# Thermal Stabilization of Poliovirus Type 3 Live Vaccine Strain by Sucrose in the Presence of Magnesium Chloride

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**Abstract:** Infectivity of highly temperature-sensitive and attenuated poliovirus type 3 vaccine strain, Leon $12a_1b$, at 35-37℃ was stabilized by 15-20% sucrose in the medium with 1 M $MgCl_2$. On the other hand, the virus stability was reduced by 10% polyethylene glycol (PEG, Mol. Wt. 6000) in the medium without $MgCl_2$, especially when the virus-PEG mixtures were incubated after freeze-drying. Fibrous cellulose CF11 apparently adsorbed majority of the virus and did not change its inactivation rate, when virus-cellulose mixtures were centrifuged and the precipitates were incubated at 37℃. When virus-cellulose mixtures were filtrated through nitrocellulose membranes, however, virus infectivity recovered from the cellulose on the membrane was poor for 0 day's specimen, with apparent stabilization of the adsorbed virus after 1-2 weeks incubation at 37℃.

*Key words:* Attenuated poliovirus, Stabilization, Sucrose

## INTRODUCTION

Poliovaccines, both inactivated and live-attenuated, have widely been used and proved to be safe and effective to reduce the incidence of paralytic poliomyelitis during the past 20-30 years, and mass-immunization with live vaccines was shown to decrease the transmission of wild strains of poliovirus (Salk, 1955, 1984; Sabin, 1959, 1977, 1980, 1985; Melnick, 1978; Fox, 1980; John, 1984). However, significant numbers of paralytic poliomyelitis have still been reported as localized epidemics among those who had received live poliovaccines as well as among unvaccinated populations in tropical areas (John, 1972; Chodanker and Dave, 1979; LaForce *et al.*, 1980; John *et al.*, 1983).

Trivalent live poliovaccines have been orally administered to confer long-lasting immunity, but these vaccine strains, especially type 3 poliovirus, are highly temperature-sen-

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sitive (Sabin, 1977; Melnick, 1984). Stabilization of the vaccine viruses has been considered as an important factor for successful immunization by oral poliovaccines in various regions of tropical and subtropical countries with warm climate. Several investigators tried to stabilize polioviruses by cations, organic and inorganic acids, or proteinaceous substances, with the best result by 1 M $MgCl_2$ (Wallis and Melnick, 1961, 1962; Melnick and Wallis, 1963; Srivastava *et al.*, 1987). While, epidemiological studies have shown that 1 M $MgCl_2$ is not sufficient to preserve immunogenicities of highly temperature-sensitive and attenuated poliovirus strains in warm-climate countries (John, 1972, 1981; Albert, 1987).

Recently, the first author reported that 15% surcose stabilized highly temperature-sensitive and live-attenuated type 3 poliovirus vaccine strain, Leon $12a_1b$, at 37℃ even in the absence of 1 M $MgCl_2$ (Srivastava, 1988). This finding would be applied to preserve immunogenicities of temperature-sensitive oral poliovaccines in tropics even when cold-chain system is not sufficient. Present report describes combined effect of sucrose and $MgCl_2$ to stabilize the same type 3 poliovaccine strain, as well as the effects of PEG and fibrous cellulose.

## Materials and Methods

*Virus and cell*: Attenuated poliovirus type 3, Sabin vaccine strain Leon $12a_1b$, was obtained from 2 different sources. One from Hoechst Co., West Germany, was passaged once in human rhabdomyosarcoma (RD) cells and the progeny virus was diluted 1:10 in physiological saline to a titer of $7.5 \times 10^5$ PFU/ml, which was used as the virus without $MgCl_2$. Another had been stabilized by 1 M $MgCl_2$ and kept in the Enterovirus Unit, Institute of Hygiene and Epidemiology, Prague, Czechoslovakia. This specimen was diluted with physiological saline containing 2 M $MgCl_2$ to the same virus titer as above and was used as the virus with $MgCl_2$. The pH of physiological saline was 7 for all experiments in this study. RD cells were grown in Eagle's minimal essential medium (MEM, Eagle, 1959) with 10% heat-inactivated calf serum at 37℃.

*Infectivity assay*: Virus specimens were serially diluted in 10-fold steps with physiological saline at room temperature. Growth medium was removed from RD cell cultures in 6 cm diameter Petri dishes, and 0.1 ml of the diluted virus was inoculated in each dish. After 1 hour of adsorption at 37℃, the cells were covered by 5 ml of overlay medium containing 1% agarose and 2% calf serum in MEM with neutral red. When the agarose was solidified, the dishes were incubated at 37℃ for 4-5 days to form plaques. Virus infectivity was shown by plaque-forming unit (PFU) per ml.

*Chemicals*: Fibrous cellulose CF11 was obtained from Whatman Chemicals Separation Ltd., England. PEG 6000 and sucrose were the products of Lachema, Brno, Czechoslovakia.

## RESULTS

*Effect of sucrose on stabilization of attenuated poliovirus type 3 vaccine strain in the presence of 1 M $MgCl_2$*

Weighed amount of sucrose was spread on a sheet of aluminium foil and sterilized overnight by ultraviolet (UV) light (4 lamps of 15 Watts, distance of 30 cm), and was dissolved in physiological saline to make 90, 40, and 30% (w/v) solutions. These solutions were added to equal volumes of the virus with $MgCl_2$ to make final sucrose concentration of 45, 20, and 15%, and virus titer of $3.75 \times 10^5$ PFU/ml. Each mixture was divided into several tubes and incubated at 37 or 35℃. A control was prepared by adding physiological saline to an equal volume of the virus with $MgCl_2$ and incubated along with the specimens with sucrose. In the first experiment, the tubes with 45%, and 15% sucrose as well as the control were incubated at 37℃, sampled at 1 week interval until 4 weeks of incubation, and kept frozen at −20℃ until infectivity was assayed. The result in Fig. 1 shows that virus infectivity in the control with $MgCl_2$ alone was exponentially inactivated (about 1 log/week) and became undetectable after 4 weeks of incubation. When 45% sucrose was included in the medium with $MgCl_2$, the virus infectivity was stabilized a little during the first 2 weeks, but it decreased to undetectable level after 4 weeks. Better stabilization of the virus infectivity was obtained by 15% sucrose with $MgCl_2$ during the first 2 weeks, then the inactivation rate became similar to the control with $MgCl_2$ alone, still infective

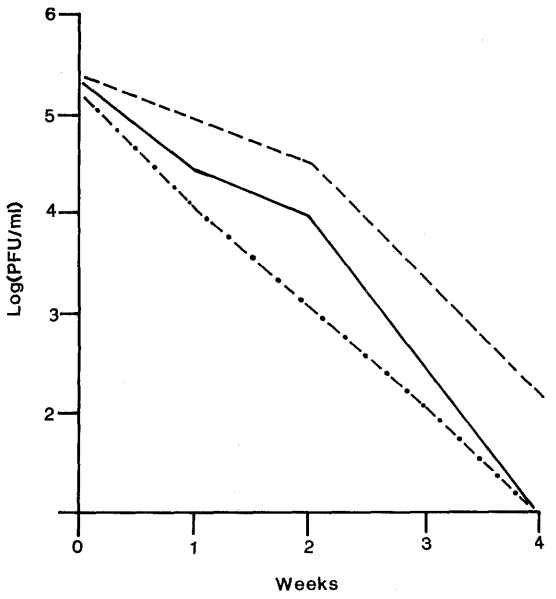


Fig. 1. Survival of attenuated poliovirus strain Leon $12a_1b$ by sucrose in the presence of $MgCl_2$ at 37℃ for 4 weeks. Full line (———) 45% sucrose; dashed line (– – – –) 15% sucrose; dotted and dashed line (—•—•—•—•—) without sucrose in 1 M $MgCl_2$.

virus was detectable after 4 weeks of incubation.

In the second experiment, the specimen with 20% sucrose and $MgCl_2$ was incubated at 35℃ along with the control of $MgCl_2$ alone and sampled every day until 8 days of incubation to assay virus infectivity as in the first experiment. The result in Fig. 2 shows that the virus infectivity was stabilized by 20% sucrose and $MgCl_2$ compared with the control of $MgCl_2$ alone during the first 3 days and on the 8th day of incubation. But the virus infectivity was almost the same between 4 to 7 days of incubation for both specimens with and without 20% sucrose.

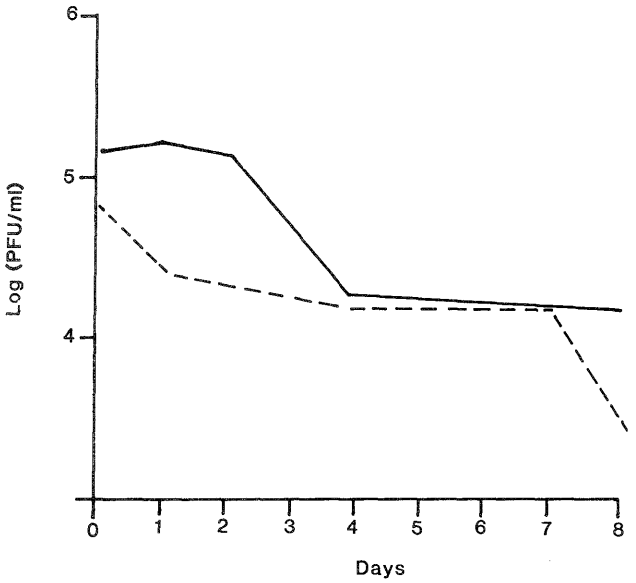


Fig. 2. Stabilization of attenuated poliovirus strain Leon $12a_1b$ by 20% sucrose in the presence of $MgCl_2$ at 35℃. Full line (————) 20% sucrose; dotted line (- - - - -) without sucrose in the presence of 1 M $MgCl_2$.

*Effect of PEG on the stability of attenuated poliovirus type 3 vaccine strain*

PEG has been used to concentrate bacteriophage (Yamamoto *et al.*, 1970) or animal virus (McSherry and Benzinger, 1970). Experiments were performed to test its stabilizing effect on the attenuated poliovirus. Twenty percent (w/v) PEG solution in physiological saline was autoclaved, and mixed with an equal volume of the virus without $MgCl_2$ to make final concentration of 10% PEG and $3.75 \times 10^5$ PFU/ml of the virus. The virus-PEG mixture was dispensed into several tubes and kept at 4℃ for 10 min followed by centrifugation at 8,000 rpm for 30 min. The supernatant was removed and the tubes with precipitated virus were tightly closed and incubated at 37℃ along with control virus without $MgCl_2$. The specimens were sampled after 0, 1, and 2 weeks of incubation and kept frozen at −20℃. Before titration of virus infectivity, PEG-precipitated virus was dissolved to the initial volume with physiological saline. The result in Fig. 3 shows that infectivity of PEG-precipitated virus was more rapidly inactivated than virus infectivity in

the control without $MgCl_2$. In another experiment, PEG was sterilized by UV light similarly to the sucrose experiment and virus-PEG mixture was prepared as described above. The mixture was dispensed into several ampoules and freeze-dried overnight at 4℃. The ampoules were divided into 2 lots; one was closed by sterile cotton plug and another was glass-sealed. All the ampoules were incubated at 37℃ and sampled after 0, 2 and 10 days of incubation to assay virus infectivity as described above. The result in Fig. 3 shows that the virus infectivity of freeze-dried specimens was inactivated with similar rate for both cotton-plugged and glass-sealed ampoules, and the rate was higher than the previous experiment without freeze-drying. Therefore, PEG-precipitation, especially followed by freeze-drying, appears to accelerate the inactivation of the attenuated poliovirus vaccine strain.

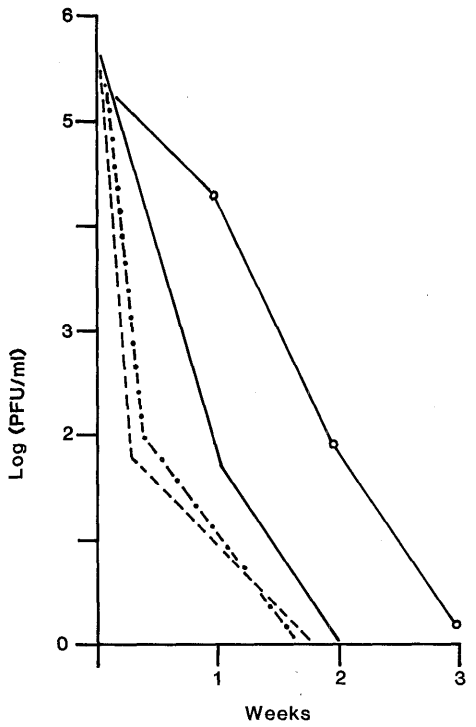


Fig. 3. Effect of PEG on the stability of attenuated polioviruis strain Leon $12a_1b$ strain without $MgCl_2$ at 37℃. Full line (————) 10% PEG autoclaved; dashed line (– – – –) PEG freeze-dried and cotton plugged; dotted and dashed line (—•—•—•—•—) PEG freeze-dried and glass-sealed; open circle and full line (—— ∘ —— ∘ ——) control without PEG.

*Effect of fibrous cellulose CF11 on the stabilization of attanuated poliovirus type 3 vaccine strain*

Several types of cellulose were reported to adsorb poliovirus (Bendová, 1982). Experiments were performed to see the effect of fibrous cellulose CF11 on the stability of poliovirus type 3 vaccine strain. One hundred milligram each of the cellulose was distributed onto 5 different aluminium foil, sterilized by UV-light as described before, and

transfered into 5 different tubes. The cellulose in each tube was suspended with 1 ml of the virus without $MgCl_2$, well-mixed for 30 min at room temperature and centrifuged at 4,000 rpm for 15 min at 4℃. Supernatent was removed and tubes with cellulose-virus precipitate were incubated at 37℃. The tubes were sampled after 0, 1, and 2 weeks of incubation and kept at −20℃. Before infectivity assay, the precipitate was resuspended by 1 ml of 0.2 M glycine buffer, pH 8.5, by vortex mixing for 5 min. The result in Fig. 4 shows that the virus infectivity was inactivated at similar rate as the control experiment in Fig. 2. So that, the cellulose apparently adsorbed the virus but did not stabilize its infectivity. Another experiment was performed using similar preparation, but the cellulose-virus mixtures were filtrated by sterilized nitrocellulose membrane (type HA, 0.45 $\mu$ pore size, Milipore, USA) instead of centrifugation. The filters retaining cellulose and adsorbed virus were transfered into tubes, which were rubber-stoppered and incubated at 37℃. The tubes were sampled after 0, 1 and 2 weeks, and virus-cellulose precipitate on the filter in each tube was resuspended in 1 ml of 0.2 M glycine buffer, pH 8.5, before assaying virus infectivity. The result in Fig. 4 indicated that the inactivation rate of virus infectivity was apparently reduced, although the titer on day 0 was almost 2 logs lower than the previous experiment. Since most of the virus infectivity on day 0 was recovered after centrifugation and resuspension of cellulose-virus mixture in the previous experiment, low virus titer on day 0 in the latter experiment cannot be explained by poor adsorption of the virus to the cellulose and passing through the filter. Rather, the result suggests that majority (99%) of the virus population might have been fixed on the membrane and cannot easily be eluted

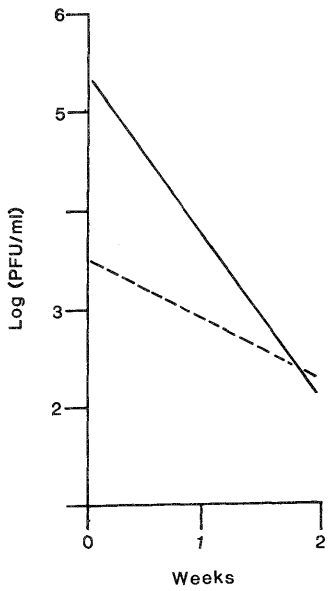


Fig. 4. Survival of attenuated poliovirus strain Leon $12a_1b$ without $MgCl_2$ at 37℃ on fibrous cellulose CF11. Full line (————) virus-cellulose mixture precipitated by centrifugation; dashed line (– – –) virus-cellulose mixture membrane-filtrated.

out because of their higher affinity to nitrocellulose membrane than to fibrous cellulose. In contrast, minority (1%) of the virus population which could be eluted out was somehow stabilized by adsorption to fibrous cellulose and/or nitrocellulose membrane.

## Discussion

Live attenuated oral poliovirus vaccines have widely been used to control epidemics of paralytic poliomyelitis with economical advantages (Sabin, 1959, 1980, 1985; Melnick, 1978), but their efficacy sometimes decreased because of their temperature-sensitivity, especially in warm-climate countries where cold-chain system for storage and transport of vaccines is not always sufficient (John, 1972; Chodanker and Dave, 1979; LaForce *et al.*, 1980; John *et al.*, 1983). It has been recommended that oral poliovaccines should be stored and transported at 4℃ to maintain its immunogenicity (Melnick and Wallis, 1963). First trial to overcome this problem was performed by stabilization of the virus infectivity by various cations and 1 M $MgCl_2$ was most effective (Wallis and Melnick, 1961). Wallis and Melnick (1962) reported that organic and inorganic acids could stabilize attenuated poliovirus type 1 LSc vaccine strain between pH 3 to 5.5. Srivastava *et al.* (1987) stabilized attenuated poliovirus type 3 Sabin vaccine strain, Leon $12a_1b$, by hydrochloric acid. Srivastava (1988) also reported that the same vaccine strain could be stabilized by 15% sucrose for 4 weeks at 35℃ even without $MgCl_2$.

This study showed that the same type 3 Sabin vaccine strain, Leon $12a_1b$, was stabilized at 35-37℃ by 15-20% sucrose in the presence of 1 M $MgCl_2$, and the effect was better than by sucrose alone which was previously reported (Srivastava, 1988). The present results are better than those reported by Mirchamsy *et al.* (1978) on the stabilization of Sabin live trivalent polio vaccine by $MgCl_2$ and sucrose. These stabilization methods may be applied to practical use of live-attenuated polio vaccines in tropical countries. On the other hand, PEG apparently decreased the virus stability and cannot be used as vaccine stabilizer. Another high molecular weight vehicle, fibrous cellulose CF11, appeared to adsorb the virus but stabilization of the virus was not observed when virus-cellulose mixture was centrifuged and resulting precipitate was incubated at 37℃. When virus-cellulose mixture was filtrated through nitrocellulose membrane, however, the result was rather intriguing. The retained virus infectivity was apparently stabilized, although the virus titer on day 0 was significantly low. The result may indicate heterogeneity of the virus population, the majority of which was firmly bound to the membrane and difficult to be eluted. While, small portion of the virus population which could be eluted appeared to be stabilized. This finding should be examined further before its practical application.

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## REFERENCES


1) Albert, M. J. (1987): Failure of live, oral virus vaccines in developing countries. J. Infect. Dis., 155, 1350.
2) Bendová, E. (1982): The problem of elimination of viruses from drinking water. PhD Thesis., Praha.
3) Chodanker, V. P. & Dave, K. H. (1979): Pattern of prevalence of paralytic poliomyelitis in Greater Bombay, India. J. Indian Med. Assoc., 73, 25-29.
4) Eagle, H. (1959): Amino acid metabolism in mammalian cell cultures. Science, 130, 432-437.
5) Fox, J. P. (1980): Eradication of poliomyelitis in the United States: a commentry on the Salk reviews. Rev. Infect. Dis., 2, 277-281.
6) John, T. J. (1972): Problem with oral polio vaccine in India. Indian J. Pediatr., 9, 252-256.
7) John, T. J. (1981): How shall we control poliomyelitis in India? Indian J. Pediatr., 48, 565-568.
8) John, T. J. (1984): Poliomyelitis in India: prospects and problems of control. Rev. Infect. Dis., 6 (Suppl), S438-441.
9) John, T. J., Pandian, R., Gadomski, A., Steinhoff, M., John, M. & Ray, M. (1983): Control of poliomyelitis by pulse immunization in Vellore, India. Brit. Med. J., 286, 31-32.
10) LaForce, F. M., Lichnevski, M. S., Keja, J. & Henderson, R. H. (1980): Clinical survey techniques to estimate prevalence and annual incidence of poliomyelitis in developing countries. Bull. WHO., 58, 609-620.
11) McSharry, J. & Benzinger, R. (1970): Concentration and purification of vesicular stomatitis virus by polyethylene glycol. "precipitation". Virology, 40, 745-746.
12) Melnick, J. L. (1978): Advantage and disadvantage of killed and live poliomyelitis vaccines. Bull. WHO., 56. 21-38.
13) Melnick, J. L. (1984): Live attenuated oral poliovirus vaccine. Rev. Infect. Dis., 6 (Suppl), S323-327.
14) Melnick, J. L. & Wallis, C. (1963): Effect of pH on thermal stabilization of oral poliovirus vaccine by magnesium chloride. Proc. Soc. Exptl. Biol. Med., 112, 894-897.
15) Melnick. J. L., Ashkenazi, A., Midulla, V. C. Wallis, C. & Bernstein, A. (1963): Immunogenic potency of $MgCl_2$ stabilized oral poliovaccine. J. Amer. Med. Assoc., 185, 144-146.
16) Mirchamsy, H., Shafyi, A., Mahinpour, M. & Nazari, P. (1978): Stabilizing effect of magnesium chloride and sucrose on Sabin live polio vaccine. Develop. Biol. Standard, 41, 255-257.
17) Sabin, S. B. (1959): Present position of immunization against poliomyelitis with live virus vaccines. Brit. Med. J., 1, 663-680.
18) Sabin, A. B. (1977): Oral poliomyelitis vaccine: achievements and problems in worldwide use. Bull. Internatl. Pediatr. Assoc., 2, 6-22.
19) Sabin, A. B. (1980): Vaccination against poliomyelitis in economically underdeveloped countries. Bull. WHO., 58, 141-157.
20) Sabin, A. B. (1985): Oral poliovirus vaccine: history of its development and use and current challenge to eliminate poliomyelitis from the world. J. Infect. Dis., 151, 420-436.
21) Salk, J. E. (1955): A concept of the mechanism of immunity for preventing paralysis in

poliomyelitis. Ann. NY. Acad. Sci., 61, 1023-1038.
22) Salk, J. E. (1984): One-dose immunization against paralytic poliomyelitis using a noninfectious vaccine. Rev. Infect. Dis., 6 (Suppl), S444-450.
23) Srivastava, A. K. (1988): Stabilization of attenuated poliovirus type 3 vaccine strain by sucrose. Acta Virol., in press.
24) Srivastava, A. K., Koza, J. & Matyásová, I. (1987): Effect of hydrochloric acid concentration on the stabilization of poliovirus at high temperature. Trop. Med., 29, 55-56.
25) Wallis, C. & Melnick, J. L. (1961): Stabilization of poliovirus by cations. Texas Rep. Biol. Med., 19, 683-700.
26) Wallis, C. & Melnick, J. L. (1962): Effect of organic and inorganic acids on poliovirus at 50℃. Proc. Soc. Exptl. Biol. Med., 111, 305-308.
27) Yamamoto, K. R., Alberts, B. M., Benzinger, R., Lawhorne, L. & Treiber, G. (1970): Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large scale virus purification. Virology, 40, 734-744.

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庶糖と塩化マグネシウムによる3型ポリオ生ワクチンの耐熱性の増大

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熱に極めて不安定な3型ポリオウイルス生ワクチン Leon $12a_1b$ 株の耐熱性を庶糖と塩化マグネシウムによって増大することができた．分子量6,000のポリオエチレングリコール（PEG）はこのウイルスの耐熱性を低下し，その効果はPEGとウイルスの混合液を凍結乾燥した場合に殊に顕著であった．このウイルスは繊維性セルロースCF11に吸着され，CF11とウイルスの混合液を遠心して得られた沈澱中のウイルスの37℃での耐熱性はCF11を加えなかった対照と変らなかった．CF11とウイルスの混合液をニトロセルロース膜で濾過した場合，膜に含まれるウイルスの37℃での耐熱性は1～2週間増大しているように見えたが，濾過直後の膜からウイルスは効率良く回収されなかった．

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