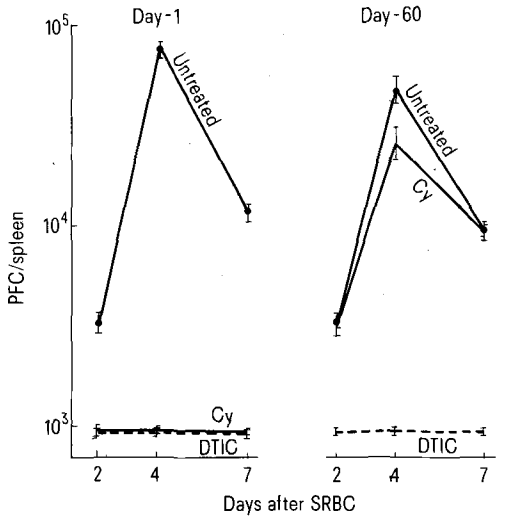

Effect of single equitoxic doses of DTIC and Cy on mouse antibody production. The drugs were given either 1 day (left side) or 60 days (right side) before SRBC and antibody production was assessed on day 2, 4 and 7 after the stimulus. ● ——— ● *Untreated;* ——— *Cy-treated animals;* -------- *DTIC-treated animals.*

L5MF-22 (H-2^b) cells 1 or 60 days later. The table shows that non-pretreated control $CD2F_1$ mice rejected tumor challenge, whereas animals pretreated with either DTIC or Cy 1 day before challenge died of generalized lymphoma with median survival times similar to those of compatible C57Bl/10 (H-2^b) mice. On the other hand, when drug treatment was performed 60 days earlier, the mice injected with Cy rejected the L5MF-22 tumor; in contrast $CD2F_1$ hosts pretreated with DTIC failed to reject the challenge and succumbed with generalized lymphoma.

Discussion. We have compared the immunodepressive activity of DTIC with that of Cy, an alkylating agent widely considered as a standard in experimental immunodepression. Primary antibody response and allograft reactivity have been examined, since previous studies[10,11] had shown that both are affected by DTIC treatment. Tests were performed at 2 different time intervals after drug treatment, 1 or 60 days, since previous observations had shown that a DTIC-induced depression of allograft response could exceed 20 days[2]. The remarkably long interval of 60 days was also chosen to rule out the possibility that the duration of the immunodepressive effects could be due to the persistence of DTIC in mouse organs after its administration. It has been repeatedly reported[13,14] that DTIC has a short halflife, as discussed elsewhere[2]. Consistent with previous reports[10,12], we have found that high equitoxic doses of DTIC and Cy abrogated the 2 primary immune responses under investigation, when the 2 drugs were given 1 day before the antigenic stimulus. But even when DTIC was given 60 days before the antigen, the immunodepression was still complete, the converse being true for Cy. The cellular bases of DTIC-induced immunodepression are still unknown. However, such long duration of the immunodepressive effects caused by DTIC suggests several hypotheses including the possibility that this agent might affect lymphoid cell populations (either suppressor or effector lymphocytes) with extremely long turnover rates. Moreover these findings suggest possible clinical applications of DTIC in the pharmacological control of graft rejection.

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2 M.C. Fioretti, Pharm. Res. Comm. *7*, 481 (1975).
3 E. Bonmassar, A. Prada, G. Giannattasio and C. Testorelli, Arch. It. Pat. Clin. Tum. *8*, 231 (1965).
4 E. Bonmassar, A. Bonmassar, S. Vadlamudi and A. Goldin, Proc. natl Acad. Sci. USA *66*, 1089 (1970).
5 E. Bonmassar, A. Nicolin, S. Vadlamudi, I. Kline and A. Goldin, Fedn Proc. *30*, 628 (1971).
6 A. Nicolin, A. Bini and E. Coronetti, Nature *251*, 654 (1974).
7 F. Campanile, D.P. Houchens, M. Gaston, A. Goldin and E. Bonmassar, J. natl Cancer Inst. *55*, 207 (1975).
8 C. Riccardi, M.C. Fioretti, A. Giampietri, P. Puccetti and A. Goldin, Transplantation, in press.
9 N.K. Jerne, C. Henry, A.A. Nordin, H. Fuji, A.M.C. Koros and I. Lefkovits, Transplantn Rev. *18*, 130 (1974).
10 A. Vecchi, M.C. Fioretti, A. Mantovani, A. Barzi and F. Spreafico, Transplantation *6*, 619 (1976).
11 E. Bonmassar, A. Bonmassar, S. Vadlamudi and A. Goldin, Cancer Res. *32*, 1446 (1972).
12 F. Spreafico, A. Mantovani, E. Menconi and A. Goldin, Fedn Proc. *33*, 572 (1974).
13 T.L. Loo, B.B. Tanner, G.E. Housholder and B.J. Shepard, J. Pharm. Sci. *57*, 2126 (1968).
14 J.L. Skibba, G. Ramirez, R.R. Beal and G.T. Bryan, Cancer Res. *29*, 1944 (1969).

Inhibition of glycolysis by L-sorbose in dog erythrocytes

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Summary. We have demonstrated previously that in vitro L-sorbose acts directly on dog erythrocytes to induce hemolysis. Here we report that L-sorbose depresses lactate formation in dog hemolysates from glucose, mannose and fructose but not from glucose-6-phosphate and galactose, suggesting that L-sorbose interacts with glycolysis at the level of the hexokinase.

Recently we reported that the ingestion of L-sorbose, a monosaccharide of the ketohexose group, causes hemolysis in dogs[2,3]. In vitro studies showed that L-sorbose acts directly on dog erythrocytes to induce hemolysis[4]. Sorbose concentrations as low as 2 mM lead to total hemolysis after 48 h of incubation. The dependence of the hemolytic effect of sorbose upon temperature and pH suggests that L-sorbose acts on the red blood cell metabolism rather than directly on the cell membrane[4].

The monosaccharide nature of L-sorbose would suggest an interaction with glycolysis. Since the energy available to erythrocytes is derived mainly from glycolysis, an inhibition of this metabolic pathway by L-sorbose could lead to a depletion of the ATP level and secondarily to hemolysis.

Materials and methods. Swiss Beagle dogs were obtained from the Institute of Biological and Medical Research (Füllinsdorf, BL). Blood samples were collected in heparinized tubes and the red blood cells washed 2-3 times with physiological NaCl solution. The red blood cells were incubated in Hanks balanced salt solution (with 20 mM Hepes-buffer, pH 7.4 instead of the bicarbonate buffer, without phenol red and supplemented with 60 µg/ml

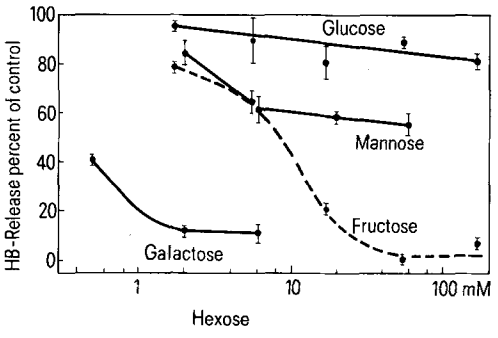

Fig. 1. Effect of D-glucose, D-mannose, D-fructose, and D-galactose on the L-sorbose induced hemolysis in dog erythrocytes in vitro. Aliquots of washed dog erythrocytes (containing 8.3–11.6 mg hemoglobin) were incubated in Hanks balanced salt solution (with 20 mM Hepes-buffer, pH 7.4 instead of the bicarbonate buffer, without phenol red and supplemented with 60 µg/ml penicillin and 100 µg/ml streptomycin) containing 1.7 mM L-sorbose without and with the indicated concentrations of the 4 hexoses for 48 h in a H_2O-saturated atmosphere at 36 °C. After centrifugation the hemoglobin content was determined in the medium. Results are the mean±SD of triplicate determinations expressed as percent of the hemoglobin release of dog erythrocytes incubated with L-sorbose alone.

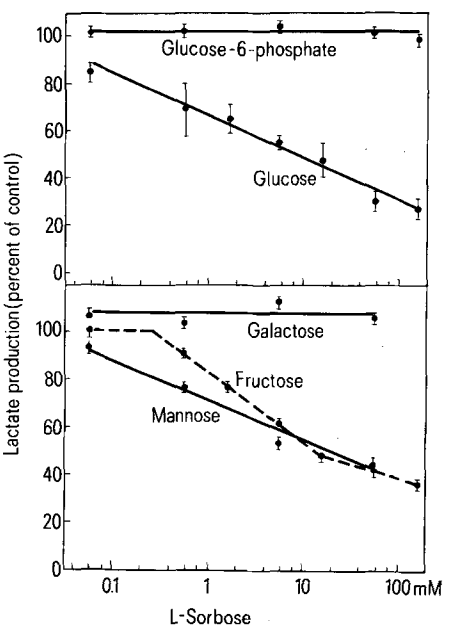

Fig. 2. Effect of L-sorbose on the lactate formation in hemolysates of dog erythrocytes using 4 hexoses and glucose-6-phosphate as substrate. Aliquots of 1.5 ml hemolysate were incubated in cell culture dishes in a final volume of 5 ml containing 1.0 mM ATP, 0.3 mM NAD, 1.5 mM $MgCl_2$, 2.0 mM K_3PO_4, 50 mM Hepes, pH 7.4 and 6 mM of the various substrates without and with increasing concentrations of L-sorbose for 24 h at 36 °C. The lactate formed was determined in the perchloric acid soluble fraction. Results are the mean±SD of duplicate or triplicate determinations expressed as percent of the lactate formed in absence of L-sorbose. Control values in µmoles lactate formed per g hemoglobin per 24 h for D-glucose, glucose-6-phosphate, D-fructose, D-mannose, and D-galactose were 90.6±7.0, 100.1±1.0, 100.2±2.9, 81.6±1.5, and 33.2±4.1, respectively.

penicillin and 100 µg/ml streptomycin) in cell culture dishes (Falcon, 5.5 cm, final volume 5 ml) in a H_2O-saturated atmosphere. After incubation, the medium together with the cells were centrifuged and the hemoglobin (Hb) concentration was determined in the supernatant using a Coulter Counter ZF.

Hemolysates were prepared by freezing and thawing of the washed erythrocytes. For the measurement of glycolysis, the hemolysate was incubated in the medium described by Chapman et al.[5] buffered with 50 mM Hepes, pH 7.4 instead of glycine buffer, and the lactate formed was determined enzymatically with lactate dehydrogenase[6] in the perchloric acid soluble fraction. L-sorbose (minimum 99.5%) obtained from Hoffmann-La Roche. All other materials were reagent grade.

Results. The sorbose-induced hemolysis in dog erythrocytes was reversed by a equimolar concentration of galactose and about 30 times higher concentrations of fructose, but was much less affected by mannose and glucose up to concentrations 35 and 100 times that of sorbose, respectively (figure 1).

Using glucose as substrate, the lactate formation in hemolysates of dog erythrocytes was linear between 6 and 24 h of incubation and was markedly inhibited by sorbose (table). An inhibition of glycolysis by sorbose of 27 and 59% was observed after 14.5 and 24 h of incubation, respectively. However, starting glycolysis with glucose-6-phosphate, sorbose did not decrease the amount of lactate formed (table).

The lactate formation in the hemolysate of dog erythrocytes was dose-dependent inhibited by sorbose using glucose, mannose or fructose as the energy source (figure 2). In contrast, increasing the sorbose concentration up to 9.5 and 28 times that of galactose and glucose-6-phosphate, respectively, had no effect on the amount of lactate formed. There was no formation of lactate when dog hemolysate was incubated with sorbose alone in concentration from 0.6 to 60 mM for 24 h.

Discussion. These studies indicate that the sorbose-induced hemolysis in dog erythrocytes may result from an interaction of sorbose with glycolysis at the level of the hexokinase resulting in a depletion of the ATP level and secondarily in hemolysis. The main catabolism of glucose and mannose is ultimately dependent upon hexokinase, whereas fructose can enter glycolysis by an alternative pathway using ketohexokinase instead of hexokinase, and the catabolism of galactose is independent of the hexokinase. That fructose may enter glycolysis by 2 pathways might explain the observation that high concentrations of fructose can reverse the sorbose-induced hemolysis in vitro and also that, compared with glucose and mannose, the inhibition pattern of the lactate formation by sorbose in hemolysates of dog erythrocytes was different when fructose was used as substrate.

Effect of L-sorbose on the time course of lactate formation in hemolysates of dog erythrocytes using D-glucose and glucose-6-phosphate as energy source

Substrate	L-Sorbose	Time of incubation (h) 6	14.5	24
D-Glucose (6 mM)	None	9.5 ± 0.1	46.5 ± 1.6	90.6 ± 2.2
D-Glucose (6 mM)	5.6 mM	9.1 ± 0.2	33.9 ± 1.6	36.9 ± 1.2
Glucose-6-phosphate (6 mM)	None	n.d.	48.0 ± 0.2	78.2 ± 3.0
Glucose-6-phosphate (6 mM)	5.6 mM	n.d.	47.2 ± 1.5	77.7 ± 1.9

Aliquots of 1.5 ml hemolysate, prepared by freezing and thawing of washed dog erythrocytes, were incubated in cell culture dishes (Falcon, 5.5 cm) in a final volume of 5 ml containing 1.0 mM ATP, 0.3 mM NAD, 1.5 mM $MgCl_2$, 2.0 mM K_3PO_4, 50 mM Hepes pH 7.4, and D-glucose, glucose-6-phosphate, and L-sorbose as indicated at 36 °C. After incubation 0.2 ml of 70% perchloric acid was added, and after centrifugation the lactate formed was measured in the supernatant with lactic dehydrogenase according to Hohorst[7]. Results are the mean ± SD of triplicate determinations expressed as µmoles lactate formed per g of hemoglobin. n.d., not determined.

In presence of an equimolar concentration of galactose, sorbose could not induce lysis of dog erythrocytes in vitro. Since the catabolism of galactose is independent of hexokinase, and the lactate formation in dog hemolysates was not inhibited at all by sorbose using galactose or glucose-6-phosphate as substrate, the view that sorbose interacts with glycolysis at the level of the hexokinase is strongly supported.

The finding that the sorbose-induced hemolysis in dog erythrocytes is dependent upon time of incubation, temperature, and pH[4] also supports an interaction of sorbose with the red blood cell metabolism rather than a direct action on the cell membrane. In addition, there was no indication of a decreased osmotic resistance of erythrocytes collected from dogs treated with sorbose for 3 weeks[3].

Possible interactions, among others, of sorbose with hexokinase are a) competitive inhibition, b) noncompetitive inhibition and c) inhibition by a metabolite of sorbose. The latter possibility is supported by the time course of inhibition by sorbose of the lactate formation from glucose (table). An increasing inhibition of glycolysis was noted after 6 h of incubation, resulting from a complete inhibition of lactate formation in presence of sorbose between 14.5 and 24 h of incubation, in contrast to the linear increase of lactate formed in absence of sorbose. Enzyme kinetic investigations with partly purified hexokinase suggest that sorbose may be phosphorylated in dog erythrocytes (Keller, unpublished) in contrast to erythrocytes of man[7]. However, sorbose is not metabolized to lactate.

Among several species tested, including man, only dog erythrocytes showed such an extraordinary high sensitivity to sorbose-induced hemolysis[4]. Sorbose did not inhibit the lactate formation in hemolysates from man using glucose, fructose, mannose, and galactose as substrate (Kistler, unpublished). However, in view of the many common forms of hemolytic anemia in humans, and considering the potential role of sorbose as a food additive, further investigations are necessary to exclude the possibility that human erythrocytes with some specific deficiencies could be sensitive to sorbose-induced hemolysis.

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2 P. Keller and A. Kistler, Experientia *33*, 1380 (1977).
3 P. Keller and A. Kistler, Schweiz. Arch. Tierheilkunde *120*, 29 (1978).
4 A. Kistler and P. Keller, Experientia *33*, 1379 (1977).
5 R.G. Chapman, M.A. Hennessey, A.M. Waltersdorph, F.M. Huennekeus and B.W. Gabrio, J. clin. Invest. *41*, 1249 (1962).
6 H.J. Hohorst, in: Methoden der enzymatischen Analyse, 2.Aufl., Bd.II, p.1425. Ed. H.U. Bergmeyer. Verlag Chemie, Weinheim 1970.
7 G. Rijksen and G.E. J. Staal, Biochim. biophys. Acta *445*, 330 (1976).

Immunomodulating effects in vitro of a hydroxythiazolobenzimidazole in the absence of mitogenicity

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Summary. Wy-13, 876, a hydroxythiazolobenzimidazole, enhanced in vitro antibody formation by mouse spleen cells immunized with sheep red blood cells. The optimal dose was 25–50 µg/culture. The compound did not have a mitogenic effect at any dose.

Recent studies suggest that substances such as bacterial lipopolysaccharides stimulate the immune response by inducing mitogenesis[1–3]. Much of the work in this laboratory has been concerned with the role of a synthetic low mol. wt hydroxythiazolobenzimidazole (Wy-13, 876 or 3(p-chlorophenyl)-2,3-dihydro-3-hydroxythiazolo[3,2-a]benzimidazole-2-acetic acid) in antibody synthesis[4]. The compound has previously been shown to inhibit Lewis lung tumor growth and to stimulate T cell activity in vivo[5,6]. The data presented in this study indicate that Wy-13,876 enhances in vitro antibody formation in mouse spleen cells without producing a mitogenic effect.

Materials and methods. Spleen cells obtained from normal 6 to 8 weeks old Balb/c mice were washed in Hank's solution and 5×10^6 cells and cultured in 0.5 ml medium (RPMI 1640) containing 5% fetal calf serum. The cells were placed on a dialysis membrane in the inner chamber of a Marbrook vessel containing 11–12 ml medium in the outer chamber exactly as described elsewhere[7]. The cultures were immunized with 0.1 ml suspensions of sheep red blood cells (SRBCs) ranging from 2×10^4 to 2×10^6 erythrocytes/ml; control cultures contained no SRBCs. The hydroxythiazolobenzimidazole, suspended in tissue culture medium immediately before use, was added at concentrations ranging from 1 to 1000 µg in 0.1 ml medium to test cultures which were then incubated at 37 °C in an atmosphere of 95% air and 5% CO_2 for 5 days. Spleen cells from at least 5 cultures per group were tested for antibody plaque forming cells (PFCs) after incubation by the direct plaque assay of Jerne as described earlier[7].

To determine mitogenic responses, 10^6 spleen cells were incubated with graded quantities (1–500 µg/0.1 ml) of the benzimidazole at 37 °C for 24–48 h and then pulsed with 0.1 ml ^{3}H-thymidine (2 µCi) followed by incubation for an additional 18 h. The amount of radioactivity in the DNA of the cells was determined by standard scintillation counting.

Results. Addition of graded amounts of the benzimidazole to normal spleen cells cultured in vitro induced a change in the antibody response (table 1). Although 1–10 µg of the compound had no significant effect on the number of antibody forming cells in cultures immunized with 2×10^6 SRBCs, a 25–50 µg dose gave an optimal increase of about 50–80%. The 100 µg dose had little effect, while the 500–1000 µg doses greatly reduced antibody formation. This appeared to be due to the toxicity of the drug at the higher levels (table 2). When lower immunizing doses of SRBCs (2×10^5 or 2×10^4) were used, fewer PFCs appeared in the cultures. Addition of graded quantities of the benzimidazole resulted in a greater response at most levels in comparison to control cultures (table 1). Although total antibody formation was less than the level achieved with the highest dose of SRBCs, the percentage increase in PFCs