TABLE 2

OXIDATION OF REDUCED DPN AND REDUCTION OF TTC BY YEAST DIAPHORASE*

Time (min)	pH 7.0			pH 8.3		
	TTC present			TTC present		
	DPN mμM	Formazan mμM		DPN mμM	Formazan mμM	
0	0	0	0	0	0	0
5	–	9	0	–	12	12
10	9	13	0	27	21	24
20	21	24	0	54	51	39
50	53	34	0	64	58	67

* System same as in Table 1.

other mechanisms, such as the cytochrome-cytochrome oxidase systems, are involved, either directly or indirectly, is at present under investigation.[3]

References

1. KUHN, R., and JERCHEL, D. *Ber.*, **74**, (Pt. 1), 949 (1941).
2. KUN, E., and ABOOD, L. G. *Science*, **109**, 144 (1949).
3. FRED, R. B., and KNIGHT, S. G. *Ibid.*, 169.
4. BLACK, M. M., and KLEINER, I. S. *Ibid.*, **110**, 660 (1949).
5. GUNZ, F. W. *Nature*, **163**, 98 (1949).
6. SEVAG, M. G., and FORBES, M. *Arch. Biochem.*, **29**, 229 (1950).
7. BIELIG, H. J., KAUSCHE, G. A., and HAARDICK, H. *Z. Naturforsch.*, **46**, 80 (1949).
8. KUHN, R. Address at Univ. Penna., Phila. (May 1950).
9. MATTSON, A. M., JENSEN, C. D., and DUTCHER, R. A. *Science*, **106**, 294 (1947).
10. MEYERHOF, O., and JUNOWICZ-KOCHOLATY, R. *J. Biol. Chem.*, **149**, 71 (1943).
11. GREEN, D. E., and DEWAN, J. G. *Biochem. J.*, **32**, 1200 (1938).
12. OHLMEYER, P. *Biochem. Z.*, **297**, 66 (1938).

[3] Since submission of this paper, the work was continued with an isolated bacterial (*E. coli*) DPN-H_2 oxidase and TTC, as well as neotetrazolium. The findings were similar and will be the subject of a future report.

The Use and Toxicity of Pontamine Sky Blue

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The need for a better method of visualizing the primary and secondary lymphatic echelons in resectional surgery in carcinoma has long been recognized. Any agent used for this purpose should have the following characteristics: (1) high water solubility, (2) high degree of contrast compared to the tissues into which it is injected, (3) high specificity for and low diffusibility from lymph and lymph nodes, (4) low toxicity, (5) color stability, particularly near pH 7.2, and (6) ability to penetrate into the surgical area within 15 min. Braithwaite (*1*) found that indigo carmine fulfilled most of these criteria, but unfortunately the rate of dye uptake and its penetration into the surgical area left much to be desired. A review of the work of McMasters and Parsons (*2*) and Hudack and McMasters (*3*) showed that Pontamine Sky Blue (sodium salt of dimethoxydiphenyl-diazo-bis-8-amino-1-naphthol-3, 6 disulfonic acid) fitted all these criteria. In a study involving 35 cases of gastric resection for carcinoma, Weinberg and Greaney (*4*) established the utility of the dye for this purpose.

Recently the dye has been employed in more than 50 cases of intrathoracic surgery to delineate the surgical anatomy of the endothoracic lymphatics. The patient is prepared for such surgery by the usual procedure, an endotracheal catheter introduced, the thoracic cage opened and 5 ml of a 4% solution of Pontamine Sky Blue injected into the lung parenchyma near the hilum. The dye spreads rapidly, outlining the lymphatics not only in the immediate area but also in the more remote areas, thus allowing more radical surgery to be performed. This has extended the scope and completeness of extirpation of the diseased and potentially diseased tissue in bronchiogenic carcinoma. During the course of this work, 8 patients showed a residual blue coloring of the subcutaneous tissues. This was of concern not only to us but to those investigators using the dye under our directions because nothing was known about the possible toxicity of the dye.

The intravenous toxicity of Pontamine Sky Blue was determined in mice, and the LD_{50} was calculated by the Litchfield-Wilcoxon (*5*) method (Table 1).

TABLE 1

LD_{50} OF PONTAMINE SKY BLUE IN MICE

No. animals	LD_{50} g/kg	Confidence limits (odds 19/20) g/kg	Slope	Confidence limits (odds 19/20)
45	2.26	2.154–2.371	1.02	0.93–1.11

The symptoms of toxicity observed were acute respiratory embarrassment and death by cardiac and respiratory failure. The respiratory phase can be readily counteracted by artificial respiration.

Thirty animals that survived doses between 1.5 and 2.5 g/kg of the dye were observed for 28 days. Slight discoloration of the skin was observed in all the animals, but no evidence of residual toxicity was observed at necropsy.

In the use of any dyestuff, there is the possibility of traces of inorganic and organic materials being carried through the process and ending up in the final product. Such materials can produce toxic symptoms unrelated to the over-all toxic symptoms produced by the dye itself. Spectrographic analysis of Pontamine Sky Blue revealed that there were minute traces of Mn, Pb, and Cr. However, these ions were in such small quantities that they could not possibly cause any of the toxic manifestations seen when the dye was injected into humans or animals.

The possibility of toxicity caused by aging of the

4% solution used in the human studies was tested by injecting 0.5 g/kg into mice. The solution used was between 6 and 12 months of age. None of the 10 animals given the dye showed any evidence of toxicity.

When one compares the usual human dose, 5 ml 4% (0.2 g), with the LD_{50} for mice (2.26 g/kg), it is evident that there is a large safety factor involved even if the dye should inadvertently be injected intravenously. Furthermore, when one considers that most of the stained area is removed during surgery, it is probable that the total amount of residual dye is of no practical importance. Applying the toxicity criteria of Hodge and Steiner (*6*) reveals that Pontamine Sky Blue would be classed as a slightly toxic material.

A full report of the clinical utility and duration of skin staining caused by Pontamine Sky Blue will be reported in detail elsewhere.

References

1. BRAITHWAITE, L. R. *Brit. J. Surg.*, **11**, 7 (1923).
2. MCMASTERS, P. E., and PARSONS, R. J. *J. Exptl. Med.*, **69**, 247 (1939).
3. HUDACK, S. S., and MCMASTERS, P. D. *Ibid.*, **57**, 751 (1933).
4. WEINBERG, J., and GREANEY, E. M. *Surg. Gynecol. Obstet.*, **90**, 561 (1950).
5. LITCHFIELD, J. T., JR., and WILCOXON, F. *J. Pharmacol. Exptl. Therap.*, **96**, 99 (1949).
6. HODGE, H. C., and STEINER, J. *Am. Ind. Hyg. Assoc. Quart.*, **10**, 93 (1949).

Ion Exchange Separation of Desoxyribonucleotides[1]

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As has been implied by Cohn (*1*), the ion exchange methods developed by him for the separation of ribonucleotides (*2*) may be readily adapted to the separation of desoxyribonucleotides. Since no procedure for this separation has been published, it would seem worth while to outline the technique developed in our laboratory.[2]

The mixed desoxyribonucleotides were prepared by a two-stage enzymatic hydrolysis of highly polymerized desoxyribonucleic acid (DRNA). Two hundred mg of DRNA, prepared from calf thymus according to the method of Mirsky and Pollister (*3*), was dissolved in 20 ml 0.5 *M* acetate buffer, pH 6.5. To this were added 10 ml 0.1 *M* $MgSO_4$ and 10 ml of a distilled water solution of crystalline pancreatic desoxyribonuclease (Worthington Biochemical), containing 400 μg/ml. The enzymatic digestion was carried out for 72 hr at 37° C, under a layer of spectroscopically purified hexane, with constant stirring by magnetic stirrer.

The second stage of enzymatic digestion was carried out according to the method of Klein (*4*), using a nuclease preparation from intestinal mucosa, in the presence of arsenate to inhibit alkaline phosphatase also present in the preparation. The pH of the digest resulting from the action of the pancreatic enzyme was adjusted to 8.5 with 2 *N* NaOH. To the digest were added 10 ml 0.36 *M* $Na_3(AsO_4)_2$, 10 ml 1 *M* $NH_4OH \cdot (NH_4)_2SO_4$ buffer, pH 8.5, and 10 ml of a solution containing 17 mg/ml of the intestinal enzyme, prepared according to Klein, as modified by Brady (*5*). Digestion was carried out for 20 hr at 37° C under hexane, with constant stirring.

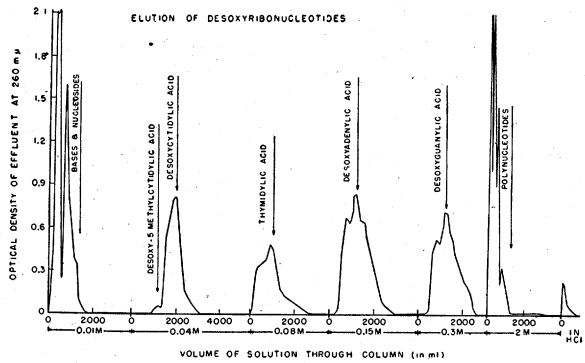

FIG. 1.

The pH of the digest was adjusted to 4.7 with glacial acetic acid to precipitate the intestinal preparation, which was then centrifuged out. The centrifugate was washed with 0.01 *M* acetate buffer, pH 4.7, and the washings were added to the previous supernatant. The pH of the combined solutions was adjusted to 9.0, and the volume made up to 250 ml.

This solution, containing mixed bases, nucleosides, nucleotides, and some undigested polynucleotides, was then added to the ion exchange column, π cm^2 × 11 cm, Dowex A-1 resin, 250–500 mesh[3] in the acetate form. Because of the marked lability of the purine desoxyribonucleotides to acid, it is desirable to employ procedures that avoid any prolonged contact with solutions of low pH. For this reason, elution from the ion exchange column was carried out in buffered solution at pH 4.3.

The elution procedure is indicated in Fig. 1. Bases and nucleosides, which accounted for 21.6% of the ultraviolet absorption of the material added to the column, were eluted with 0.01 *M* acetate buffer, pH 4.3. Desoxy-5 methyl cytidylic acid and desoxycytidylic acid were eluted, with incomplete separation, with 0.04 *M* buffer. If it is desired to separate these completely, they may be recycled on an acetate column, using buffer at pH 4.7 for elution (*1*). Thymidylic acid was removed with 0.08 *M* buffer, desoxyadenylic with 0.15 *M* buffer, and desoxyguanylic with 0.3 *M* buffer.

Subsequent passage of 2 *M* acetate buffer, pH 4.3, through the column removed a number (at least four) of different substances with varying absorption curves, presumably polynucleotides. These are being further investigated. Finally, treatment with 1 *N* HCl removed

[1] This investigation was supported in large part by a grant from the Rockefeller Foundation.

[2] Since submission of this paper, analogous procedures have been described by Hurst *et al.* (*J. Biol. Chem.*, **188**, 705 [1951]) and by Volkin *et al.* (*J. Am. Chem. Soc.*, **73**, 1533 [1951]).

[3] This resin was obtained through the courtesy of L. Matheson, of the Dow Chemical Company.