Note 2. Triethylamine in the above experiment can be substituted with pyridine (39 ml); yield 23 g (32%).

Note 3. According to the procedure of Breslow, *et al.*[18] in the preparation of *n*-octadecyl azidoformate, sodium azide (50 g) and H_2O (60 ml) were added to the solution of *tert*-butyl chloroformate prepared in ether in the presence of pyridine as stated above. Yield of the azide was 6.5 g to 11 g (9 to 15%).

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18) D.S. Breslow, T.J. Prosser, A.F. Marcantonio, and C.A. Genge, *J. Am. Chem. Soc.*, **89**, 2384 (1967).

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New Color Reaction of Hexosamines

The practical methods for the determination of hexosamines were the colorimetric methods developed by Elson,[1] Morgan[2] and Dische.[3] A new color reaction has been deviced for the microdetection and determination of hexosamines, based on a principle completely different from that of the Elson, Morgan or Dische method and having the advantage of being considerably more simple and selective. *p*-Nitrobenzaldehyde (*p*-NBA) reacted readily with hexosamines in pyridine solution to yield Schiff bases, and exhibited a blue color by addition of tetraethylammonium hydroxide solution. A possible mechanism of this color reaction was shown in Chart 1.

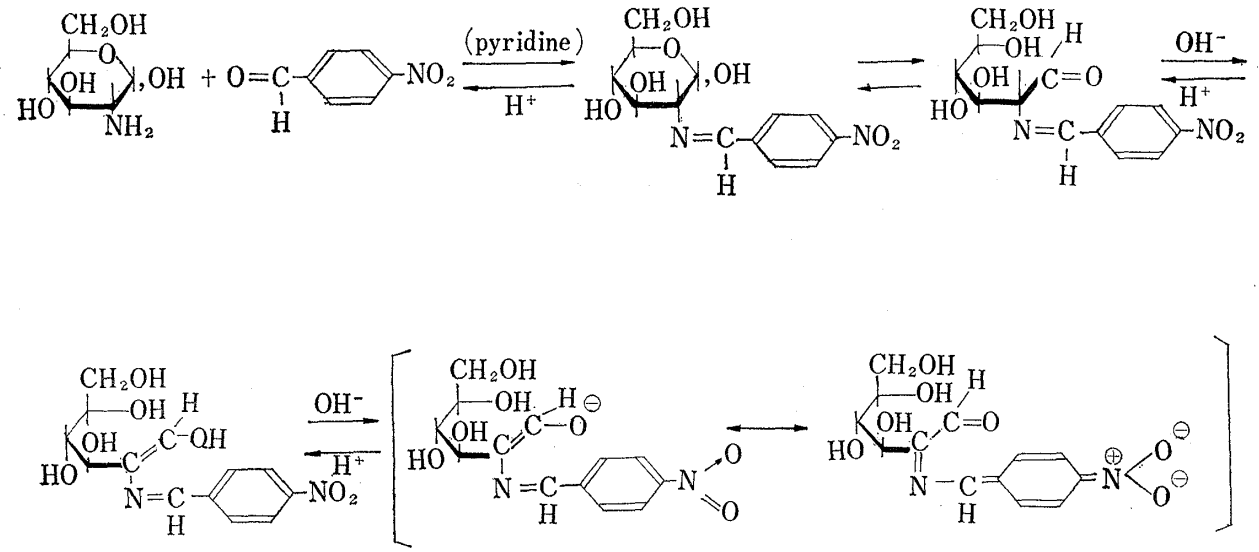

Chart 1. Mechanism of Color Reaction

1) L.A. Elson and W.J.T. Morgan, *Biochem. J.*, **26**, 1824 (1933).
2) W.J.T. Morgan and L.A. Elson, *Biochem. J.*, **28**, 988 (1934).
3) Z. Dische and E. Borenfreund, *J. Biol. Chem.*, **184**, 517 (1950).

By the use of glucosamine, the detection and determination of hexosamines with *p*-NBA were examined. The optical conditions found were as follows:

Reagents——

a) 2% and 1% *p*-NBA solution in pyridine.

b) 10% tetraethylammonium hydroxide.

c) 0.3% tetraethylammonium hydroxide: 3.0 ml of 10% aqueous tetraethylammonium hydroxide was diluted to 100 ml with ethanol.

d) 10% HCl

e) Standard solution of glucosamine: 10 mg of glucosamine HCl was dissolved in 0.5 ml of water and diluted to 20 ml with pyridine. From the above stock solution, standard solutions of suitable concentrations (ranging from 25 to 125 μg/ml) were prepared.

Procedure——

Microdetection of hexosamines

Method A: To 1 drop of the aqueous test solution containing hexosamines 0.3 ml of pyridine and 3 drops of 2% *p*-NBA solution were added, mixed throughly, and allowed to stand for several minutes in ice water. The mixture was made alkaline with a drop of 10% tetraethylammonium hydroxide. A blue color appeared instantly.

Method B: One drop of the aqueous test solution or a little amount of the solid sample containing N-substituted hexosamines, hexosaminides or mucopolysaccharides was mixed with a drop of 10% HCl, and heated in a boiling water bath for about 20 minutes. When cooled, the mixture was neutralized by addition of $NaHCO_3$, and then examined in the same manner above mentioned.

Colorimetric method of hexosamines

To 1.0 ml of the standard solution or the sample solution, 0.5 ml of 1% *p*-NBA solution was added, mixed throughly and allowed to stand for 20 minutes at 27—28°. The mixture, after cooling for about 5 minutes in an ice-water bath, was diluted to 10 ml with ice-cold

Table I. Color Reaction of Hexosamines and Various Compounds by Method A and Method B

Compound	Method A	Method B
Glucosamine HCl	+++	
Galactosamine HCl	+++	
Mannosamine HCl	+++	
N-Acetylglucosamine	−	+++
N-Acetylmannosamine	−	+++
Ethyl N-acetyl-β-D-glucosaminide	−	+++
Phenyl N-acetyl-β-D-glucosaminide	−	+++
Tetra-O-acetyl-D-glucosamine	+	+++
Pentaacetyl-D-glucosamine	−	+++
Tetra-O-actyl-N-methyl-D-glucosamine	−	+++
Chondroitine sulfate A (Na-salt)	−	+++
Chondroitine sulfate C (Na-salt)	−	+++
Hyaluronic acid (K-salt)	−	+++
Heparine (Na-salt)	−	+++
Hyalobiuronic acid	+	+++
Chondrosine	+	+++
Muramic acid	+	+++
3-O-Methyl-D-glucosamine	+++	
3,4,6-Tri-O-methyl-D-glucosamine	+++	
3-Amino-3-deoxy-D-allouronic acid	−	
Methyl 3-amino-3-deoxy-α-D-glucopyranoside	−	
Methyl 6-amino-6-deoxy-α-D-glucopyranoside	−	−
Glucose, Xylose, Mannose, Glucuronic acid	−	−
Amino acids (19 samples)	−	

0.3% tetraethylammonium hydroxide. The maximum absorbancy of the resultant red color was measured at 504 mμ against the reagnet blank between 10 and 25 minutes.

The calibration curve was linear in the range of 25—130 μg/ml of glucosamine HCl solution. Glucosamine and the other 2-amino-2-deoxy sugars showed similar colors under these conditions (Method A and Colorimetric method), while various N-substituted derivatives, glucosaminides, mucopolysaccharides, most of the other sugars and amino acids have no color by Method A as indicated in Table I. After hydrolysis with HCl, various derivatives of 2-amino-2-deoxy sugars and mucopolysaccharides gave a blue color as shown in Table I.

Details of the experiment and the reaction mechanism will be reported in the near furture.

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A Contact Antitumor Activity of *Bacillus natto* on Solid Type Ehrlich Carcinoma Cells

We have investigated extensively the specificity of metabolic and hydrolytic activities of soil bacteria and *E. coli* on benzoic acid derivatives, amino acids and acyl-amino acids and also on the acylase activity of ascites tumor cells.[1,2] Based on these reuslts, we have been trying to search for some familiar bacteria, which have high selective toxicity on human tumor cells. The first choice was the bacillus of 'Natto' (fermented Japanese beans) which is a popular and cheap daily food for Japanese. Here we wish to report the contact antiutmor effect of a strain (tentatively called KMD 1126) of *Bacillus natto* newly isolated from 'Natto'.

To test the antitumor effect, a conventional screening method was avoided. The bacterial cells were given directly to the established solid type Ehrlich carcinoma cells. The control tumor cells were formed in the same animal, since a direct contact effect of the bacillus on the tumor cells is to be expected, canceling a possible stimulation of defense mechanism in the tumor-bearing animal by this bacillus.

Biological Test: a) Experimental animal: Male inbred mice (20—22.5 g body weight) of ICR-JCL strain were used throughout.

b) Preparation of Ehrlich carcinoma cell suspension: A mouse was transplanted intraperitoneally with approximately 10^7 Ehrlich ascites carcinoma cells. After 7 days, the sedimented cells from the ascitic fluid were diluted with Dulbecco buffer A to the concentration of 2.5×10^7 tumor cells per ml.

c) Preparation of washed *Bacillus natto* KMD 1126 suspension: 10 ml of a 6-hour broth culture of KMD 1126 at 37° by shaking was centrifuged, and the sedimented cells, after

1) Y. Kameda, *et al.*, *Nature*, **169**, 1016 (1952); **170**, 888 (1952); **181**, 1225 (1958); **182**, 453 (1958); **191**, 1122 (1961); **192**, 468 (1961); **197**, 314 (1963).
2) Y. Kemeda, *et al.*, *Chem. Pharm. Bull.* (Tokyo), **15**, 1573, 1578, 1586 (1967); *ibid.*, in press.