# STUDIES ON THE EXPERIMENTAL CHRONIC POISONING OF NITROBENZENE

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# STUDIES ON THE EXPERIMENTAL CHRONIC POISONING OF NITROBENZENE.

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## Introduction.

The aromatic nitrocompounds have been utilized as the precursors of various dyes and other chemicals for decades. The poisoning due to the use of these chemicals in factories has been reported by many such as Renshaw,[1] Schwanke[2] and others, and in Japan recently by Shizume,[3] Matsushima,[4] Nomura,[5] Higashida and Hara,[6] Hara,[7] Fujimoto,[8] Otsuka,[9] Nishida[10] and Shiraishi.[11] Besides these reports, experimental studies on the poisoning of these compounds have been carried out by Gelfins,[12] Aleksandrov,[13] von Oettingen,[14] Kubota,[15, 16] Fukue,[17, 18] Mizunoe,[19] Yoshida[20] and others. Reports by Williams,[21–24] Fishman,[25] Parke,[26] Nakajima,[27–30] Sato[31, 32] and Takemura[33] were the principal ones on the detoxication mechanism of such compounds as nitrobenzene and the concerned enzyme, co-enzyme and sulfa-containing substances, and those by Yamaga[34, 35] and Bodansky[36] were on the nature of enzyme in case of detoxication of aromatic hydrocarbon. The recent studies on the poisoning tend to be carried out not only from biochemical aspect but also from neurological or endocrinological standpoint, and the sexual and individual differences are studied by Hirokawa[37, 38] and Harashima.[39] Since the publication of "stress theory" by Selye,[40–42] the concept of stress has been introduced in the field of toxicology as shown in the researches by Smyth,[43] Essellier,[44] Nishiyama[45] and others.

In spite of quite a number of studies made already, few reference is available on the study of chronic poisoning of aromatic nitrocompounds, and it seems that the recent papers by Fukue[18] and Yoshida[29] are the only references available at present. This means that there are many problems yet to be studied on chronic poisoning such as allowable dose, nature of resistance against poisoning, detoxication mechanism and so on. In an attempt to investigate the chronic poisoning of nitrobenzene, experimental studies were made on rabbits.

## Materials and Methods.

Five mature male rabbits for each experiment, including one as the contrast, were fed on bean-curd-leavings and green vegetables for about three months.

0.7ml. of nitrobenzene per kilogram of body weight was daily injected subcuta-

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neously before feeding. This dosis was considered from the result of the preparatory experiment to be the maximum amount of not to cause the death of animal from acute poisoning.

Blood was examined before the injection once a week on the following items :– 1) red blood cell count, 2) content of total haemoglobin (KCN–$K_3$ Fe (CN)$_6$ method), 3) reticulocytes (Schilling's method), 4) basophilic stippled red blood cells (Manson-Schwarz's staining), 5) methaemoglobin ratio,[46] 6) white blood cell count, 7) haemogram, 8) mean nuclear number by Sugiyama's method, 9) cobalt-test and bromsulphalein test on the serum.

The following were examined once a week on the 24-hour urine collected after the injection :– 1) total $NO_2$ (titanous chloride method modified by Musha),[47] 2) aromatic primary amine in the original and the hydrolyzed urine by 0.5 N–HCl 60 minutes at 100 °C. (Bratton-Marshall's method),[48, 49] 3) total glucuronide in the original urine and glucosideform glucuronide in the hydrolyzed urine by 0.2N–NaOH five minutes at 100 °C. (Nose's modified carbazol method),[50, 51] 4) ethereal sulphuric acid (benzidine method),[52] 5) chromatgraphy (Giri's method),[53, 54] 6) protein, reducing substance, urobilinogen and coproporphyrine (Brugsch's modification of Fisher's method).

Among many studies on the detoxication of nitrobenzene in rabbit by R. T. Williams and his co-workers,[21–24] T. Sato,[31, 32] T. Nakajima[27, 28] and so on, D. V. Parke[26] reported that more than a half of intaken nitrobenzene is excreted in urine, mostly as p-aminophenol (31%), while in small quantity as m-(4%) and o-aminophenol (31%), while in small quantity as m-(4%) and o-aminophenol (3%), para-(9%), meta-(9%) and orthonitrophenol (0.1%), 4-nitrocatechol (0.7%), nitroquinol (0.1%) and p-nitrophenylmercapturic acid (0.3%). These amino compounds are produced through the amination of nitroradical and further more will undergo the acetylation. On the other hand, hydroxylation occurs on para, meta or ortho position to nitro or amino radical, and still more conjugation with glucuronic acid or sulphuric acid may occur. Based on these considerations ethereal glucuronic acid and ethereal sulphuric acid are measured for the index of hydroxylation.

Aromatic primary amines develop colour by Bratton-Marshall's method[48, 49] easily, but the tone and density of colour vary considerably depending upon difference of the side chain or the conjugated compounds. Therefore this method is considered to be inadequate to this examination of urine which includes various kinds of detoxication products. However, Bratton and Marshall determined originally the quantity of detoxication products of sulfamine excreted in urine. So this method may be applicable to presumption of the outline of detoxication of nitrobenzene. For the standard of photometry, the conjugated compound of p-aminophenol, chief detoxication product of nitrobenzene, is hardly available in pure state, therefore, p-aminobenzoic acid is applied because of its colour tone. But since the obtained value using p-aminobenzoic acid as the standard is about one fifth of the actually obtained by analysis of the conjugated compounds of aminophenol, the results obtain-

ed photometrically are corrected and shown in the Table.

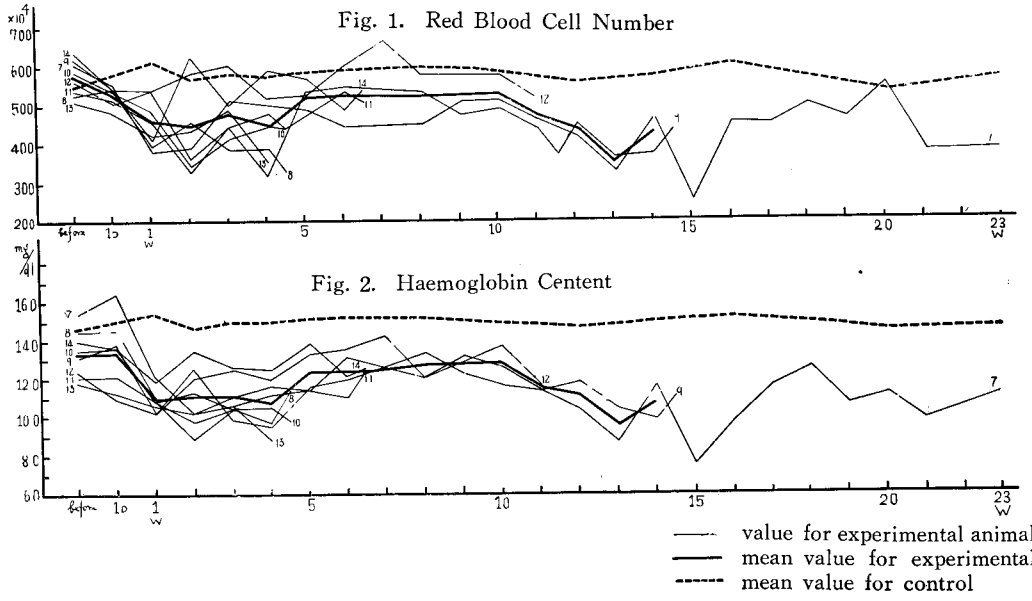
The hydrolysis of urine with hydrochloric acid in concentration of 0.5 N at 100 °C. for 60 minutes decomposes the acetyl group of detoxicated compounds completely and liberates aromatic primary amines which can be estimated by this method. This procedure decomposes completely ethereal sulphate too at the same time, but not ethereal glucuronic acid.

*Chromatography.*

Circular paper chromatography is carried out on "Toyo" No. 51 filter paper (30 cm. in diameter) after Giri,[53] using n-butanol: glacial acetic acid: water (4 : 1 : 1) as the solvent. In this procedure the elimination of colloidal substance in urine is not carried out because of non-interference in the separation and the colour reaction. This process takes six to seven hours. For the quantitative meaning the volume of urine to be spotted on the paper is corrected according to the actually excreted amount based on the assumption that urine is excreted one ml. per 10 gm. of body weight a day in case of rabbit. In order to concentrate the urine on the paper, 0.01 ml. of ordinary urine is spotted five times. p-Dimethylaminobenzaldehyde for amine especially aminophenol, Bratton-Marshall's diazo reagent for aromatic primary amine, $FeCl_3 + K_3Fe(CN)_6$ for phenol and the aniline hydrochloride for reducing substance are used as the spraying reagent.

## Results.

Some of the data obtained on blood corpuscle and urine are shown in the Fig. 1–10. The bold line in the figures indicates the mean value of each item excluding



those of distinctly different from others in quality. The findings on urine vary considerably according to individuals but which is not the case on blood.

Among the eight experimental rabbits, three died spontaneously in the fourth week, two in the sixth and one each in the 11th and the 14th. The last one was slaughtered in the 24th experimental week. For the sake of discussion the whole experimental period could be divided into three stages, the first stage is from the beginning through the fifth week of the experiment, the second from the sixth through the fifteenth week and the rest is the third.

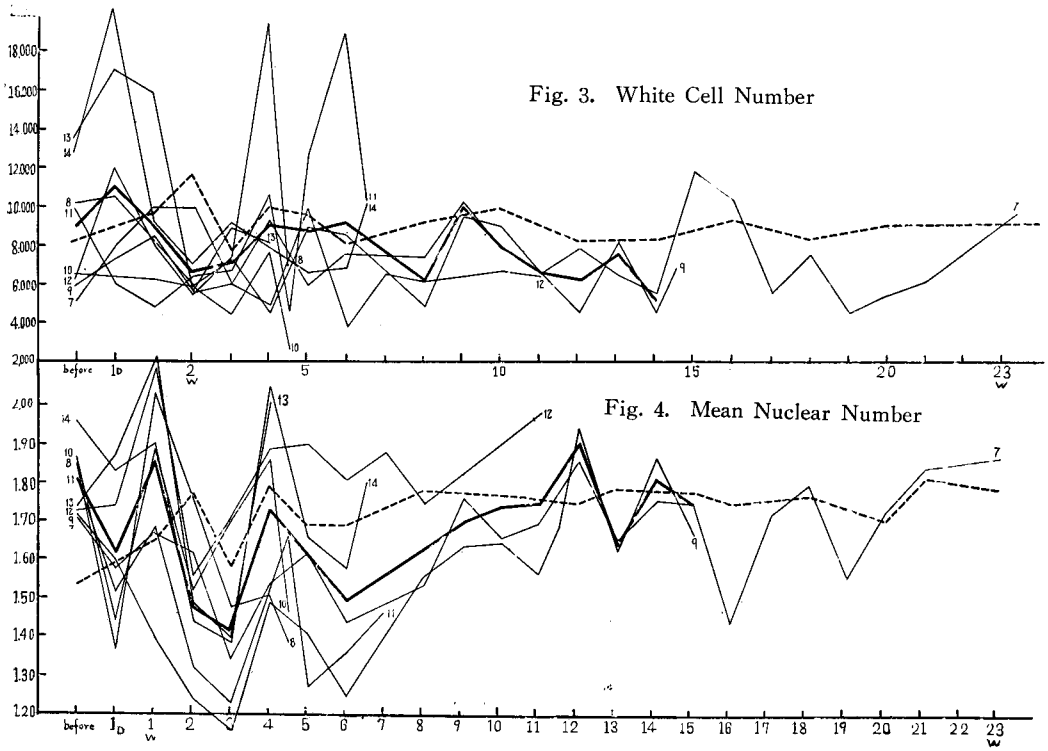



Fig. 3. White Cell Number




Fig. 4. Mean Nuclear Number

*Blood:*

Curves of mean values for the red blood cell and the content of total haemoglobin are almost parallel with each othre (Fig. 1, 2). As to the individual cases, change in the haemoglobin content appears slightly after that of the red blood cell number. The variation of the haemoglobin content is not so large as that of the red blood cell. Thus, in the first stage anaemia occurs, which is almost recovered in the second stage, and recurs at the end of that stage. The process of changes in the third stage resembles to that in the second stage. Basophilic stippled red blood cells appear only during the period between the first and the third week and never in other weeks; in seven out of the eight rabbits, of which four show as high as about 30 per cent. Number of reticulocytes increases in all rabbits during the whole course of experiment. The normoblast and the polychromasia are also seen. Met-haemoglo-

bin content increases in all rabbits, but not so much to present any significant difference to the contrast.

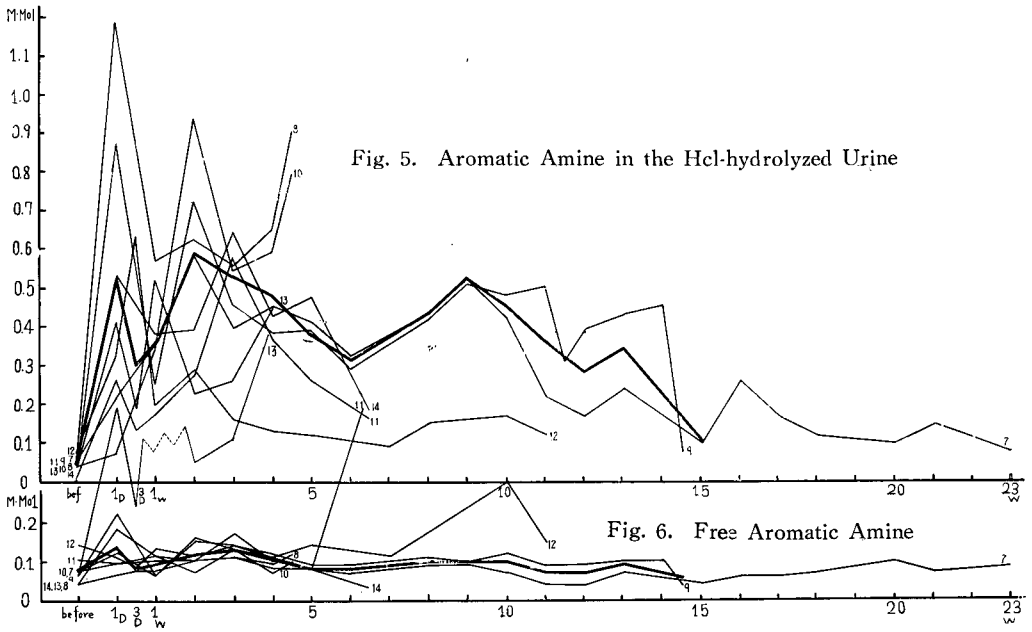



Fig. 5. Aromatic Amine in the Hcl-hydrolyzed Urine




Fig. 6. Free Aromatic Amine

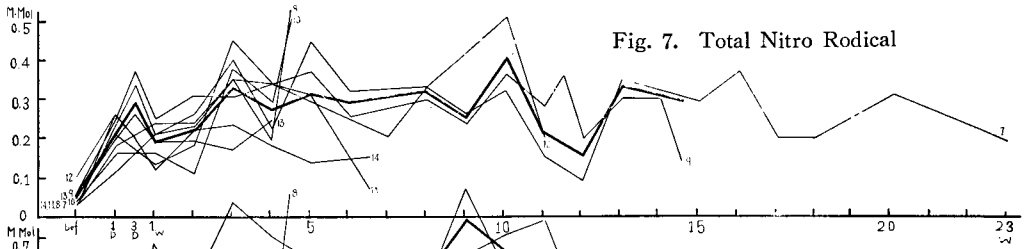



Fig. 7. Total Nitro Rodical

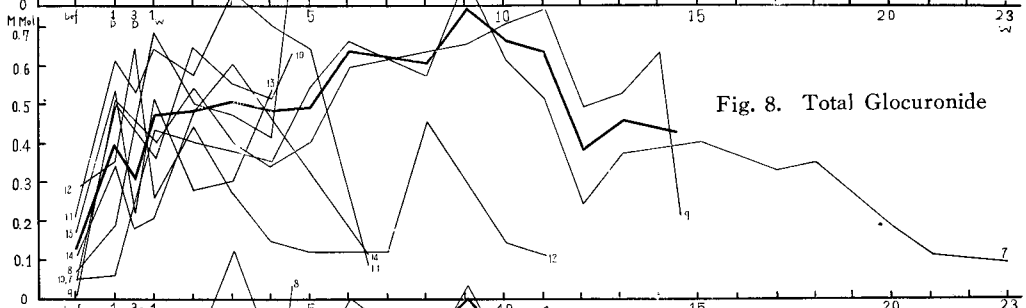



Fig. 8. Total Glocuronide

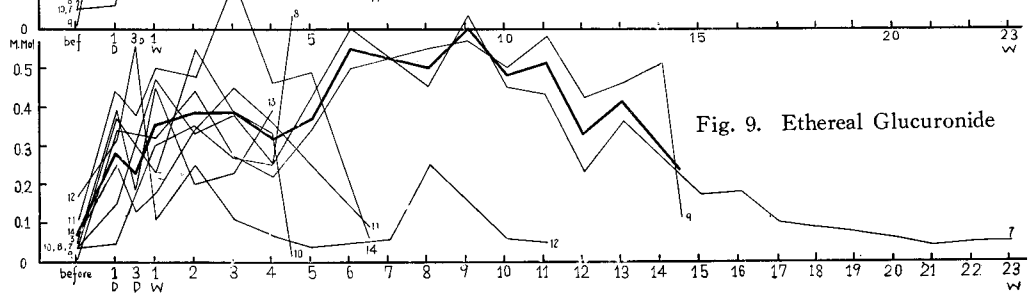



Fig. 9. Ethereal Glucuronide

Number of the white blood cell varies considerably but does not show definite figures (Fig. 3), and no significant difference could be seen between the experimental rabbits and the contrasts. Mean nuclear number also varies considerably, but the inclination of change resembles with one another (Fig. 4), left-shift in general and does not significantly differ from the contrasts. No definite change is observed in number of lymphocyte, monocyte, eosinophilic and basophilic leucocyte.

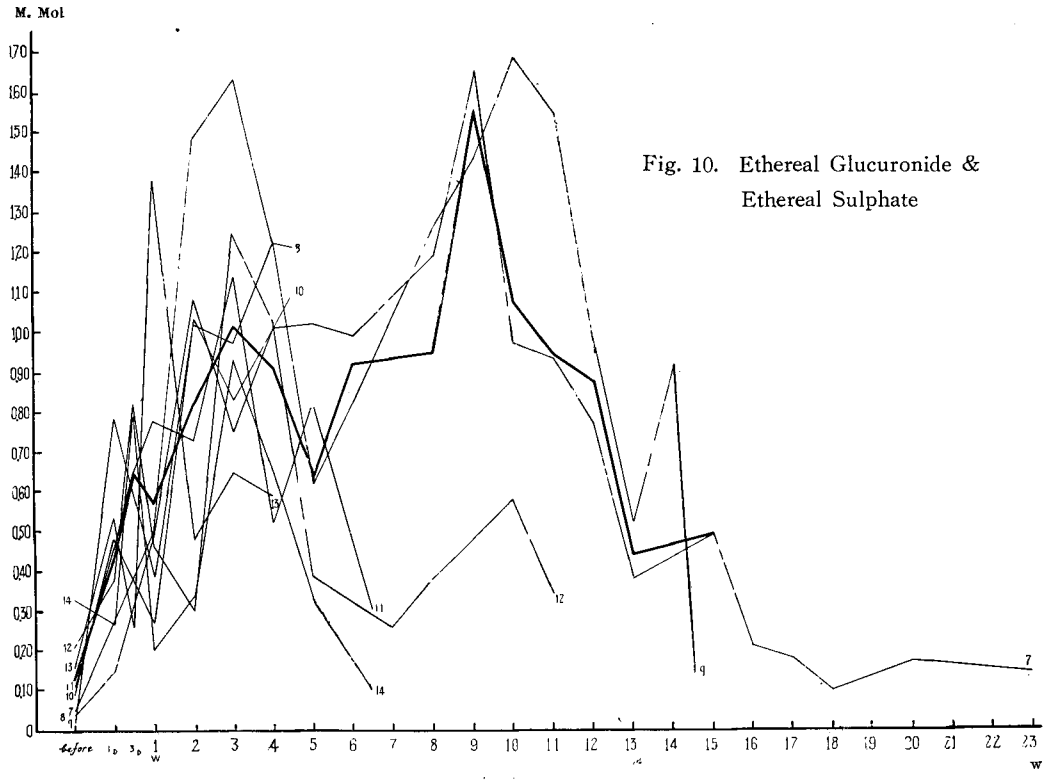



Fig. 10. Ethereal Glucuronide & Ethereal Sulphate

*Urine :*

The amount of excreted substances mentioned in the chapter on the methods is expressed in millimol.

The curve of total $NO_2$ (Fig. 7) shows biphasic peak during the first stage, rapidly increasing its amount after the first injection, followed by relatively small variation afterwards. Aromatic amine in the Hcl-hydrolyzed urine has a similar curve (Fig. 5) to that of tatal $NO_2$, but the fluctuation in the first stage is large and the peak of curve in the second stage is lower than that in the fiirt stage. Its amount excreted in the third stage decreases gradually to the normal level. The fluctuation of the amount of free aromatic amine (Fig. 6) is little throughout the whole course, except one example showing a curve with law biphasic peak in the first stage corresponding to the increase of aromatic amine in the Hcl-hydrolyzed urine. Ethereal glucuronic acid is a form which occupies naturally the most amount of the total glucuronic acid,

and its change is parallel to that of the latter (Fig. 8, 9). Both curves have peaks, much higher in the second stage than in the first stage contrary to the curve of aromatic amine in the Hcl-hydrolyzed urine, and begin to descend gradually at the end of the second stage and reach to the normal level. On the change in the amount of ethereal sulphuric acid the similar inclination is seen. Thus, the curve for hydroxylation (Fig. 10) quite resembles to that of the glucuronic acid.

In all of three rabbits Nos. 8, 10 and 13, which were dead in the fourth experimental week, unusual amount of total $NO_2$, aromatic amine in the Hcl-hydrolyzed urine and total glucuronic acid are excreted simultaneously into urine suddenly before death contrary to the survived ones. In two of them, the amount of ethereal glucuronic acide increases too, parallel to the total glucuronic acide, while it decreases in another rabbit No. 10 against the increase of the total glucuronic acid. The amount of free aromatic amine excreted by No. 13 rabbit is always higher than that of amine in the Hcl-hydrolyzed urine in contrast to the other cases. Immediately before death the rabbits Nos. 11 and 12 excreted a larger amount af free aromatic amine than that in the Hcl-hydrolyzed urine. These findings can be explained by the fact that a certain substance, which reacts in the Bratton-Marshall's test and is supposed to be aminophenylsulphate, is detected chromatographically in the original urine. Excretion of total and ethereal glucuronic acid in No. 12 rabbit is considerably little against others.

*Chromatography :*

Some of the chromatographical findings in the urine are shown in the Table 1, where the substances estimated by the aldehyde reagent or the phenolic reaction are expressed according to their Rf value respectively. Data on the substances estimated

Table 1

| | Original Urine | | Hcl-hydrolyzed Urine | | NaOH-hydrolyzed Urine | |
|---|---|---|---|---|---|---|
| Reaction / Rf | Amine | Phenol | Amine | Phenol | Amine | Phenol |
| 0.83 | | b) ± | c) ± | b) ± | | b) ± |
| 0.74 | | | a) ++ | a) ++ | c) ± | c) ± |
| 0.63 | e) +++ | c) ± | e) +++ | c) ± | e) +++ | c) ± |
| 0.60 | c) ± | c) ± | b) + | b) + | c) ± | c) ± |
| 0.51 | b) + | b) + | b) + | b) + | b) + | b) + |
| 0.48 | b) + | b) + | c) ± | c) ± | c) ± | c) ± |
| 0.45 | a) ++ | a) ++ | c) ± | c) ± | a) ++ | a) ++ |
| 0.38 | d) ++ | d) ++ | | | d) ++ | d) ++ |
| 0.31 | c) ± | c) ± | c) ± | c) ± | c) ± | c) ± |
| 0.24 | | | a) + | a) + | | |
| 0.22 | b) + | b) + | a) + | a) + | b) + | b) + |
| 0.15 | | | c) + | c) + | | |

r) detected always, b) appeared nearly every time, c) appeared sometimes, d) detected in the urine of the rabbit No. 13 through the whole period, e) Urea.

by the Bratton-Marshall's test or the reduction test are omitted from the table because constant data can not be obtained.

Three substances of Rf 0.48, 0.45 and 0.38 in the original urine fade away when hydrolyzed with hydrochloric acid and five substances appear newly at Rf 0.74, 0.60, 0.24, 0.22 and 0.15. These are supposed to be the substances which lose the sulphuric acid or the acetyl group. No difference is seen between the chromatogram of the original urine and that of NaOH-hydrolyzed except Rf 0.74 and 0.48. This is due to scanty amount of unstable glucuronide. According to the condition of experimental animal, number and size of spots, as well as the tone of colour may vary. They decrease in the emaciated state, especially the spot at Rf 0.24 in rabbits Nos. 7, 9 and 12 and at Rf 0.22 in rabbits Nos. 7 and 9 disappear in the last stage of the experiment. The spot at Rf 0.24 is not seen or too feeble to be seen on the first experimental day.

Change in quantity of ethereal and total glucuronide in ths urine of No. 10 rabbit in prelethal stage was described above. In this case a chromatogram of four distinct spots is developed by the reduction reagent and one of them (Rf 0.47) is supposed to be glucuronic acid and the others (Rf 0.61, 0.32 and 0.24) are to be sugars.

The findings in chromatographical study will vary in parallel with those of quantitative estimation in urine, chromatography is, therefore, assumed to be a convenient method to know the state of detoxication in the process of poisoning.

*Other symptoms :*

Generally speaking, the losses of appetite and body weight are seen in their emaciated state, especially noticeable in their last stage of poisoning. Emaciation is observed generally at the end of each stage of poisoning mentioned above. In agonal stage, rabbits are restless, irritable, and react spastically to stimulus.

The positive aldehyde reaction in urine is seen on the rabbits Nos. 8, 9, 10, 11 and 13 in their last stage, and the Nylander's reaction is positive on rabbits Nos. 10 and 13 in the prelethal stage.

Results of the cobalt-reaction on the serum of all rabbits show the shift to left in their last stage of poisoning, while little change is seen on the bromsulphalein-test. These findings signify the disturbed functions of liver and the pancreas in the last stage of poisoning, having verified by the histopathological findings described in the Table 2.

*Histopathological Findings :*

See the Table 2.

Table 2

| | |
|---|---|
| Liver | Yellowish appearance, intense stasis.<br>Fatty degeneration, irregular cellular cord, turbid swelling and karyolysis of liver cells.<br>Slight hyperplasia of connective tissues, considerably severe cellular in-filtration.<br>These findings are slight in degree on the rabbits Nos. 5, 7, 10 and 13. |

| | |
|---|---|
| Kidney | Swelling and adhaesion of glomeruli, sçanty fluid in glomeruli, slight cellular infiltration.<br>Slight hyaline droplet degeneration of tubular cell.<br>These findings are more remarkable on the rabbits Nos. 8, 9, 10 and 14 than the others. |
| Spleen | Enlargement is seen only on the rabbits Nos. 7, 9 and 12 which survived more than six weeks.<br>Intense stasis, opening of sinus and appearance of megakaryocytes, hypertrophy and hyperplasia of reticuloendothelial cells, yellowish brown pigments are seen in many cells.<br>Hypertrophia and hyaline degeneration of vascular wall.<br>Malpighian follicle is clearly observed. |
| Adrenal | Rabbits Nos. 8, 10, 11, 13 and 14 : The fascicullar layer is narrow in width. Degenerated darkish and small cellular groups with pyknotic nucleus, disorder of cellular cord and the lack of intracellular minute vacuole are seen in the fascicular layer.<br>Rabbits Nos. 7, 9 and 12 : The findings described above are rare. Formation of submembranous cortical nodules is seen.<br>In both groups of rabbits : Few findings in the reticular and glomerular layer and in the medulla. |
| Heart | Slight wax and vacuole degenerarion, atrophy of muscle. |
| Lung | Hypertrophy of alveolar wall and megakaryocytes in alveolar vessel are seen only on the rabbit No. 14. |
| Bone-Marrow | Increase in megakaryocyte. |
| Pancreas | Degeneration of Langerhans' Insule is seen in high grade only on the rabbit No. 13. |

## Discussions.

*A. Symptomatological Views :*

Aggravation of general symptoms such as loss of appetite and reduced body weight have been observed around the fifth and fifteenth week. Some of the experimental animals would die in the fifth week, but most of them are healthy in appearance and survive until the 15th week. Together with the aggravation of general symptoms, changes in blood and urine are observed.

With decrease in number of red blood cells and haemoglobin content, these experimental animals usually suffer with anaemia in the first four weeks, followed by recovery, and around the 15th week anaemia again recurs. The basophilic stippled red blood cells are seen only in the initial three weeks. No definite change in number of white blood cells is observed. The mean nuclear number of pseudoeosinophilic cells fluctuates markedly during the first five weeks and shows a biphasic depression of curve, indicating a high grade shift to left, followed by gradual recovery to the normal level.

The contents of total $NO_2$, total $NH_2$, total-and ethereal-glucuronic acid and sulphuric acid in urine are quite high, and show respective two peaks in the first four weeks. These high levels continue until the 15th week with a little fluctuation and descend.

These findings of the blood and urine are interpreted as signs of pronounced

reaction against poison in living being. For first five weeks, they react unstable. In the next ten weeks the body function will become a steady active state, characterized by hyperfunction such as hydroxylation. After this stage, the general condition gets continue bad to worse until death takes place, however, a transient recovery in blood findings has been noted.

Judging from the changes in general symptoms and the findings such as on blood and urine, the detoxication process of chronic poisoning, such as in this experiment is seemed to change its nature twice at the fifth and fifteenth week. So the whole course could be divided into three stages :— the initial response stage, the resistance stage and the exhaustion stage.

*B. Biochemical Views :*

Much has been reported on the detoxication of acute nitrobenzene poisoning and on some substances in urine such as ethereal sulphuric acid in case of the chronic form of poisoning in rabbit.[21-32] However, there are few which dealt more minutely with detoxication in chronic poisoning of nitrobenzene. For the minute study, the detoxication products in chronic nitrobenzene poisoning are pursued in this experiment with a special emphasis on the amination, acetylation and hydroxylation.

The variation and the change of the amount of total $NO_2$ and free aromatic amine in each animal are less throughout the whole course of the experiment. The amount of total aromatic amine excreted in urine which is regarded as the index of acetylation and amination varies remarkably in the initial stage of poisoning. The graphic curve shows biphasic peak in the initial stage and its higher one is somewhat higher than the peak in the second stage, which varies less than that in the first stage. In the third stage, the amount is scanty. On the other hand, the chromatographical detection of aromatic amine shows the same change as described above, and the spot at Rf 0.22 or 0.24 which is ascertained to be the glucuronide fades away when aromatic amine is scanty.

The excreted amount of ethereal-glucuronic acid and sulphuric acid regarded as the index of hydroxylation varies but not so large as that of aromatic amine. The peak of the curve in the second stage is about one half higher than the higher one in the first stage. Excretion in the third stage is scanty as in case of aromatic amine.

The above mentioned variation in the quality of detoxication products suggests that the mode of detoxication changes during the whole course; namely, the amination and acetylation surpass the hydroxylation in the initial stage, while the relation is reversed in the second stage, and a marked decline takes place in the latter stage.

From P. R. Venkataraman, A. Venkataraman and M. B. Lewis' interesting observations,[33] the degree of acetylation of p-aminobenzoic acid is increased and its conjugation with glucuronic acid is decreased by simultaneous administration of glycine or malic acid. R. T. Williams[24] recognized the existence of a possible relation between glucuronic acid conjugation and acetylation.

*C. Pathological Views :*

Histopathological findings show a definite change caused by nitrocompound poisoning, although some differences are observed in the liver, spleen and adrenal cortex between the animals died around the fifth week, which are refered as Group A hereafter for the convenience sake, and the ones survived after that week, which are referred as Group B hereafter.

In the liver, the fatty degeneration and destruction of liver cells are principal findings in the Group B, and are slightly stronger than those in the Group A. The only difference among the findings on the spleen between the Group A and B is the enlargement which may be caused by the continuous hyperfunction of spleen. This can be deduced from the micropathological findings mentioned above. The difference in micropathological findings in the adrenal cortex between the Group A and B is significant. Hypofunction in the Group A is deduced from the intense regressive degeneration and the lack of fatty granule in the fascicular layer, while hyperfunction in the Group B is due to a little or non degeneration of the cortex and the expansion of fascicular layer accompanied with numerous minute vacuoles.

Considering from the fact that under 3 to 5-fold longer term of poisoning the state of adrenal cortex in the Group B is sound, and the degree of injury of other organs is the same or less with that of the Group A, the Group B seems to be superior in the resistance against poisoning to the Group A, although it is not yet clear whether the resistance is congenital or acquired. Many investigators have studied on the activity of adrenal glands in an emregency, and Selye explained especially the activity in stress in relation with the hypophyseal gland. In this experiment also, the adrenal cortex is noticed to play an important role in the defense of body. Still it is a question whether or not any relation exists between the function of adrenal glands and the three stages of a chronic poisoning such as the present experiment. From the foregoing view, the course of chronic nitrobenzene poisoning in the present experiment can be divided into three stages, and the identical symptoms and with the state of sxcretion of detoxication products are seen on every experimental animal of the same poisoning stage, and no difference has been observed between the animals died in the early stage and ones survived. Animals tend to die at the change of stages. From the characteristic symptoms and detoxication products excreted in urine in each stage, one could decide as to what stage of poisoning experimental animals belong.

The specific feature which would indicate the degree of resistance to poisoning could not be found in the present experiment, althought the pathological findings on the adrenal cortex were the only difference between the animal died early and the other. However. the function of adrenal cortex and of some other endocrine organs and activities of nervous system or enzymsystem may suggest the registance to poisoning.

## Summary and Conclusion.

In order to study the detoxication mechanism and the bodily reaction in chronic

poisoning, the chronic nitrobenzene poisoning on rabbit was experimented and the changes have been pursued symptomatologically, biochemically and pathologically. The results are as follows:

1) Number of the red blood cell and the haemoglobin content decrease markedly in the early stage resulting in anaemia but the majority recover later, though not to the normal level. Also in the early stage, number of the white blood cell, percentage of the pseudoeosinophilic cell and of the lymphocyte, and the mean nuclear number vary greatly.

2) The excretion of detoxication products in urine is heavy and fluctuates in the early stage, while not so in the other stages. As to the detoxication process, amination, acetylation and hydroxylation are intense in the early and middle stage of poisoning, especially hydroxylation in the middle stage. They all become weak in the last stage of poisoning.

3) Judging from the above mentioned data, the whole process of poisoning could be divided into three stages, namely, the initial response stage, the resistance stage and the exhaustion stage.

4) The symptoms and the state of detoxication during the whole course of poisoning change from stage to stage and showed the characteristic feature of the respective stage.

5) Comparing the pathological findings of animals died early with those of survived ones, a marked difference is found only in the adrenal cortex, while it is not so much in other organs. Considering from these facts, the resistibility against poisoning in the latter animals seems to surpass that of the former ones, though it is a question whether it is congenital or acquired.

6) The detoxication mechanism and the resistibility against poison in chronic poisoning ought to be studied from the viewpoints of biochemistry, enzymology, neurology and endocrinology.

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