多くなる。又ベンゼンを確認することが不可能な場合もある。今回は先に分溜を行つているため，各成分が比較的，或溜分に濃縮されているから此の様な心配は殆ど考えられなかつた。勿論他の分析法についても同様な事が言える。

(5) 計　算　法

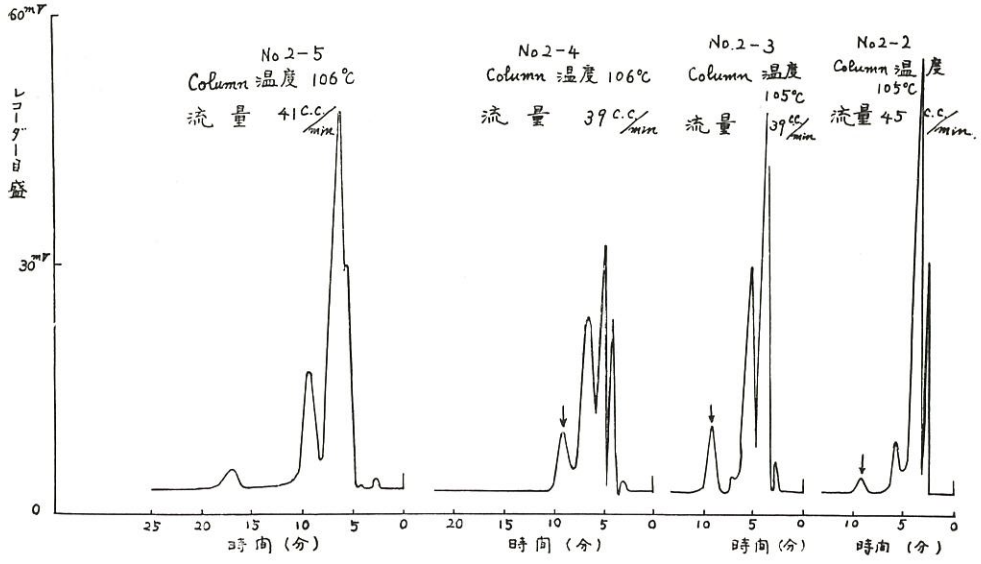
各溜分毎にベンゼン量を決定し，その合計量を全溜分合計量で割つてベンゼンの重量パーセン

トを算出した。

ガスクロマトグラフ又は紫外吸収スペクトルによつて決定される各溜分中ベンゼン量は一定容積の試料中にあるベンゼンの容積である。即ちガスクロマトグラフの場合を例にすれば，純粋ベンゼン 0.05 cc の波高値（数回の測定の平均値）で各溜分クロマトグラム中のベンゼンの山の波高値を割つて先ずそのパーセント x_i を算出する。各溜分は今回，その重量のみの測定を行い，容積測定を行わなかつたので，可能な有機溶剤の比重の平均として 0.88 を各溜分の比重と見な



第 1 図 (a)



第 1 図 (b)

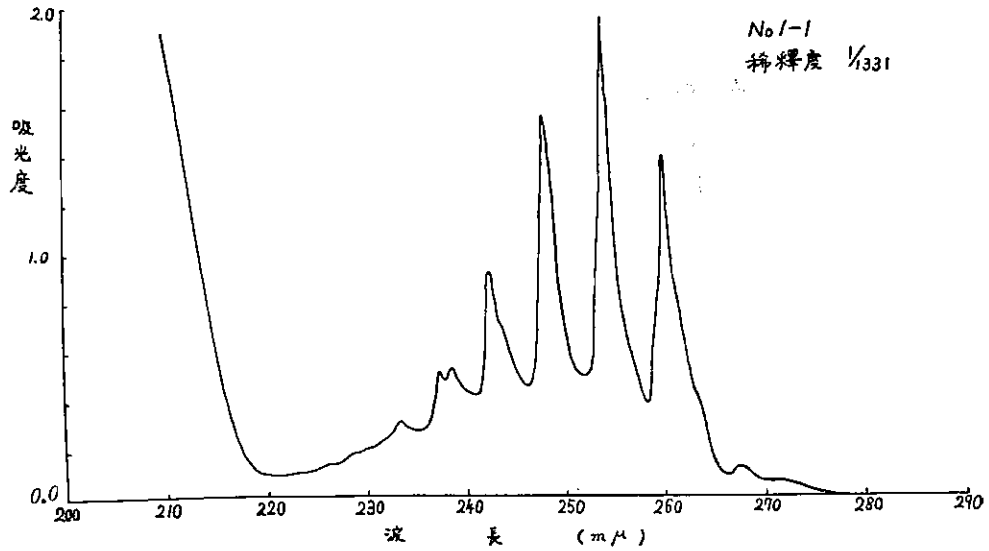
して，その容積を算出した。各溜分の重量を w_i と書けば容積は $w_i/0.88$ である。この溜分中に含まれるベンゼンの重量はベンゼンの比重を d とすれば

$$(w_i/0.88)\times x_i \times d$$

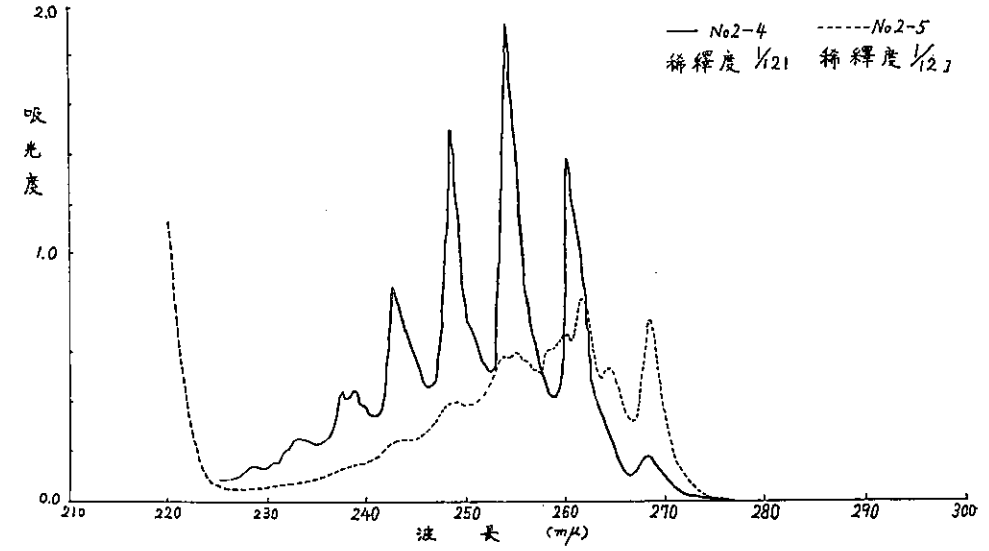
となる。ベンゼンの比重は 0.8787 であるから近似的に $d=0.88$ としてゴム糊一試料の溶剤に含まれるベンゼンの重量パーセントは

$$\frac{\sum_i w_i \times x_i}{\sum_i w_i}\times 100\%$$

として算出した。



第 2 図 (a)



第 2 図 (b)

第 1 表　分溜，溜分の分析結果

試　料	溜出温度 (°C)	重　量 (グラム)	ベンゼン		備　考
			(%)	重　量 (グラム)	
No. 1-1	80	51.13	100	51.13	
2	80	58.78	100	58.78	
3	80	57.07	100	57.07	
4	81～110	17.31	43	7.44	
5	残溜分	30.83	1.0	0.31	
No. 2-1	33～40	12.24	0	0	
2	40～62	13.60	3.53	0.48	
3	62～69	14.36	15.3	2.20	
4	69～90	26.45	12.9	3.41	
5	90～97	10.30	0	0	
6	97～115	23.79	0	0	
7	残溜分	27.80	0	0	
No. 3-1	32～60	15.25	1.18	0.18	
2	60～74	35.73	17.65	6.31	
3	74～110	39.98	24.2	9.68	紫外吸収スペクトルから決定
4	110～115	32.53	0	0	
5	残溜分	37.21	0	0	
No. 4-1	72～76	3.23	66.3	2.14	
2	76～79	16.24	85.6	13.80	
3	79～80	4.64	98	4.55	
4	80～81	54.53	100	54.53	
5	80～81	63.69	100	63.69	
6	80～81	30.38	100	30.38	
7	82～109	13.93	49.5	6.90	
8	109～114	45.34	7.5	3.40	
					分溜前全重量 245 グラム (減少 5.3%)
No. 5-1	70～72	46.56			
2	70～72	49.64			
3	72	46.18	13	23.9	
4	72～73	41.41			
5	73～101	26.46	27	7.14	
6	残溜分	33.33	0	0	
No. 6-1	41～83	26.35	7.1	1.87	
2	83～89	22.74	0.6	0.14	
3	89～93	15.39	1.2	0.18	
4	93～96	34.73	2.4	0.83	
5	残溜分	28.31	0	0	
					分溜前全重量 135.5 グラム (減少 5.9%)

試　料	溜出温度 (°C)	重　量 (グラム)	ベンゼン		備　考
			(%)	重　量 (グラム)	
No. 7-1	77～111	16.37	2.8	0.46	
2	111～114				
3	111～114	192.47	0	0	
4	111～114				
5	110～111	46.56	0	0	
6	残溜分	48.70	0	0	
No. 8-1	87～112	13.11	31	4.06	
2	112～115	52.18	1.2	0.63	
3	115	53.64	0	0	
4	115	58.67	0	0	
5	残溜分	27.18	0	0	
No. 9	上　層	78.9	3.46	2.73	分溜による効果なし (分離不完全)
	下　層	35.3	0	0	

第2表 分 析 総 結 果

試　料	全量 (グラム)	ベンゼン重量 (グラム)	ベンゼン量 (%)	他の成分 (推定を含む)
No. 1	215.1	174.7	81±8	トルエン
No. 2	128.5	6.1	4.7±0.5	石油エーテル，エステル，アルコール類，トルエン
No. 3	160.7	16.2	10.1±1.0	石油エーテル，エステル，シクロヘキサン？　トルエン
No. 4	232.0	179.4	77±8	石油エーテル，トルエン
No. 5	243.6	31.0	12.7±1.3	石油エーテル，トルエン
No. 6	127.5	3.02	2.4±0.3	石油エーテル，エステル，アルコール類？
No. 7	304.1	0.46	0.15±0.02	(石油エーテル) トルエン
No. 8	204.8	4.69	2.2±0.2	トルエン
No. 9	114.2	2.73	2.4±0.2	エステル，石油エーテル？

(6) 誤　　差

注射器によるガスクロマトグラフ装置への試料の注入に際して生ずる誤差は最大 5% に達する。比重の値を 0.88 にとつたが大体 0.86 から 0.90 の間に溜分の比重は分散していると考えられるので，そのために起り得る誤差の最大は 2% 前後と見なされる。分溜塔による分溜及び最初の単蒸溜に際しての重量の減少は大体最高 5% であつた。此等可能性のある誤差の原因を考えに入れて計算すると今回の測定結果の分析値の 10% 以内に真値が存在していることを示している。

測定結果

以上の分析法に基き測定した結果をまとめると以下の如くになる。

第1図には試料のガスクロマトグラムを，又第2図には紫外吸収スペクトルを測定したもののスペクトルを示した。(ガスクロマトグラム中矢印がベンゼンの山を示す)。

これらのデーターをもとに計算した結果を第1表に示した。

以上を総括して第2表に示した。

労 衛 研 報 第2号, 70～75, (1959)

日本産ケイソウ土の調査並びにケイソウ殻の結晶化に関する一考察

浜 田 晃 島 津 正 司

INVESTIGATIONS ON DIATOMACEOUS EARTH FROM JAPAN AND A STUDY OF THE CRYSTALLIZATION OF FRUSTULE

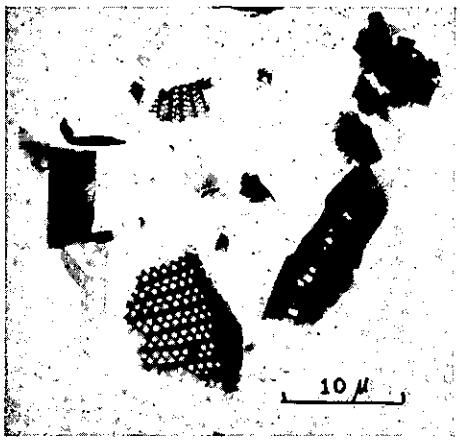
Akira HAMADA and Masaji SHIMAZU

日本産ケイソウ土 (diatomaceous earth) 30種 (原土29種, 焼成品1種) の鉱物組成および遊離けい酸量を表示し, その大要を述べ, ケイソウ殻に関して一考察を試みた。

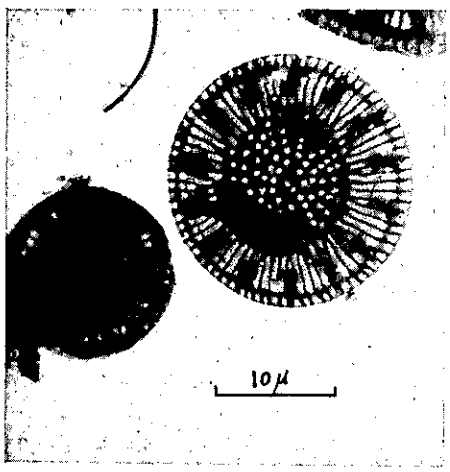
1) 粒度および形状

電子顕微鏡観察によるとケイソウ土は一般に数ミクロン程度の粉末粒子からなつていて, 主成分はケイソウ殻 (frustule) であり, その他に不純物として若干の粘土などを混えることが多い (図版1参照)。ケイソウ殻の形態または形状は試料によつて異なり必ずしも同種ではないが, 破壊されていないケイソウ殻単粒子の大きさは一般に20ミクロン前後であつた (図版2参照)。このようにケイソウ土は主としてケイソウ殻の集積した生物源岩石である。

図版 1
ケイソウ殻の破片および他の不純物
(電子顕微鏡写真)



図版 2
ケイソウ殻の単粒子
(電子顕微鏡写真)



2) 遊離けい酸量

りん酸分析法によつて定量した遊離けい酸量は付表の如く大略15パーセントを超えないが, その値は試料によつて異なり一定していない。X線回折像の回折強度所見による半定量的結果を

加味して考えると(付表参照)，遊離けい酸総量は非晶質けい酸量の多少によつてはそれ程左右されないで，結晶質無水けい酸量の多少によつて大きく左右されるように思われる。すなわち，りん酸処理によつて非晶質けい酸は可成り溶解されるが，結晶質無水けい酸（ここでは石英，クリストバル石）は殆んど溶解されない為と考えられる。付表の No. 27 試料が極めて高い遊離けい酸量を示しているが，これは X 線回折像により明らかにクリストバル石に変化しているものであつて当然の結果である。このクリストバル石は回折線の様相から推して結晶度が多少劣つていると考えられる。

りん酸分析法は加熱法にシュミットの法[1]を適用させた。その結果従来よりも恒温保持が容易で所定の温度に ±2°C の範囲内で制御し得た。

3) 鉱物成分

ケイソウ土の鉱物の同定は主として X 線回折法によつて行い，必要に応じて岩石顕微鏡観察，電子顕微回折，示差熱分析などを行つた。その結果，付表に示したようにケイソウ土の主成分は非晶質けい酸であり，その他若干のクリストバル石，石英，長石，モンモリロナイト群鉱物，加水ウンモ型粘土鉱物，カオリン鉱物，混合層粘土鉱物などを含んでいることが判つた。

ケイソウ土に含まれている非晶質けい酸，クリストバル石，および石英の存在並びにそれらの関係についての詳細は他の機会にゆずるが，二三の点について簡単に述べると：

従来ケイソウ土の主要構成物質であるケイソウ殻は含水非晶質けい酸とされていたが[2,3]，筆者らの検討の結果ケイソウ殻には全く非晶質のものと，不完全なクリストバル石構造を示すものとがあることが判つた。このクリストバル石に結晶化したケイソウ殻の結晶度は極めて低い。更に石英に結晶化しているケイソウ殻も存在する可能性が考えられるが，現在の処検討中で明らかではない。

クリストバル石化ケイソウ殻について説明する，上記 29 種天然ケイソウ土の X 線回折像を観察すると二群に大別しうる。すなわち一群は約 4Å に頂点を有する丘陵状のブロード像を示し，他の一群はこのブロード像に重なつて約 4Å に弱いが明らかにピークと認め得る回折線が重なつている。前者は明らかに非晶質けい酸の存在を意味し，後者（4Å 型）は非晶質けい酸のみならず結晶性物質の共存を意味する。この約 4Å 線はクリストバル石の最強回折線の面間隔に近似的に一致する。4Å 型試料のケイソウ殻を制限視野電子回折像として捉えると，数少い回折斑点を認め，その回折点の面間隔はクリストバル石のそれにほぼ一致する。しかし回折斑点の数や種類から推して極めて不完全なクリストバル石であることがわかる。以上により 4Å 型ケイソウ殻には不完全な α-クリストバル石に結晶化しているものがあることが判つた。

4Å 型試料のケイソウ殻であつても必ずしもクリストバル石化しているものばかりとは限らず，クリストバル石ケイソウ殻と非晶質ケイソウ殻が混在していることが多い。またケイソウ殻一粒子全体がクリストバル石化しているというよりは粒子の一部のみが結晶化していて，その部分が浮き出して観察される（電子顕微拡大像において）のが普通であつた。

岩石顕微鏡下にケイソウ殻の屈折率を検べると，その値の低いものから異常に高い値を示すものまであるのはケイソウ殻の結晶度に対応する現象と思われる。

次にケイソウ土に含まれる石英について；試料によつては石英を含むものと含まないものとがあるが，付表に示すように大部分の試料が石英を含有している。この石英は不純物として混入し

ているのか，ケイソウ殻が一部結晶化して石英に変化しているのか明らかではない。しかしケイソウ殻が天然の地質条件下においてクリストバル石に変化するものみならず石英にも変化する可能性を考えることができる。というのは筆者らはケイソウ土の人工加熱処理によつて次の様な結果を得たからである。すなわち29種のケイソウ原土のうち非晶質けい酸のみの回折像を示すと思われる試料1種（付表，No. 4）を選び，大気中において毎分 8°C の上昇率で加熱し後所定の温度において1時間加熱して，直ちに常温大気中に急冷させた試料を用意した。ここに所定の温度とは 1200°，1100°，1000°，900°C，その他の温度である。

これら人工処理試料の X 線回折像を検討した結果，1000°C 処理試料ではブロードな非晶質けい酸の回折像に約 4 Å の小さなピークの発達を認め，1100°C 処理試料では約 4 Å ピークおよび 3.34 Å ピークの発達を認め，1200°C ではブロード像は消失し，クリストバル石の殆んど全回折線と 3.34 Å（石英）線を認めた。すなわちクリストバル石と少量の石英の成長を認めた。

ケイソウ土の試料によつては加熱によつて石英の生成を全々認めないものもある。このように石英への変化が認められるものとそうでないものとがある原因は，実は原試料のケイソウ殻の性質の相異にあると考えられ，石英の成長を認めた原試料（加熱前）の X 線回折像を注意深く観察すると 3.34 Å（石英回折線の位置に一致）にピークの徴候を認めることができる。すなわちケイソウ殻自体がすでに石英構造の結晶核を有していて，加熱により石英に成長したと思われる。以上の石英への変化の確認を得るために今後電子顕微回折，示差熱分析により検討したいと考えている。なお上記に関するデータは他の機会に発表する予定である。

以上のような非晶質けい酸が天然においてクリストバル石に変化している例は蛋白石[4]において周知の事実であるが，ケイソウ土も蛋白石に照して興味あることであろう。また珪肺問題に関連してケイソウ殻の結晶学的性質を検討することは意義あることと考えている。すなわち遊離けい酸鉱物の結晶度または結晶の不完全性と有害度との関係は考察すべき一面ではあるまいかと考えている。換言すると，一般に鉱物の個性は地質的生成条件によって異なるが，このような個性の相異が生体内反応においても差異を生ずる結果になるのではあるまいかという疑問をもっている。

文　　献

1) Schmidt, K. G. (1954): Berichte der Deutschen Keramischen Gesellschaft e. v., **31**, 402-404.
2) 日本鉱産誌 IV（昭 29），p. 89.
3) 窯業工学ハンドブック（1952），p. 203（技報堂）
4) Sudo, T. (1950): 地質雑，**56**, No. 654.

付　表：ケイソウ土の遊離けい酸量および鉱物成分

整理番号	試料名その他	X線回折像所見		遊離けい酸量(%)
1	北海道小樽市	非晶質けい酸 モンモリロナイト鉱物，石英，長石，雲母型粘土鉱物	多量 微量	6.80
2	北海道網走市能取(砕粉)	非晶質けい酸 石英 クリストバライト，長石，モンモリロナイト鉱物	多量 小量 微量	14.33
3	北海道網走市呼人(原石)	非晶質けい酸 石英 クリストバライト，長石，モンモリロナイト鉱物	多量 小量 微量	11.00
4	北海道倶知安町 (粘土分を含まず)	非晶質けい酸 石英(?)，長石(?)	多量 微量	10.04
5	〃 (粘土分を含む)	非晶質けい酸 石英(?)，長石(?)，その他(?)	多量 微量	3.80
6	午海道瀬棚，けいそう土原土	非晶質けい酸，モンモリロナイト鉱物，石英 クリストバライト，長石	中量 微量	9.17
7	秋田県午浦町真山黒滝 秋田鉱業(株)	非晶質けい酸 モンモリロナイト鉱物 石英	多量 中量 少量	7.53
8	秋田県米内沢町浦田 浦田けいそう土改良かまど製造組合	非晶質けい酸 石英 クリストバライト，モンモリロナイト鉱物	多量 少量 微量	10.35
9	青森県十和田市	非晶質けい酸 石英，クリストバライト，雲母型粘土鉱物，長石 モンモリロナイト鉱物，混合層粘土鉱物	多量 少量 微量	7.50
10	宮城県大河原町 東京保温材大河原工場	非晶質けい酸 石英，クリストバライト，モンモリロナイト鉱物，雲母型粘土鉱物 混合層粘土鉱物，長石	多量 小量 微量	2.61
11	新潟県佐渡郡沢根町 沢根鉱山けいそう土	非晶質けい酸，石英 モンモリロナイト鉱物 クリストバライト 雲母型粘土鉱物，カオリン型粘土鉱物	中量 中量 中量 微量	9.51
12	長野県上水内郡三水村赤塩	非晶質けい酸 クリストバライト，石英，長石 雲母型粘土鉱物，モンモリロナイト鉱物	多量 中量 微量	4.67
13	〃　下水内郡岡上村	非晶質けい酸，石英 クリストバライト カオリン型鉱物，モンモリロナイト型鉱物	中量 少量 微量	9.95

整理番号	試料名その他	X線回折像所見		遊離けい酸量(%)
14	長野県上田労基署	非晶質けい酸 石英，長石，クリストバライト，其他	多量 少量	9.80
15	〃	非晶質けい酸 石英，モンモリロナイト鉱物 雲母型粘土鉱物，カオリン型粘土鉱物	多量 少量 微量	7.05
16	長野県小諸市	非晶質けい酸，石英，クリストバライト，モンモリロナイト鉱物 長石 雲母型粘土鉱物，カオリン型粘土鉱物	中量 少量 微量	7.61
17	岐阜県八幡町	非晶質けい酸 長石 石英，クリストバライト	多量 中量 微量	2.10
18	石川県七尾市和倉駅前 イソライト工業(株)	非晶質けい酸 石英，長石 モンモリロナイト鉱物，クリストバライト 雲母型粘土鉱物，カオリン型鉱物	多量 中量 少量 微量	11.52
19	石川県輪島市小峰山	非晶質けい酸 石英 クリストバライト，長石，モンモリロナイト鉱物，雲母型粘土鉱物	多量 少量 微量	12.96
20	石川県珠洲市正院町	非晶質けい酸 モンモリロナイト，石英 長石，クリストバラトイ 雲母型粘土鉱物	多量 中量 少量 微量	13.22
21	島根県周吉郡西郷町加茂 日の丸窯業加茂工場	非晶質けい酸 石英，モンモリロナイト鉱物 長石，クリストバライト	多量 少量 微量	9.22
22	島根県西郷町	非晶質けい酸 石英，モンモリロナイト鉱物	多量 少量	13.00
23	熊本県人吉市	非晶質けい酸 クリストバライト	多量 微量	1.80
24	大分県湯布院町若杉	雲母型粘土鉱物，石英 非晶質けい酸，クリストバライト カオリン型鉱物	多量 中量 少量	14.00
25	大分県庄内町南庄内 大野原けいそう土原土	非晶質けい酸 クリストバライト 長石 カオリン型鉱物，雲母型粘土鉱物	多量 少量 微量 微量	2.30

整理番号	試料名その他	X線回折像所見		遊離けい酸量(%)
26	大分県庄内町阿蘿野 井手下けいそう土原土	非晶質けい酸 クリストバライト，長石	多量 少量	1.50
27	大分県九重町右母 白山工業(株)， 野上右田混合焼成	クリストバライト(構造不整又は不完全結晶)		59.30
28	大分県九重町右田 白山工業(株)，右田原土	非晶質けい酸 石英，クリストバライト，長石， モンモリロナイト鉱物	多量 微量	8.32
29	大分県山香町野原 土けいそう土	非晶質けい酸 クリストバライト，石英，長石 モンモリロナイト鉱物，雲母型粘土鉱物， カオリン型鉱物	多量 少量 微量	6.51
30	大分県野上町 岩尾生産分原土	非晶質けい酸 モンモリロナイト鉱物 長石，クリストバライト 雲母型粘土鉱物，カオリン型鉱物	多量 中量 少量 微量	5.62

(?) は回折線と認めてよいか否か明らかでない程弱い線であったことを意味する

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