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**BIOCHEMICAL DECOMPOSITION OF COAL-TAR DYES I.
BIOCHEMICAL DECOMPOSITION AND IDENTIFICATION OF DECOMPOSED PRODUCTS**

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Abstract.....Biochemical degradation test of food coal-tar dyes using sludge was studied in order to evaluate their safety.

It was found that some dyes were little decomposed under aerobic condition but four azo dyes were readily decomposed under anaerobic condition. These were Food Yellow No.4, No.5, and Food Red No.2, No. 102.

Decomposed products of these four azo dyes by sludge under anaerobic condition were identified as sulfonilic, naphthionic acids and so on.

Keywords: aerobic decomposition, anaerobic decomposition, activated sludge, food additive, coal-tar dye, sulfanilic acid, naphthinic acid.

INTRODUCTION

There are suspicions of carcinogenesis of some tar dyes (Boffey, 1976). The toxicity of food additives should be evaluated with both food additives themselves and their decomposed products. But, the toxicity studied for food additives have scarcely been conducted. Only Matsui et al (1976) reported metabolism of cyclamates with the action of intestinal micro-organisms in connection with the toxicity of the decomposed products. Food additives may be transformed not only in human body but also in environment. There is no administrative control for color of effluent by the Water pollution Control Law, and the risk of mass discharge of dye stuffs into the public water from the effluents. When food additives factories deliver a

part of the products to the effluents, they are subjected to microbial action.

Therefore, it is very important to study metabolism of food tar dyes in the environment. The elucidation of mechanism of tar dye metabolism in the environment and the assessment of toxicity of the decomposed products are the ultimate purposes for our investigation. In this report the microbial decomposition of tar dyes by sludge and the chemical structure of decomposed products are studied.

EXPERIMENTS

1. Materials

(1) Sludge : Return activated sludge was obtained from the municipal sewage treatment plant, Nakahama, Osaka. The return sludge was acclimated to the synthetic sewage for a week or longer, and it was used for the aerobic and anaerobic decomposition experiments.

(2) Synthetic sewage : Glucose, pepton and potassium dihydrogen phosphate, 30g each, were dissolved in 1 liter water and the pH was adjusted to pH 7.0 with sodium hydroxide.

(3) Seeded dilution water : To 1 liter, 10 ml of supernatant of sludge was added.

(4) Tar dyes and relating compounds : Ten kinds of authentic compounds were obtained from the National Institute of Hygienic Sciences. Sodium sulfanilate and sodium naphthionate (Guaranteed reagents) were obtained from Nakarai chemical Co. Ltd., and recrystalized three times from ethanol. Amino-R acid*¹ salt, amino-G acid*² salt, and amino-S acid*³ salt were synthesized with ponceau'R (C.I. 16150), Orange G (C.I. 16230) and Brilliant Orange H (C.I. 16020), respectively (Namikura, 1976) and recrystallized from ethanol. All the other chemicals were guranteed reagents.

2. Instruments

(1) Thin layer chromatography (TLC) : Thin layer plates were prepared with Kieselgel GF₂₅₄ (TYP 60 from Merck) and avicel SF cellulose from Funakoshi Chemicals Co. Ltd., and developed with n-BuOH : EtOH : water : 28 % ammonia (80 : 20 : 10 : 20 : 1), and with ethyl acetate : glacial acetic acid : water (40 : 20 : 20), respectively.

(2) Spectrophotometer : Hitachi double-beam spectrophotometer, type 565.

(3) Warburg's smnometer : Asahi Riken Industry Co. Ltd.

(4) Dissolved oxygen meter : Beckmann type 10800 apparatus.

(5) High performance liquid chromatography (HPLC) : Typek-8860 apparatus, Kyowa Seimitsu Co. Ltd., was equipped with U. V. detector at 254 nm and an

*1 Amino-R acid : 1-amino-3-naphtho1-3,6-disulfonic acid

*2 Amino-G acid : 1-amino-2-namino1-6,8-disulfonic acid

*3 Amino-S acid : 1-amino-2-naphto1-9-sulfonic acid

anion exchange column AAX (1m×2.1mm ϕ I. D.).

2. Method

2.1 Aerobic decomposition

2.1.1 Aerobic decomposition of dyes with sludge – To 750 ml of sludge (MLSS ca. 3,000 ppm) 250 ml of 0.03 M dye solution was added, and bubbled with air sufficiently at 20°C. 5 ml sample was taken out once a day. After sampling 5 ml of synthetic sewage was added to the mixture. Each sample was filtered through filter paper and diluted twenty times prior to the spectrophotometric measurement at the absorption maximum within the visible range. The decrease of dyes concentration was expressed in terms of percent to the initial absorption.

2.1.2 Oxygen uptake of sludge – 2.0 ml of sludge, 0.2 ml of 1,000 ppm dye solution, and 0.2 ml of 20% potassium hydroxide were pipetted into the vessel, the side arm and central well, respectively. The sludge and the dye solution were mixed and the vessel was shaken at 25°C. The oxygen uptake was measured. The oxygen uptake by sludge alone was subtracted from the these by dyes addition.

2.1.3 Determination of BOD – Dye solutions (10, 20, and 40 ppm) were prepared with the seeded dilution water and kept at 20°C (Japanese Industrial Standards Committee, 1971). The dissolved oxygen contents were measured by a dissolved oxygen meter, because of the coloration of the solutions.

2.2 Anaerobic decomposition

2.2.1 Anaerobic decomposition of dyes with sludge – To 750 ml of sludge (MLSS ca. 3,000 ppm), 250 ml of 0.03 M dye solution was added and stood in room with stirring by a magnetic stirrer, 5 ml of the solution was taken out once a day for the measurement described in 2.1.1. After every sampling, nitrogen gas was introduced into the solution and the vessels were closed tightly to shut the air. For anaerobic decomposition experiments, generally, digested sludge will be used, but in this experiment, sewage acclimated sludge was used, because the digested sewage gives too large blank values and interfere to spectra absorption.

2.2.2 Quantitative determination of anaerobic decomposed products – The reaction mixture was filtered and concentrated upto 25 ml under reduced pressure at 50°C. The concentrates were subjected to subsequent separation and identification. Method I : Each concentrate was spotted on a silica gel GF₂₅₄ thin layer plate, and the plate was developed with a solvent mixture of n-butanol : ethanol : water : 28% ammonia (80 : 20 : 10 : 1) and examined under U. V. lamp. For the coloration by coupling reaction, on the plate 5 % NaNO₂ - 1 % HCl (1 : 1) was sprayed and then 1-maphol-4-sulfonic acid (NW acid) saturated ethanol prior to exposure to ammonia vapour.

Method II : After separation a coupling reagent containing 1-(4-sulfophenyl)-3-methyl-5-pyrazolone instead of NW acid was sprayed on the silica gel thin layer

plate.

Method III : A cellulose (avicel SF) thin layer plate was developed with a ethyl acetate : acetic acid : water (40 : 20 : 20) mixture and examined under U. V. lamp. For the coloration by coupling reaction, the plate was sprayed with 2.5% CuCl_2 , 2.5% NaNO_2 , 1% Na_2CO_3 , and 1% resorcinol solutions, successively.

2.2.3 Quantitative determination of anaerobic decomposed products - Concentrate of the reaction mixture was charged on a thin layer plate by the previous method and developed. Each spot was scraped from the plate and extracted with water. 10 ml of the extract was charged on high performance liquid chromatography. The column was eluted with 0.1 M KH_2PO_4 - K_2HPO_4 buffer (PH 8.0) at 60 kg/cm². The chart speed was 5 mm/min. The calibration curves obtained were linear upto 0.0001 M for both sodium sulfanilate and sodium naphthionate. The recovery rates were 80% and the coefficients of variation were less than 5%.

RESULTS AND DISCUSSION

1. Aerobic decomposition

1.1 Aerobic decomposition of dyes with sludge.

During 10 days aerobic experiment, the absorbance of dye solution was measured once a day for 10 kinds of dyes. The results are shown in Fig. 1. Red No.3, No. 104, No.105 and Blue No.2 were scarcely decreased, so the results of these dyes were omitted.

All the examined dyes were decomposed extremely slowly. The decomposition of

Fig. 1. Aerobic Decomposition of Dyes by Sludge

* estimated by absorbance at λ_{max} of each dye. 250 ml of dye sol. (0.003M) and 750 ml of sludge sol. (3000 ppm) were mixed.

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Food Blue No. 1 and Red No. 106 were essentially negligible. Furthermore commercial azo dyes in the most popular used at present, such as Yellow No. 4 and No. 5 and Red No. 2 and No. 102 were decomposed only about 20% during the 10 days. Free acids of xanthine dyes, such as Red No. 3, No. 104, and No. 105 were precipitated due to the slight acidity (PH 6-7) of the solutions.

1.2 Effects of dyes on oxygen uptake of sludge

A time course of oxygen uptake of sludge was measured for 5 hours in order to evaluate the action of dyes on microorganisms. The results are shown in Table 1. Extremely low oxygen uptake by sludge was observed in all the experiments. Even the highest value observed for Food Yellow No. 4 was merely 3.81 O₂-mg/h·g of sludge. The negative oxygen absorption was observed on xanthine dyes, suggesting possible inhibition of microbial respiration by the dyes. Sato and Akiyama (1972) reported that acetate, which might be located pretty in natural environment, gave 34.5 O₂-mg/h·g of sludge for oxygen uptake and 88.4 mg-O₂/h·g of sludge for the decomposition rate, respectively. Compared with these values, the food tar dyes tested in this report seem to be resistant to oxydative decomposition.

1.3 Determination of BOD

In all experiment, the dissolved oxygen contents on the 5th day were essentially the same to initial ones. The low reactivity of aerobic sludge towards dyes was confirmed.

2. Anaerobic decomposition of dyes

2.1 Anaerobic decomposition with sludge

The anaerobic decomposition of several dyes was measured for 10 days. The results are shown in Fig. 2. Xanthine dyes, Red No. 3, No. 104 and No. 105, were not used because of their precipitation.

Table. 1. Effects of dyes on Oxygen uptake by Sludge

Food dyes added to sludge		Oxygen absorption (O ₂ -mg/hg-sludge)*				
		1	2	3	4	5 (hrs)
Food Yellow	No. 4	3.22	2.34	2.38	1.72	3.81
"	No. 5	1.60	3.24	2.19	3.67	2.82
Food Red	No. 2	2.38	1.60	1.90	1.29	2.00
"	No. 102	2.19	2.12	1.69	1.38	2.34
Food Red	No. 3	1.79	0	-0.86	-2.12	-1.17
"	No. 104	2.26	1.93	0.55	0	-0.07
"	No. 105	0.79	1.22	-0.23	-0.67	-1.24
"	No. 106	0.65	1.43	1.22	0.73	-0.10
Food Blue	No. 1	3.05	1.98	2.38	1.74	3.34
"	No. 2	2.86	1.34	1.67	0.76	2.15

* These values were estimated by Warburg method. 0.2 ml of dye sol. (1000 ppm) were added to 2.0 ml of sludge (3000 ppm).

Fig. 2. Anaerobic Decomposition of Food Dyes by Sludge

* estimated by absorbance $\lambda_{\max}$ of each dye. 250 ml of dye sol. (0.003 M) and 750 ml of sludge sol. (3000 ppm) were mixed.

The dyes decomposed more rapidly, under the anaerobic condition than the aerobic complete fading took place within 3-6 days in the solutions of azo dyes. The fading might be attributed to the partial reduction of azo compound at the double bond in anaerobic circumstance (Singh, 1970).

2.2 Mode of the anaerobic decomposition of azo dye

In order to study the time course of the decomposition, the change of ultraviolet and visible spectra were measured for 4 kinds of azo dyes which were decomposed remarkably in the anaerobic circumstance. The typical spectra of Food Yellow No. 5 is shown in Chart 1.

The absorbance at 480 nm was decreased gradually and that of 260 nm was increased with isosbestic points at 250, 270 and 360 nm. This phenomenon might be occurred by cleavage of the conjugated double bond and the production of a new compound with maximum absorption at 260 nm. The similar alteration pattern were noted in spectra obtained during the decomposition of the other dyes. The new maximum absorption were at 250, 240, and 245 nm for Food Yellow No.4, Red No.2, and Red No.102, respectively. Yamabe (1972) reported that the most of dyes were reduced by microorganisms in intestine and formed aromatic amines which were excreted into feces. The first step of anaerobic decomposition of azo dyes with sludge was supposedly the reductive cleavage of azo bond.

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Chart. 1. Influence of Anaerobic Decomposition of Absorption Spectra of Food Yellow No.5

* Number (0, 2, 3, 4, 5) shows culture periods (day). 250 ml of dye sol. (0.003 M) and 750 ml of sludge sol. were mixed.

Fig. 3. Thin-Layer Chromatogram of Decomposition Products of Food Dyes

Adsorbent : Kieselgel GF₂₅₄ (Typ 60)
Solvent ; n-Butanol : Ethanol : Water
28%-Ammonia=80 : 20 : 10 : 1

Avicel SF (Cellulose powder) ;
Ethyl acetate ; Acetic acid : Water
=40 : 20 : 20

Sample 1 : Food Yellow No.4
3 : Food Red No.2
5 : Mixture of standard

2 : Food Yellow No.5
4 : Food Red No.102

The Shadowed spots are those which have fluorescence under UV irradiation

3. Thin layer chromatography of anaerobic decomposed products

3.1 Identification on a silica gel thin layer plate

After anaerobic decomposition for 10 days, each reaction mixture was concentrated and analyzed. The results are shown in left side of Fig. 3.

Two spots were observed for each compound and one spot remained at the original place. The spots A and B were pale black and had the same R_f value to those of sulfanilic acid. The spots C and D were blue white and had the same R_f values to those of naphthionic acid, when coupling reaction by method I was applied to the plate (Kamikura, 1967), spots A and B colored orange, and spots C and D colored red. By method II, spots A and B colored yellow and spots C and D did yellowish brown. By R_f value and color reaction, spots A and B were identified as sulfanilic acid and spots C and D were as naphthionic acid.

3.2 Identification on a cellulose thin layer plate

The spots remained at the original place on a silica gel plate fluorescent, so they should be highly polar substances and were supposed to be relating compounds to R, G, and S acids which were formed through cleavage at the azo bond of dyes. In order to separate these polar substances, each concentrate was spotted on a cellulose thin layer plate and developed by suitable solvent for the separation of polar substances. The results are shown in right side of Fig. 3. The spots of those observed under U. V. lamp might correspond to the spots remained at the original place. By R_f values and coloring coupling reagents, the fluorescent substances contained in No. 2, No. 3, and No. 4 were identified as amino-S, amino-R, and amino-G acids, respectively. The lower spot of No. 1 colored orange red by Method III was identified as 1-(4'-sulfophenyl-3-carboxy-4-amino-5-hydroxypyrazole) (Kamikura, 1967). All of these substances were proved to be the compounds formed from original dyes by reduction at azo bond.

4. Quantitative determination of anaerobic decomposed products of dyes by high performance liquid chromatography

The high performance liquid chromatography (HPLC) was utilized for the quantitative determination of sulfanilic and naphthionic acid (Singh, 1974) in the reaction mixture of Food Yellow No. 4 of No. 5, and Food Red No. 2 or No. 102, after 10 days incubation. Recoveries with the authentic samples for these dyes were about 80%. The coefficient of variations were less than 5%. The results are shown in Table 2.

Theoretically one mole of these azo dyes may produce one mole of either sulfanilic or naphthionic acid. The yield estimated by HPLC under correction for the recovery accounted to about 50%. The fading of the solution was complete, so that the other 50% were possibly subjected to further decomposition or microbial incorporation into the sludge. The details are under investigation.

5. Pathways of anaerobic decomposition of azo dyes by sludge

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Table. 2. Quantitative determination of Biochemical Decomposition Products of Azo dyes used by anaerobic Sludge

Decomposion product	Prodction ratio to added dye (%)*			
	Exp. 1	Exp. 2	Exp. 3	Average
sulfanic acid produced from				
Food Yellow No. 4	48.2	55.8	59.7	54.5
Food Yellow No. 5	43.7	39.5	38.5	40.6
naphthionic acid produced from				
Food Red No. 2	70.3	59.6	67.1	65.7
Food Red No. 102	43.9	60.7	42.0	48.9

* μg of sample sol. (0.03 M) were spotted on TLC plates from which test solution was made by extraction with 1 ml, and sulfanilic and naphthionic acids were determined by HPLC.

Chart. 2. Hypothesis of Reductive Decomposition Pathway of Azo Dyes.

The results of the absorption spectra change (Chart 1) and the thin layer chromatogram (Fig. 3) led us to propose the anaerobic decomposition pathways as shown in Chart 2 for these 4 dyes.

By these observations, it was concluded that aerobic decomposition of azo dyes hardly took place in the environment, whereas anaerobic microbial decomposition proceeds considerably. The first step of anaerobic decomposition appears to be reduction at the azo bond, that is, splitting off a compound with amino group via an unidentified intermediate of hydrozo compounds.

CONCLUSION

Microbial decomposition of food coal-tar dyes by the sludge was investigated as a step in the evaluation of toxicity of tar dyes and the following results were obtained.

1. The dyes were hardly decomposed aerobically. From the oxygen uptake by Warburg's manometer and from BOD determination, the low activity of the sludge to dyes was shown.
2. In anaerobic decomposition experiments with the sludge, complete fading was observed in all the solutions of azo dyes including Food Yellow No. 4 No. 5, and Food Red No. 2, No. 102, within 3-6 days.
3. The first step of the anaerobic decomposition may be the reductive cleavage at the azo bond, because compounds produced by reduction at the azo bond were identified by TLC.
4. The decomposed products of the dye by sludge under anaerobic condition were determined quantitatively by high performance liquid chromatography. The amounts of sulfanilic or naphthionic acids formed by decomposition during the 10 days accounted for about 50% of the initial contents of the dyes on a molar basis.

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