Effect of bis-(*p*-nitrophenyl) phosphate on the biosynthesis and the utilization of lipid-intermediates

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(Received 1 April 1982/Accepted 8 June 1982)

Incubations of rat spleen lymphocytes with the required labelled nucleotide sugars lead to the formation of the various lipid-intermediates involved in the *N*-glycosylation of proteins. The effect of bis-(*p*-nitrophenyl) phosphate on the different reactions involved in the dolichol pathway has been studied. Although dolichyl phosphate mannose, dolichyl phosphate glucose and dolichyl diphosphate *N*-acetylglucosamine synthesis is not affected at all by bis-(*p*-nitrophenyl) phosphate (20 mM), this product inhibits completely the addition of the second *N*-acetylglucosamine residue on the dolichyl diphosphate *N*-acetylglucosamine acceptor. The addition of the five innermost mannose residues from GDP-mannose as donor is also strongly abolished. However, the addition of the more distal sugars, i.e. the four mannose residues using dolichyl phosphate mannose as donors and the additional glucose residues are only slightly affected. The reactions involved in the utilization of dolichyl diphosphate oligosaccharide, i.e. transfer to the proteins or degradation into soluble phospho-oligosaccharides, are also strongly inhibited. Thus bis-(*p*-nitrophenyl) phosphate appears to affect only the reactions involving the presence of dolichyl diphosphate sugar as substrate.

N-Glycosylation of proteins requires the synthesis of a precursor oligosaccharide containing glucose, mannose and *N*-acetylglucosamine linked to dolichol by a diphosphate group. This aspect has been widely investigated (for review see Hemming, 1977; Parodi & Leloir, 1979; Hanover & Lennarz, 1981; Hubbard & Ivatt, 1981). This dolichol pathway has been described in various systems and has been characterized also by using whole lymphocytes (Hoflack *et al.*, 1980). In this system, it has been demonstrated that oligosaccharide-*PP*-Dol can be utilized in two ways: either their transfer 'en bloc' on to protein acceptors or their degradation into soluble phospho-oligosaccharides (Cacan *et al.*, 1980).

Many steps of this lipid-mediated glycosylation process can be specifically inhibited by various substances, most of them being sugar analogues and antibiotics (Schwartz & Datema, 1980) and are useful tools in glycoprotein research. More recent studies (Kato *et al.*, 1980, 1981) have shown that

Abbreviations used: Glc-*P*-Dol, dolichyl phosphate glucose; Man-*P*-Dol, dolichyl phosphate mannose; GlcNAc-*PP*-Dol, dolichyl diphosphate *N*-acetylglucosamine; oligosaccharide-*PP*-Dol, dolichyl diphosphate oligosaccharide.

aryl phosphates could be used as water-soluble analogues of dolichyl phosphate, thus preventing the glycosylation of the natural substrate. In this paper, we investigate the possible inhibitory effect of bis-(*p*-nitrophenyl) phosphate, which is known to be a phosphodiesterase substrate and could interfere with enzymes dealing with phosphate or diphosphate groups, such as those involved in some steps of the dolichol cycle.

Experimental

Materials

All reagents were of analytical grade. GDP-[¹⁴C]mannose and UDP-[¹⁴C]glucose were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. UDP-[³H]*N*-acetylglucosamine was from New England Nuclear Corp., Boston, MA, U.S.A. GDP-mannose, UDP-glucose, UDP-*N*-acetylglucosamine, AMP, mannose, glucose, *N*-acetylglucosamine and bis-(*p*-nitrophenyl) phosphate sodium salt were obtained from Sigma Chemical Co., St Louis, Mo, U.S.A. *NN'*-Diacetylchitobiose was from Pharmindustrial, Villeneuve La Garenne, France. Amphomycin and tunicamycin

were kindly provided by the Laboratoires Bristol, Paris, France, and by Dr. W. F. J. Cuthbertson, Glaxo Research, Stoke Poges, Berks., U.K., respectively.

Cell preparations

Spleen lymphocytes were prepared from 6-week-old Wistar rats as follows. Briefly, the spleen was dissociated and cells were suspended in medium described by Cox & Peters (1979). After centrifugation (200 g for 10 min) erythrocytes were selectively lysed by 0.154 M-NH₄Cl. The lymphocytes were washed twice in the medium described by Cox & Peters (1979).

Standard assays

The incubations were carried out in the medium described by Cox & Peters (1979) containing 5 mM-MgCl₂, 2 mM-MnCl₂ and 5 mM-AMP. Labelled nucleotide sugars used as precursors were at a final concentration of 6 μ M at the following specific radioactivities: GDP-[¹⁴C]mannose, 180 c.p.m. $\cdot$ pmol⁻¹; UDP-[¹⁴C]glucose, 280 c.p.m. $\cdot$ pmol⁻¹; UDP-[³H]*N*-acetylglucosamine, 1250 c.p.m. $\cdot$ pmol⁻¹. To avoid intracellular utilization of freed radioactive sugar (Verbert *et al.*, 1976) 1 mM of the corresponding unlabelled sugar was added. Incubations were at 30°C with 10⁸ cells in a final volume of 100 μ l during 20 min. Modifications of these standard conditions are mentioned in the legend to each Figure.

Sequential lipid extraction

After incubation, labelled lipid intermediates were extracted as previously described (Hoflack *et al.*, 1980), leading to the formation of a chloroform/methanol extract, a chloroform/methanol/water extract and a residual protein pellet. In addition, this procedure gave aqueous phase containing precursors, precursor-degradation products and the water-soluble material released by the degradation of lipid intermediates as previously demonstrated (Cacan *et al.*, 1980). Counting of radioactivity was carried out in scintillation medium.

Mild acid treatment of lipid intermediates

The saccharidic moiety of the lipid intermediates was released by mild acid treatment as previously described (Richards & Hemming, 1972): 0.01 M-HCl in methanol/water (1:1, v/v) at 100°C for 10 min.

Chromatographic techniques

The integrity of the nucleotide-sugars was checked by paper-chromatographic analysis of the degradation products as described by Spik *et al.* (1979).

N-Acetylglucosamine and *NN'*-diacetylchitobiose released from their lipid derivatives were

separated by chromatography on Whatman no. 3 paper using solvent A [butan-1-ol/pyridine/water (6:4:3, by vol.)] and with authentic standards as markers.

Separation of Man₉(GlcNAc)₂ from (Man)₅(GlcNAc)₂ oligosaccharides derived from their lipid-intermediates has been achieved by t.l.c. on a silica gel plate in solvent B [ethanol/butan-1-ol/pyridine/water/acetic acid (100:10:10:30:3, by vol.)] with, as markers, oligomannosidic type oligosaccharides obtained from the urine of patients with mannosidosis, which were a generous gift from Dr. G. Strecker, Université des Sciences et Techniques de Lille, Villeneuve d'Ascq, France.

Separation of Glc-*P*-Dol from glucosyl ceramide was performed by DEAE-paper chromatography (Whatman DE 81) with solvent C [chloroform/methanol (2:1, v/v)]. Glc-*P*-Dol stays at the origin and glucosyl ceramide co-migrates with the solvent front as indicated by authentic glucosyl ceramide purified from the spleen of a patient with Gaucher's disease (obtained from Dr. G. Strecker).

Preparation of purified [¹⁴C]Man-*P*-Dol

[¹⁴C]Man-*P*-Dol was prepared by scaling-up standard incubations of lymphocytes with GDP-[¹⁴C]mannose. After sequential lipid extraction, the [¹⁴C]Man-*P*-Dol recovered in the chloroform/methanol extract was purified by DEAE-cellulose (Whatman DE 52) column chromatography as described by Barr & Hemming (1972).

Results

Inhibition of lipid-linked oligosaccharide synthesis by bis-(*p*-nitrophenyl) phosphate

We have previously shown that incubations of whole lymphocytes with UDP-*N*-acetylglucosamine, GDP-[¹⁴C]mannose and UDP-glucose led to the formation of [¹⁴C]Man-*P*-Dol and [¹⁴C]mannose-containing oligosaccharide-*PP*-Dol, which are either transferred to proteins or degraded into phospho-oligosaccharides (Hoflack *et al.*, 1981). Fig. 1 shows that when incubations are performed in the presence of various concentrations (from 0 to 20 mM) of bis-(*p*-nitrophenyl) phosphate, the radioactivity incorporated into oligosaccharide-*PP*-Dol, protein and phospho-oligosaccharides is largely decreased (more than 80% inhibition at 10 mM). The presence of the required donors was checked: on the one hand, UDP-*N*-acetylglucosamine, UDP-glucose and GDP-mannose whose integrity was maintained, owing to the presence of both 5 mM-AMP and bis-(*p*-nitrophenyl) phosphate, which have a protective effect on the degradation of nucleotide-sugars; on the other hand, [¹⁴C]Man-*P*-Dol, whose synthesis is not affected (see Fig. 1). As many steps are

Fig. 1. Effect of bis-(*p*-nitrophenyl) phosphate concentration on the incorporation of [¹⁴C]mannose from GDP-[¹⁴C]mannose into lipid intermediates, proteins and phospho-oligosaccharides

Standard assays were performed with GDP-[¹⁴C]-mannose, UDP-*N*-acetylglucosamine and UDP-glucose as described in the Experimental section, in the presence of increasing amount of bis-(*p*-nitrophenyl) phosphate. After 20 min of incubation at 30°C, the transfer of radioactive mannose was measured into dolichyl monophosphate mannose (●), dolichyl diphosphate oligosaccharides (○), proteins (▲) and soluble phospho-oligosaccharides (■).

Fig. 2. Effect of bis-(*p*-nitrophenyl) phosphate concentration on the incorporation of [³H]*N*-acetylglucosamine from UDP-[³H]*N*-acetylglucosamine into dolichyl diphosphate *N*-acetylglucosamine and dolichyl diphosphate chitobiose

Standard assays were performed with UDP-[³H]-*N*-acetylglucosamine as the only precursor as described in the Experimental section, in the presence of increasing amounts of bis-(*p*-nitrophenyl) phosphate. After 20 min of incubation at 30°C, the relative proportion of dolichyl diphosphate *N*-acetylglucosamine (●) and dolichyl diphosphate chitobiose (○) was determined after mild acid treatment of the chloroform/methanol extract and the separation of [³H]*N*-acetylglucosamine and [³H]chitobiose by paper chromatography in solvent A.

involved in the formation of oligosaccharide-*PP*-Dol, the question arises as to which steps are inhibited by bis-(*p*-nitrophenyl) phosphate.

Effect of bis-(*p*-nitrophenyl) phosphate on the synthesis of (GlcNAc)₁₋₂-*PP*-Dol

The first two steps of the dolichol pathway can be examined by incubating lymphocytes with UDP-[³H]GlcNAc as the only precursor (Hoflack *et al.*, 1982). This leads to the formation of GlcNAc-*PP*-Dol and (GlcNAc)₂-*PP*-Dol in the ratio 3:17 (see Fig. 2). As shown in Fig. 2, addition of bis-(*p*-nitrophenyl) phosphate to the incubation medium caused a marked decrease in the synthesis of (GlcNAc)₂-*PP*-Dol, although the formation of (GlcNAc)₁-*PP*-Dol was not affected at all. This result indicates that reactions requiring nucleotide-sugars (UDP-*N*-acetylglucosamine and GDP-mannose) as donors and dolichyl phosphate as acceptor are not affected by bis-(*p*-nitrophenyl) phosphate. In contrast, the further addition of a glucosamine residue on GlcNAc-*PP*-Dol is markedly inhibited. As the further elongation will proceed on the same GlcNAc-*PP*-Dol-containing acceptor, we investigated whether mannosylation of the chitobiosyl-*PP*-Dol was also affected.

Effect of bis-(*p*-nitrophenyl) phosphate on the mannosylation process

To examine the mannosylation steps [³H]-(GlcNAc)₂-*PP*-Dol was first synthesized by incubating lymphocytes with UDP-[³H]*N*-acetylglucosamine. As demonstrated elsewhere (Hoflack *et al.*, 1982) the major [³H]*N*-acetylglucosamine-containing lipid-intermediates consist of (GlcNAc)₂-*PP*-Dol and very few oligosaccharide-*PP*-Dol species are recovered in the chloroform/methanol/water extract. After incubation for 15 min, if GDP-mannose is added together with tunicamycin, which blocks the formation of new [³H]*N*-acetylglucosamine lipid intermediates, the mannosylation of the previously synthesized ([³H]GlcNAc)₂-*PP*-Dol can be observed, since subsequent [³H]*N*-acetylglucosamine-labelled material becomes extractable in chloroform/methanol/water, as shown in Fig. 3. However, if bis-(*p*-nitrophenyl) phosphate is added together with the GDP-mannose and tunicamycin, the mannosylation does not occur, as no more [³H]GlcNAc-labelled oligosaccharide-*PP*-Dol species are recovered in the chloroform/methanol/water extract (see Fig. 3). This result indicates that the mannosylation is inhibited by

Fig. 3. Effect of bis-(*p*-nitrophenyl) phosphate on the formation of dolichyl diphosphate oligosaccharide from dolichyl diphosphate [^3H]chitobiose and GDP-mannose. Standard assays were performed with UDP-[^3H]-*N*-acetylglucosamine but without GDP-mannose as described in the Experimental section. After 15 min incubation at 30°C , 0.1 μmol of GDP-mannose and 0.5 μg of tunicamycin were added (arrow) in the absence (●) or in the presence (○) of 20 mM bis-(*p*-nitrophenyl) phosphate. The amount of [^3H]-*N*-acetylglucosamine recovered in the chloroform/methanol/water extract was measured.

bis-(*p*-nitrophenyl) phosphate and that, at least, the linkage of the innermost mannose is prevented.

It is now established that in various systems the mannosylation occurs first from GDP-mannose, leading to the formation of a $(\text{Man})_3(\text{GlcNAc})_2\text{-PP-Dol}$, which can be elongated with Man-*P*-Dol to give a typical $(\text{Man})_9(\text{GlcNAc})_2\text{-PP-Dol}$ (Chambers *et al.*, 1977; Kang *et al.*, 1978; Chapman *et al.*, 1980). Moreover it has been shown that amphomycin can be used to synthesize *in vitro* $(\text{Man})_5(\text{GlcNAc})_2\text{-PP-Dol}$ (Banerjee *et al.*, 1981). As amphomycin blocks the synthesis of Man-*P*-Dol and (GlcNAc)-*PP*-Dol, the $(\text{Man})_5(\text{GlcNAc})_2\text{-PP-Dol}$ was obtained by first incubating lymphocytes with UDP-*N*-acetylglucosamine for 15 min to allow the formation of chitobiosyl-*PP*-Dol and then GDP-mannose was added together with amphomycin (200 $\mu\text{g}/\text{ml}$). That the size of the saccharidic moiety of the oligosaccharide-lipid formed under these conditions corresponding to $(\text{Man})_5(\text{GlcNAc})_2$ was confirmed by its migration rate on t.l.c. in solvent B compared with oligomannosides of known structures.

By using this endogenous $(\text{Man})_5(\text{GlcNAc})_2\text{-PP-Dol}$ synthesized for 10 min as indicated above, the further elongation was studied by adding 50 000 c.p.m. of purified [^{14}C]Man-*P*-Dol solubilized in the presence of 0.25% Triton X-100. The mannosylation process via [^{14}C]Man-*P*-Dol as the

Fig. 4. Effect of bis-(*p*-nitrophenyl) phosphate on the incorporation of [^{14}C]mannose from dolichyl phosphate [^{14}C]mannose into endogenous $(\text{Man})_5(\text{GlcNAc})_2\text{-PP-Dol}$.

Assays were first performed with 20 μM -UDP-*N*-acetylglucosamine; then, after 15 min, 2 nmol of GDP-mannose was added with 20 μg of amphomycin. After 10 min the $(\text{Man})_5\text{GlcNAc}_2\text{-PP-Dol}$ formed were elongated in the presence of 50 000 c.p.m. of purified dolichyl phosphate [^{14}C]mannose solubilized in the presence of 0.25% Triton X-100. The mannosylation process was measured in the absence (●) or in the presence of 20 mM bis-(*p*-nitrophenyl) phosphate (○) by measuring the radioactivity of the chloroform/methanol/water extracts.

only labelled precursor was measured by counting the radioactivity of the chloroform/methanol/water-extractable material. Fig. 4 shows that in these conditions, [^{14}C]mannose is effectively incorporated from [^{14}C]Man-*P*-Dol into an oligosaccharide-*PP*-Dol. This mannosylation process is slightly reduced (around 20%) if performed in the presence of 20 mM bis-(*p*-nitrophenyl) phosphate. Thus, it appears that although bis-(*p*-nitrophenyl) phosphate leads to a strict inhibition of the transfer of the innermost mannose residues, it has a slight effect on the mannosylation of the more distal mannoses.

Effect of bis-(*p*-nitrophenyl) phosphate on the glucosylation of the oligosaccharide-lipid

Since the work of Li *et al.* (1978), it has been established that lipid-linked oligosaccharides may be found in glucosylated forms. We previously demonstrated (Hoflack *et al.*, 1981) that a typical $(\text{Glc})_3(\text{Man})_5(\text{GlcNAc})_2\text{-PP-Dol}$ could be obtained by incubating lymphocytes with UDP-glucose, GDP-mannose and UDP-*N*-acetylglucosamine. We thus examined the effect of bis-(*p*-nitrophenyl) phosphate on the synthesis of glucosylated lipid intermediates, i.e. Glc-*P*-Dol and glucosylated oligosaccharide-*PP*-Dol. Incubations of lymphocytes with UDP-[^{14}C]glucose produced two chloroform/

Fig. 5. Effect of bis-(*p*-nitrophenyl) phosphate concentration on the incorporation of [^{14}C]glucose from UDP-[^{14}C]glucose into dolichyl phosphate glucose

Standard assays were performed with UDP-[^{14}C]glucose as described in the Experimental section, in the presence of increasing amounts of bis-(*p*-nitrophenyl) phosphate. After 20 min of incubation at 30°C, the radioactivity of the chloroform/methanol extract was measured and the relative proportion of dolichyl phosphate glucose (●) and glucosyl ceramide (○) was determined by using DEAE-cellulose paper chromatography in solvent C as described in the text.

Fig. 6. Effect of bis-(*p*-nitrophenyl) phosphate on the glucosylation of dolichyl diphosphate oligosaccharide with UDP-[^{14}C]glucose

Standard assays were performed as described in the Experimental section in the presence of 20 μM -GDP-mannose and 20 μM -UDP-*N*-acetylglucosamine during 15 min at 30°C. The dolichyl diphosphate oligosaccharides formed are then glucosylated in the presence of UDP-[^{14}C]glucose and 0.5 μg of tunicamycin in the absence (●) or in the presence (○) of 20 mM-bis-(*p*-nitrophenyl) phosphate and the incorporation of [^{14}C]glucose into oligosaccharide-PP-Dol was monitored by the radioactivity recovered in the chloroform/methanol/water extract.

methanol-extractable products that were identified as Glc-*P*-Dol and glucosyl ceramide. Fig. 5 shows that the synthesis of glucosyl ceramide is completely abolished in the presence of 20 mM-bis-(*p*-nitrophenyl) phosphate, although, as has been observed for the formation of the dolichol derivatives of monosaccharides, the synthesis of Glc-*P*-Dol is not affected.

The glucosylation of oligosaccharide-PP-Dol has been observed as follows. In a first step, (Man)₅(GlcNAc)₂-PP-Dol was synthesized by incubating lymphocytes with unlabelled UDP-*N*-acetylglucosamine and GDP-mannose for 15 min. Then, in a second step, together with tunicamycin (5 $\mu\text{g}/\text{ml}$), UDP-[^{14}C]glucose is added to obtain [^{14}C]glucose-containing oligosaccharide-PP-Dol as monitored by the amount of labelled material extractable by chloroform/methanol/water. As reported in Fig. 6, one can observe that the glucosylation of oligosaccharide-PP-Dol is poorly influenced by the presence of 20 mM-bis-(*p*-nitrophenyl) phosphate.

Effect of bis-(*p*-nitrophenyl) phosphate on the fate of oligosaccharide-PP-Dol

The experiments described above indicate that bis-(*p*-nitrophenyl) phosphate affects mainly the first steps of the elongation of the oligosaccharide-lipid intermediate. Referring to Fig. 1, the decreased incorporation on to proteins and into phospho-

oligosaccharides could be due to the lack of the required oligosaccharide-PP-Dol, whose synthesis was blocked, but a direct effect on the 'en-bloc' transfer reaction or on the degradation process cannot be excluded. To investigate this point, synthesis of oligosaccharide-PP-Dol was allowed to proceed for 15 min by incubating lymphocytes with UDP-*N*-acetylglucosamine, GDP-[^{14}C]mannose and then bis-(*p*-nitrophenyl) phosphate (20 mM) was added and the fate of the synthesized oligosaccharide-PP-Dol was followed by measuring the subsequent incorporation into proteins or the amount of phospho-oligosaccharides released into the aqueous phase. The results of such experiments are shown in Fig. 7. As expected from the above results, the synthesis of [^{14}C]Man-*P*-Dol does not vary (Fig. 7a) and the synthesis of [^{14}C]mannose-labelled oligosaccharide-PP-Dol is blocked (Fig. 7b). The amount of oligosaccharide-PP-Dol formed before the addition of bis-(*p*-nitrophenyl) phosphate is not utilized to glycosylate the protein acceptors as shown by the plateau observed in Fig. 7(c). The degradation of oligosaccharide-PP-Dol is also strongly, but not totally, inhibited by the presence of bis-(*p*-nitrophenyl) phosphate as shown in Fig. 7(d). The slight increase in the phospho-oligosaccharide level that is observed corresponds to the small decrease in the level of oligosaccharide-PP-Dol shown in Fig. 7(b).

Fig. 7. Effect of bis-(*p*-nitrophenyl) phosphate on the fate of dolichyl diphosphate oligosaccharides

Standard assays were performed as described in the Experimental section with GDP-[^{14}C]mannose and $20\ \mu\text{M}$ -UDP-*N*-acetylglucosamine at 30°C . The [^{14}C]mannose incorporated in the dolichyl phosphate mannose (a), dolichyl diphosphate oligosaccharide (b), proteins (c) and soluble phospho-oligosaccharides (d) was measured without (●) or with addition of $2\ \mu\text{mol}$ of bis-(*p*-nitrophenyl) phosphate (○) after 15 min incubation (arrow).

Discussion

From the various effects of bis-(*p*-nitrophenyl) phosphate observed on the glycosylation process of *N*-glycosylated protein, the following features may be pointed out. (1) Bis-(*p*-nitrophenyl) phosphate does not affect any reactions involving as substrates dolichyl phosphate and sugar-nucleotides such as the ones leading to the synthesis of GlcNAc-PP-Dol, Man-*P*-Dol and Glc-*P*-Dol. (2) Bis-(*p*-nitrophenyl) phosphate affects reactions in which a dolichyl diphosphate-containing substrate is involved, such as elongation, transfer or degradation. (3) Concerning the elongation, the inhibitory effect is much weaker when the sugar addition occurs far from the dolichyl diphosphate glucosamine group: addition of the second *N*-acetylglucosamine is completely abolished as well as addition of the innermost mannose residues, although the last steps of mannosylation and the glucosylation are poorly affected. (4) Concerning transfer or splitting of oligosaccharide-PP-Dol, where enzymes must cleave a phosphate bond, bis-(*p*-nitrophenyl) phosphate interferes strongly with these reactions, leading to a total block of the transfer and to a high inhibition of the degradation.

Specific inhibitors are widely used in elucidating the multiple enzymic steps involved in the *N*-glycosylation of proteins. Among the whole set of inhibitors available, bis-(*p*-nitrophenyl) phosphate may obtain special attention as it blocks the oligosaccharide-lipid formation without affecting the synthesis of GlcNAc-PP-Dol, Man-*P*-Dol and Glc-*P*-Dol. Although the major effects concern the steps involving the presence of dolichyl diphosphate-containing substrates, no definite conclusions can be drawn concerning the specific mode of action of this molecule. However, it may be a useful exogenous probe to explore the normal pathway of lipid-linked oligosaccharide and glycoprotein synthesis.

This research was supported in part by the Centre National de la Recherche Scientifique (Laboratoire associé no. 217: Relation structure-fonction des constituants membranaires), by the Institut National de la Santé et de la Recherche Médicale (C.R.L. 81-2015) and by the Délégation Générale à la Recherche Scientifique et Technique (contract 79-7-0669). We thank the Director of the Laboratory, Professor J. Montreuil, for his constant interest in the development of our research and for providing research facilities. We are indebted to Dr. W. F. J. Cuthbertson of Glaxo Research for his gift of tunicamycin, to Dr. G. Strecker for glucosyl ceramide and oligosaccharides used as markers and to the Laboratoires Bristol for giving amphomycin.

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