Studies on the Origin of Mutagenic Impurities in Commercial Lithol Rubine B

MAMORU MIYAGOSHI, YUMIKO HAYAKAWA, MINORU NAGATA

and TOMIO NAGAYAMA

Tokyo College of Pharmacy, 1432-1, Horinouchi, Hachioji, 192-03, Japan

(Received September 16, 1986)

In order to clarify the origin of mutagenic impurities in the commercial cosmetic Lithol Rubine B (C.I. 15850) and Lithol Rubine BCA (C.I. 15850 : 1), several dye samples were synthesized under various conditions and their ethyl acetate extracts were tested for mutagenic activities in the Ames test. When the amount of diazotized 2-amino-5-methylbenzenesulfonic acid in the diazo solution was more than that of 3-hydroxy-2-naphthalenecarboxylic acid in the coupling solution in terms of the theoretical amount, mutagenic compounds were produced as reaction intermediates or by-products without exception. However, mutagenic compounds were never produced when the amount of the former was less than that of the latter in terms of the theoretical amount. The reaction temperature did not affect the production of mutagenic compounds. The mutagenic compounds in the synthetic Lithol Rubine BCA could be removed from the calcium lake by rinsing with water.

Keywords—mutagenicity; Ames test; commercial azo-dyes; impurity; Lithol Rubine B; Lithol Rubine BCA

Introduction

Intensive studies have been carried out on the mutagenic activities of azo-dyes.¹⁾ However, only a few papers²⁾ have referred to the mutagenic activity of the impurities contained in commercial dyes, such as starting materials, reaction intermediates and by-products. In our previous studies,³⁾ commercial samples and purified chemical samples were tested for mutagenic activities in the Ames test and the results made clear whether the mutagenic activities of the commercial samples were due to impurities or not. In the case of the commercial Sudan III (C.I. 26100; C.I. Solvent Red 23) and Scarlet Red (C.I. 26105; C.I. Solvent Red 24), the starting materials of the azo-dyes, *i.e.* *p*-aminoazobenzene and *o*-aminoazotoluene, were found to be the main mutagenic contaminants.^{3a)} On the other hand, the results suggested that the

mutagenic activities of all commercial Lithol Rubine B (C.I. 15850; C.I. Pigment Red 57; D & C Red No. 6) dyes were not due to contamination with the starting materials, *i.e.*, 2-amino-5-methylbenzenesulfonic acid (2A5MBSA) and 3-hydroxy-2-naphthalenecarboxylic acid (3H2NCA), but due to reaction intermediates or by-products contained in the samples, because both the purified chemical sample and the starting materials had no mutagenic activity.^{3b)} The azo-dye was discovered in 1903 by Gley and Siebert.^{4a)} The dye was prepared by coupling diazotized 2A5MBSA with 3H2NCA,^{4a)} and it has been primarily used as a pigment in the form of the alkaline earth metal salts, especially the calcium salt. The calcium lake is used in printing ink (rich bluish undertone and bronze overtone with good strength and covering power), in paints and is also

Lithol Rubine B

Fig. 1. Synthesis of Lithol Rubine B

used for cosmetics.^{4b)} Since the mutagenic activities were found in all samples of commercial Lithol Rubine B,^{3b)} it was suggested that the mutagenic principles might be reaction intermediates or by-products inevitably produced during the manufacture of the dye. In the case of Lithol Rubine BCA (C.I. 15850 : 1; C.I. Pigment Red 57 : 1; D & C Red No. 7), only two out of nine commercial samples were mutagenic (22.2%), in marked contrast to the samples of commercial Lithol Rubine B (100%).^{3b)}

In the present investigation, we studied the effects of various conditions on the production of the mutagenic compounds during the synthesis of Lithol Rubine B, and established the reason for the low frequency of positive mutagenicity in commercial Lithol Rubine BCA.

Materials and Methods

Commercial Dye Samples—The commercial dyes collected previously^{3b)} were re-used in this study.

Synthesis of Lithol Rubine B⁵⁾—An outline of the synthesis of the dye is shown in Fig. 1. The starting materials were extra-pure reagents without further purification (they were confirmed to be homogeneous on thin layer chromatography). At first, 11.6 g of 2A5MBSA (0.062 mol) was added to 200 ml of water containing 2.7 g of NaOH (0.067 mol) and completely dissolved at 35°C with stirring. Then 13 g of HCl (0.125 mol) was added and the mixture was stirred for 20 min, then cooled to 0–5°C with ice. A solution of 4.4 g of NaNO₂ (0.064 mol) in 50 ml

of water was slowly added over a period of 15 min and a diazo solution was obtained after stirring for 30 min at below 5°C. On the other hand, 11.8 g of 3H2NCA (0.063 mol) was added to 200 ml of water containing 5.4 g of NaOH (0.125 mol) and dissolved at 40°C with stirring to provide a coupling solution, after cooling with ice to 10–15°C. The diazo solution was slowly added to the cooled coupling solution over a period of 30 min with stirring and the mixture was stirred for an additional 1.5 h at a temperature below 15°C. The pH was adjusted to 8.3–8.5 during the additional stirring. After the coupling reaction, a crude product was separated from the reaction mixture by centrifugation and the precipitate was completely dried in an electric oven at 80°C.

When the effect of the ratio of the starting materials on the production of the mutagenic impurities was to be studied, the synthetic conditions were the same as above except for changing the amount of the starting materials in the diazo and coupling solutions.

Synthesis of Lithol Rubine BCA⁵⁾—The laking reaction of Lithol Rubine B was performed as follows. First, 7 g of the completely dried dye was dissolved in 1000 ml of water and the solution was heated at 90°C. Next, a solution of 3 g of CaCl₂ in 100 ml of water was added to the dye solution and the mixture was stirred. After an hour, the calcium lake was separated from the reaction mixture by filtration with suction, and dried at 80°C.

Extraction of Impurities—The impurities in 5 g of the commercial dyes and 15 g of authentic samples synthesized under various conditions were extracted in a Soxhlet extractor with 500 ml and 1500 ml of ethyl acetate, respectively, until the leachings were practically colorless.⁶⁾ The ethyl acetate was removed under reduced pressure, and the extracts obtained were dissolved in DMSO for the Ames test.

Mutagenesis Assay—The assay was carried out according to the modified Ames test procedure.⁷⁾ *Salmonella typhimurium* tester strains were kindly provided by Prof. Isao Tomita, Shizuoka College of Pharmacy, Shizuoka, Japan. S9 was prepared from the liver of male rats of SD strain treated with PCB.⁸⁾ 4-Nitroquinoline-1-oxide (4NQO) and benzo[*a*]pyrene

(B[a]P) were used as controls in each experiment to monitor the sensitivity of TA 98 and TA 100.

Results and Discussion

1. Lithol Rubine B

Table I showed the results of the Ames test of the impurities in commercial Lithol Rubine B. Three out of six commercial dye samples which contained impurities in amounts sufficient to be extracted with ethyl acetate from the samples used in the previous study^{3b)}

were tested. All the ethyl acetate extract were mutagenic. The mutagenic impurities were concentrated by the extraction procedure, because the mutagenic activities were stronger than those of the commercial samples themselves, whereas the residues had no mutagenic activity. Therefore, ethyl acetate extraction with a Soxhlet extractor was available to separate the impurities from the samples, as found by Kozuka *et al.*⁶⁾ Since all the impurities in the

TABLE I. Mutagenic Activities of Commercial Lithol Rubine B Dyes and Their Ethyl Acetate Extracts

Test compound	Concentration ($\mu\text{g}/\text{plate}$)	Number of His ⁺ revertants/plate			
		TA 98		TA 100	
		- S9 ^{a)}	+ S9 ^{a)}	- S9	+ S9
DMSO ^{b)}	-	24.9(16) ^{e)}	43.1(12)	103.3(11)	105.8(8)
4NQO ^{d)}	0.05	-	-	412.8(12)	-
	0.10	165.1(16)	-	558.8(12)	-
	0.20	209.6(16)	-	-	-
B[a]P ^{d)}	2	-	311.5(8)	-	394.5(6)
	5	-	904.3(7)	-	864.3(6)
Lithol	5	20 ^{f)}	36	124	107
Rubine B-1 ^{e)}	50	27	40	117	117
(original) ^{g)}	500	73 ^{h)}	44	98	113
	1000	115	48	119	118
Lithol	1	56	41	118	103
Rubine B-1	10	485	490	118	111
(extract) ^{g)}	100	1181	1100	160	135
Lithol	5	33	35	115	109
Rubine B-1	50	28	37	94	111
(residue) ^{g)}	500	35	33	108	110
	1000	39	40	119	115
Lithol	5	24	33	83	125
Rubine B-4	50	38	35	93	115
(original)	500	58	29	84	109
	1000	115	31	71	112
Lithol	1	66	53	118	119
Rubine B-4	10	404	356	146	132
(extract)	100	612	653	172	163
Lithol	5	26	35	113	121
Rubine B-4	50	26	32	100	104
(residue)	500	37	34	83	111
	1000	39	35	101	122

TABLE I. (continued)

Test compound	Concentration ($\mu\text{g}/\text{plate}$)	Number of His ⁺ revertants/plate			
		TA 98		TA 100	
		- S9 ^{a)}	+ S9 ^{a)}	- S9	+ S9
Lithol	5	23	24	101	115
Rubine B-5	50	29	33	92	121
(original)	500	30	37	89	83
	1000	<u>78</u>	35	91	84
Lithol	1	37	40	124	118
Rubine B-5	10	<u>155</u>	<u>134</u>	121	120
(extract)	100	<u>514</u>	<u>603</u>	144	128
Lithol	5	31	30	121	113
Rubine B-5	50	32	29	115	109
(residue)	500	31	33	100	115
	1000	31	22	103	120

a) -S9, without S9 mix ; +S9, with S9 mix.

b) DMSO : solvent control.

c) Mean (number of experiments).

d) 4NQO, reference compound without S9 mix ; B[a]P, reference compound with S9 mix.

e) The numbers indicate the reference numbers of commercial dye samples used in Ref. 3b.

f) The numbers of revertant colonies are the averages of two assays.

g) Original : commercial intact dye sample (data from Ref. 3b). Extract : impurities extracted with ethyl acetate from the commercial dye. Residue : commercial dye sample after removing the impurities by extracting with ethyl acetate.

h) Underlining indicates that the sample was mutagenic at the concentration tested.

commercial samples were mutagenic, it was thought likely that the mutagenic impurities were inevitably formed in the commercial dyes during manufacture, rather than being simply extraneous contaminants.

Reaction Ratio The effect of the ratio of the diazotized solution contained 2A5MBSA as a diazo component (diazo solution) to the solution containing 3H2NCA as a coupling component (coupling solution) on the formation of the mutagenic compounds was tested. The results are shown in Table II. It is clear that the mutagenic compounds were always formed when the amount of the diazotized 2A5MBSA in the diazo solution was more than that of 3H2NCA in the coupling solution in terms of the theoretical amount (see Materials and Methods), whereas no mutagenic activity was detected when the amount of the former was less than that of the latter. Since a diazonium salt in water is only stable at low temperature, an

increase of the temperature causes decomposition of the salt.⁹⁾ Thus, the mutagenic impurities may be formed as a result of the use of a greater-than-theoretical amount of the diazo compound in the commercial synthesis of Lithol Rubine B.

Reaction Temperature The effect of the reaction temperature on the formation of the mutagenic compounds was tested. The dyes were synthesized under the same conditions except that the reaction temperature was 40°C during the diazo-coupling reaction. Since the Ames test of the dyes synthesized at 40°C and their extracts with ethyl acetate gave results approximately similar to those in Table II (data not shown), the temperature of the diazo-coupling reaction did not affect the formation of the mutagenic compounds.

In the case of TA 98, there was little difference between the mutagenic activities of commercial dyes (Table I) and those of the syn-

TABLE II. Mutagenic Activities of Synthetic Lithol Rubine B Dyes Synthesized under Various Conditions and Their Impurities

Test compound	Concentration ($\mu\text{g}/\text{plate}$)	Number of His ⁺ revertants/plate			
		TA 98		TA 100	
		- S9 ^{a)}	+ S9 ^{a)}	- S9	+ S9
Lithol Rubine B (3 : 2) ^{b)} (original) ^{d)}	1	24 ^{c)}	42	91	89
	10	33	58	90	102
	100	57	83	91	101
	1000	<u>67^{e)}</u>	57	99	103
Lithol Rubine B (3 : 2) (extract) ^{d)}	1	34	42	98	110
	10	<u>131</u>	44	102	113
	100	<u>788</u>	<u>176</u>	<u>291</u>	<u>213</u>
Lithol Rubine B (6 : 5) (original)	1	29	36	113	103
	10	24	43	119	96
	100	41	44	121	79
	1000	<u>63</u>	29	151	93
Lithol Rubine B (6 : 5) (extract)	1	30	50	111	111
	10	<u>109</u>	73	115	119
	100	<u>507</u>	<u>537</u>	<u>281</u>	<u>244</u>
Lithol Rubine B (2 : 3) (original)	1	21	41	81	110
	10	22	57	87	112
	100	23	35	98	104
	1000	24	44	100	109
Lithol Rubine B (2 : 3) (extract)	1	24	38	101	111
	10	28	47	103	108
	100	28	48	110	103

a) -S9, without S9 mix; +S9, with S9 mix.

b) The ratio in parenthesis is that of the amounts of diazotized 2A5MBSA and 3H2NCA in the diazo and coupling solutions, respectively, in the reaction system.

c) The numbers of revertant colonies are the averages of two experiments.

d) Original: intact dye sample before the ethyl acetate extraction. Extract: impurities extracted with ethyl acetate from the sample.

e) Underlining indicates that the sample was mutagenic at the concentration tested.

thesized samples (Table II). In the case of TA 100, however, the extracts of synthesized samples were more mutagenic than those of commercial dyes. We may speculate that another mutagenic impurity was formed in the synthetic samples under the conditions used.

Thin layer chromatograms of ethyl acetate extracts of 3 commercial and 3 synthetic samples of Lithol Rubine B are shown in Fig. 2. The ethyl acetate extract of synthetic Lithol Rubine B (2 : 3) contained only one substance away from the origin, and this was

not mutagenic (Table II). The other samples, however, contained many substances. Therefore, these results suggest that various mutagenic impurities may be formed, depending on the conditions of preparation.

2. Lithol Rubine BCA

In the case of commercial Lithol Rubine B, only two out of nine commercial samples showed mutagenic activities (22.2%).^{3b)} Therefore, we investigated whether the calcium in the lake controlled the mutagenic activities or whether the mutagenic impurities were remo-

Fig. 2. TLC Patterns of Ethyl Acetate Extracts
Samples were applied to a Silica-gel 60F₂₅₄ TLC plate (20 × 20 cm, 0.25 mm thickness, purchased from Merck Co., Ltd.) and the plate was developed with chloroform-methanol (9 : 1, v/v) at room temperature. The spots were detected under ultraviolet irradiation. The shadowed spots are those of colored substances.

ved during the laking reaction.

Laking Reaction The mutagenic activities of Lithol Rubine BCA (3 : 2) synthesized from mutagenic Lithol Rubine B (3 : 2) and the ethyl acetate extract of the lake were examined by means of the Ames test (Table. III). The results showed that Lithol Rubine BCA (3 : 2) was not mutagenic at the concentration tested, though its ethyl acetate extract was mutagenic, indicating that calcium did not inhibit the mutagenic activities of impurities in the lake.

Rinsing with Water A sample which had been rinsed well with water after the laking reaction and its ethyl acetate extract were tested for mutagenic activities. These samples showed no mutagenic activity, suggesting that the mutagenic compounds were water-soluble.

Generally, commercial Lithol Rubine BCA is rinsed with water after the laking reaction,⁵⁾ and the water-soluble impurities would be washed away from the reaction mixtures. This might account for the low positive response rate of commercial Lithol Rubine BCA (22.2%)

TABLE III. Mutagenic Activities of Calcium Lake Synthesized from Lithol Rubine B (3 : 2) and Its Ethyl Acetate Extract

Test compound	Concentration ($\mu\text{g}/\text{plate}$)	Number of His ⁺ revertants/plate			
		TA 98		TA 100	
		- S9 ^{a)}	+ S9 ^{a)}	- S9	+ S9
Lithol Rubine BCA (3 : 2) ^{b)} (original) ^{d)}	1	20 ^{c)}	46	102	106
	10	24	52	110	110
	100	27	45	106	114
	1000	25	56	100	109
Lithol Rubine BCA (3 : 2) (extract) ^{d)}	1	29	53	99	114
	10	96 ^{e)}	71	103	102
	100	<u>450</u>	<u>230</u>	<u>216</u>	159
	1000	<u>1479</u>	<u>689</u>	<u>599</u>	<u>385</u>

a) -S9, without S9 mix ; +S9, with S9 mix.

b) The ratio in parenthesis is that of the amounts of diazotized 2A5MBSA and 3H2NCA in the diazo and coupling solutions, respectively, in the reaction system.

c) The numbers of revertant colonies are the averages of two experiments.

d) Original : synthetic intact calcium lake sample. Extract : impurities extracted with ethyl acetate from the calcium lake.

e) Underlining indicates that the sample was mutagenic at the concentration tested.

in the Ames test as compared with Lithol Rubine B (100%). The positive results in two commercial samples might reflect insufficient rinsing with water.

A significant relation between mutagenicity and carcinogenicity has been recognized,¹⁰⁾ so the presence of mutagenic compounds in commercial dyes cannot be disregarded, even though mutagenic compounds are not always carcinogenic.¹⁰⁾ Since we have shown here that mutagenic compounds can easily be formed as reaction intermediates or by-products in the production of Lithol Rubine B, depending upon the ratio of the starting materials in the diazo and coupling solutions, we conclude that the manufacturing process of commercial Lithol Rubine B should be carefully controlled.

We are now investigating whether the mutagenic compounds obtained experimentally are identical with the mutagenic impurities in the commercial Lithol Rubine B or not. Subsequently, we hope to establish the chemical structures of the mutagenic compounds and to

clarify the mechanism of mutagenesis.

Conclusion

(1) Mutagenic compounds were always produced as reaction intermediates or by-products of synthetic Lithol Rubine B and BCA when the amount of the diazotized 2A3MBSA in the diazo solution was more than that of 3H2NCA in the coupling solution in terms of the theoretical amount. The results suggest that the mutagenic activities of commercial Lithol Rubine B and BCA depend on the reaction conditions, especially the ratio of the starting materials in the diazo and coupling solutions.

(2) The temperature of the diazo-coupling reactions did not affect the formation of the mutagenic compounds.

(3) The mutagenic compounds were removed from the synthetic Lithol Rubine BCA by rinsing with water after the laking reaction. This accounts for the low rate of positive mutagenicity (22.2%) of the commercial samples in the Ames test.

References

- 1) A.E. Auletta, J.M. Kuzava and A.S. Parmar, *Mutat. Res.*, **31**, 347 (1975); *idem, ibid.*, **56**, 203 (1977); S. Venitt and C.T. Bushell, *ibid.*, **40**, 309 (1976); R.C. Garner and C.A. Nutman, *ibid.*, **44**, 9 (1977); J.P. Brown, P.S. Dietrich and C.M. Bakner, *ibid.*, **66**, 181 (1979); E.J. Lazear, J.G. Shaddock, P.R. Barren and S.C. Louie, *Toxicol. Lett.*, **4**, 519 (1979); M.R. Green and J.V. Pastewka, *J. Natl. Cancer Inst.*, **64**, 665 (1980).
- 2) J.P. Brown, G.W. Roehm and R.J. Brown, *Mutat. Res.*, **56**, 249 (1978).
- 3) a) M. Miyagoshi, Y. Hayakawa, M. Nagata and T. Nagayama, *Eisei Kagaku*, **31**, 79 (1985); b) M. Miyagoshi, Y. Hayakawa and T. Nagayama, *ibid.*, **29**, 212 (1983).
- 4) a) "Color Index," 3rd ed., Vol. 4, ed. by The Society of Dyers and Colorists and ed. by The American Association of Textile Chemists and Colorists, Lund Humphries, Bradford and London, 1971, p.4058; b) *Idem, ibid.*, Vol. 3, p.3309.
- 5) K. Tanaka, M. Haginoya and M. Sato, *Shikizai Kyokaishi*, **38**, 51 (1967).
- 6) T. Kozuka, M. Tashiro, Y. Okumura, K. Fujimoto, Y. Nakamura, S. Hashimoto and G. Nakaminami, *Hifu*, **19**, 191 (1977); T. Kozuka, M. Tashiro, S. Sano, K. Fujimoto, Y. Nakamura, S. Hashimoto and G. Nakaminami, *Contact Dermatitis*, **5**, 297 (1979).
- 7) T. Yahagi, *Tampakushitsu Kakusan Koso*, **20**, 1178 (1975).
- 8) B.N. Ames, J. McCann and E. Yamasaki, *Mutat. Res.*, **31**, 347 (1975).
- 9) M. Yokote and F. Shibamiya, "Gousei Senryou," Nikkan Kogyo Shinbun Inc., Tokyo, 1978, pp.35-38.
- 10) J. McCann and B.N. Ames, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 950 (1976).