available data suggested that the CF antigen is an integral part of the virus particle (non-infectious).

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Viral Oncolysis with Host Survival.* (28284)

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Viruses sometimes are very selective as to the type of cells in which they multiply. It has long been hoped that this specificity could be used for the destruction of cancer cells. The many efforts in this field have been reviewed by Moore(1). A number of viruses, mainly from the Arbor group, have shown quite striking oncolytic powers against transplantable tumors of the mouse. Unfortunately, a high degree of oncolytic activity was usually associated with extreme mouse virulence, so that tumor regression had to be bought at the price of the animal's death from viral invasion. The extent of tumor destruction was estimated from transplantation of the virus-treated tumor in animals previously immunized against the virus.

The existence of strains of mice genetically insusceptible to Arbor B viruses(2,3) offered the opportunity of studying viral oncolysis without killing the host. Thus, Moore(4) investigated the effects of Russian Spring-Summer Encephalitis virus on tumors transplanted into Arbor-B-resistant PRI mice; no detailed account of this work has been published (Moore, personal communication). Koprowski *et al.*(5) used West Nile virus on ascites tumors in PRI mice. The following interesting features were noted: Complete recovery from a well-established ascites tumor could be obtained; extremely low or high doses of virus were less effective than intermediate doses; mice which had survived viral oncolysis proved resistant to challenge with the same tumor and with a number of other transplantable tumors.

It is difficult to draw general conclusions from a system as unique as the PRI mouse-Arbor B virus host-parasite pair. The observation that inbred A2G mice showed a high degree of resistance against a number of Myxoviruses(6,7) offered an opportunity of using a similar approach with a completely different system. The following is an account of work done with a tumor-adapted strain of neurotropic influenza A virus(8) and the Ehrlich ascites tumor growing in A2G mice.

Materials and methods. Mice. ICR mice (fully sensitive to mouse-adapted Myxoviruses) were purchased from Dublin Laboratories, Dublin, Va. A colony of A2G mice was started from a litter obtained in April, 1962, from the Swiss Federal Office of Public Health, Berne, Switzerland. These mice were propagated by brother-sister matings partly

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at the Dept. of Microbiology, College of Medicine, University of Florida, partly at Dublin Laboratories.

Tumor. A fast-growing aneuploid line of the Ehrlich ascites carcinoma was received by courtesy of Dr. J. L. Edwards, Dept. of Pathology, University of Florida. This tumor was carried in ICR mice by weekly intraperitoneal passages of 10^6 washed tumor cells. The generation time during the exponential phase of growth had previously been found to be 13 to 14 hours (Edwards, personal communication).

Viruses. The Stuart-Harris strain of neurotropic influenza A virus, NWS, had been obtained in 1957 from the World Influenza Centre in London, Great Britain. Stock virus was prepared from infected allantoic fluid and was stored at –70°C. The tumor-adapted line of neurotropic influenza A virus(8) was received by courtesy of Dr. W. W. Ackermann, Dept. of Epidemiology, School of Public Health, University of Michigan. This strain of virus will be referred to as WSA. After one allantoic and one intracerebral passage in ICR mice a stock virus suspension was prepared in the following manner: ICR mice bearing 6-day-old Ehrlich ascites tumors were infected intraperitoneally with 100 EID_{50} of the brain passage material. After 3 days, the peritoneal exudates and aggregated cellular masses were collected from 3 mice, pooled, frozen-thawed twice, ground in a mortar, and centrifuged at 2000 rpm for 30 minutes. The supernatant was distributed in ampoules. The ampoules were sealed and kept at –70°C; each ampoule was used only once.

Virus titrations. Intracerebral titrations were done by inoculating 0.03 ml of suitable dilutions into the left hemispheres of mice anaesthetized with ether. For titration in eggs, 0.2 ml volumes of suitable dilutions were inoculated allantoically into 10-day old eggs. The inoculated eggs were incubated for 3 days at 35°C and the allantoic fluids checked for the presence of chick red cell agglutinins. Titers were expressed as 50% egg infective doses (EID_{50}) per ml.

Results. Growth of Ehrlich ascites tumor in A2G mice. Groups of A2G and ICR mice were inoculated with 10^6 washed Ehrlich ascites cells obtained from an ICR mouse. Two A2G and 2 ICR mice were sacrificed daily, their peritoneal cavities were drained and washed with 10 ml of saline solution and total number of tumor cells per mouse thus obtained was calculated from hemocytometer counts. Fig. 1 shows the results of this experiment.

No difference in growth rate of the tumor was observed between the 2 groups. Tumor-bearing mice died between the 8th and the 20th day after tumor inoculation, usually with accumulations of large amounts of ascitic fluid. The proportion of bloody ascitic fluids, taken by Hartveit(9) as an index of immunologic response of the host to the tumor, was not greater in A2G than in ICR mice. Histologically, the same pattern of invasiveness was found in both groups; for instance, invasion of the pancreas became conspicuous around the sixth day in both A2G and ICR mice.

When groups of A2G mice were inoculated with decreasing numbers of Ehrlich ascites cells taken from an ICR mouse, as few as 100 tumor cells could initiate tumor formation with eventual death of the host (Table I).

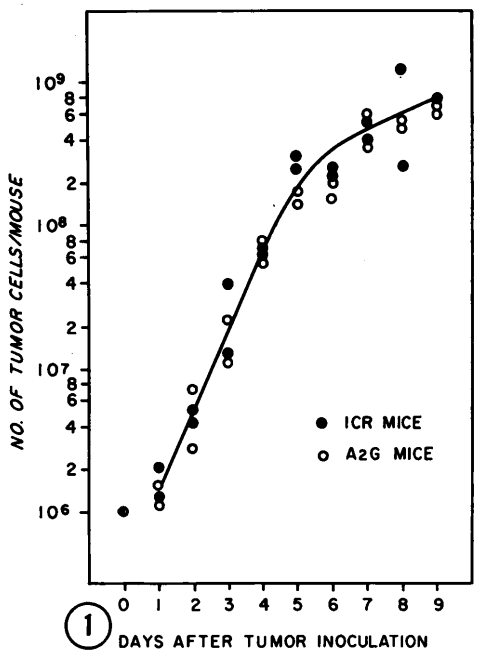
Since in mice dying from the tumor a substantial proportion of the body weight was made up of ascites, daily weighings could be used as a crude measure of tumor growth(10). Fig. 2 shows the weight curves of 5 tumor-bearing ICR mice, Fig. 3 of 5 A2G mice. Both sets of curves have similar slopes; from Fig. 3 it is also apparent that the animal's

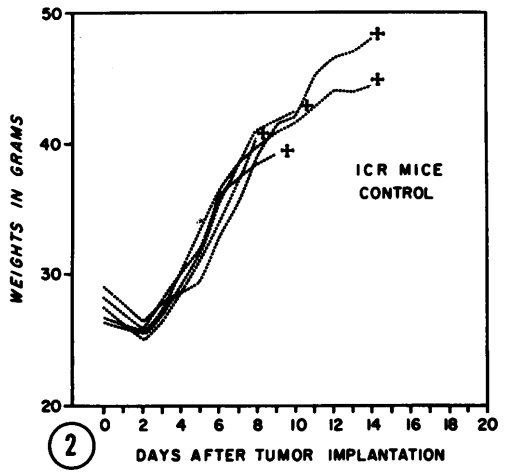
TABLE I. Response of A2G Mice to Graded Doses of Ehrlich Ascites Tumor.

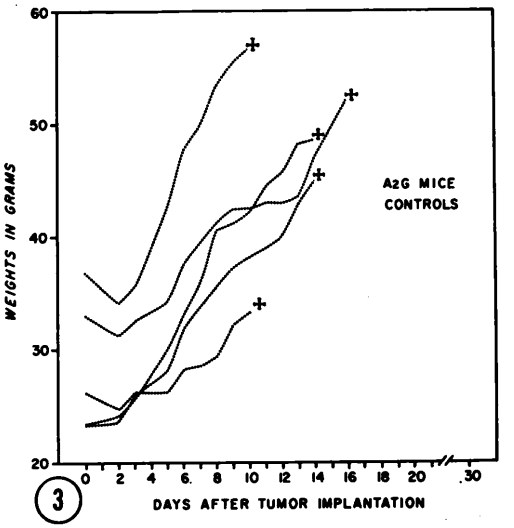
Estimated No. of tumor cells inj*	No. of mice dying with ascites tumor†
10^6	7/7
10^5	7/7
10^4	7/7
10^3	7/7
10^2	6/7

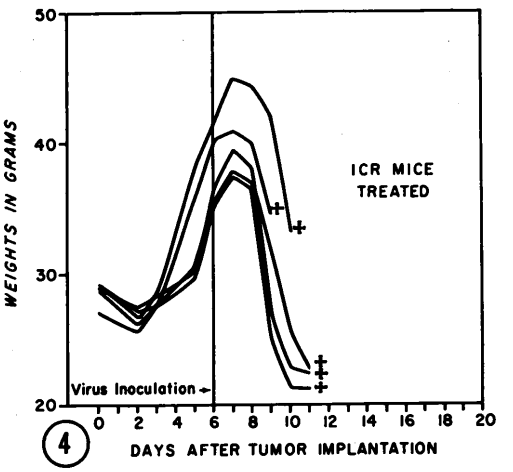
* Intraperitoneal inoculations in 0.5 ml. Washed tumor cells from an ICR mouse. Cell number estimated from hemocytometer counts.

† Numerator: No. of mice dying with ascites tumor. Denominator: Total No. of mice in group.









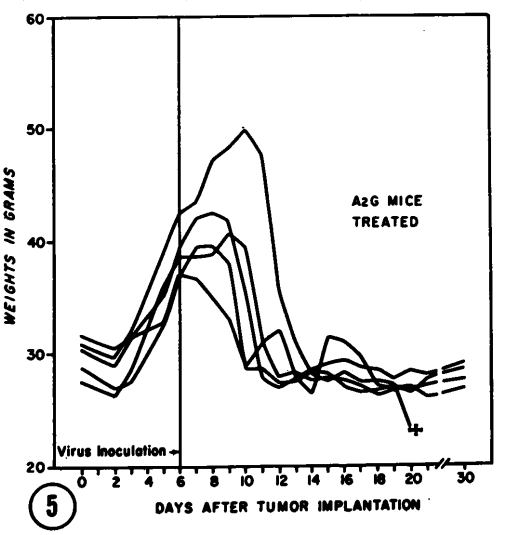


FIG. 1. Growth of Ehrlich ascites tumor in ICR mice and A2G mice. Each point represents total tumor cell count from one mouse. Zero day point represents initial inoculum.

FIG. 2. Weight curves of individual ICR mice inoculated on day zero with 10^6 Ehrlich ascites tumor cells. Crosses mark day of death.

FIG. 3. Weight curves of individual A2G mice inoculated on day zero with 10^6 Ehrlich ascites tumor cells. The large scatter in initial weight is due to the fact that few A2G mice were available for study.

FIG. 4. Weight curves of individual ICR mice bearing Ehrlich ascites tumors and treated with WSA virus.

FIG. 5. Weight curves of individual A2G mice bearing Ehrlich ascites tumors and treated with WSA virus. The 4 survivors shown on this graph were healthy 4 mo after tumor implantation.

TABLE II. Parallel Titrations of WSA Virus in Eggs, ICR Mice and A2G Mice.

Virus dilution	Eggs*	ICR mice†	A2G mice†
10^{-2}	5/5	5/5	0/5
10^{-3}	5/5	5/5	0/5
10^{-4}	5/5	5/5	0/5
10^{-5}	5/5	5/5	0/5
10^{-6}	5/5	5/5	0/5
10^{-7}	5/5	4/5	0/5
10^{-8}	2/5	0/5	0/5
10^{-9}	0/5	0/5	0/5

* Ten-day eggs, inoculated allantoically with 0.2 ml of indicated dilution. Numerator: No. of eggs showing positive hemagglutinin reaction. Denominator: Total No. of eggs in group.

† Intracerebral inoculations of 0.03 ml of indicated dilution. Numerator: No. of mice dying from infection. Denominator: Total No. of mice in group.

weight at time of tumor inoculation had little influence on tumor growth.

Resistance of A2G mice to intracerebral inoculation of WSA virus. WSA virus was titrated in parallel in 10-day eggs by the allantoic route, and in ICR and A2G mice by the intracerebral route. The results are shown in Table II.

Even the largest dose tested failed to kill A2G mice, while the lethality endpoint in ICR mice was similar to the infectivity endpoint in eggs. All surviving ICR mice were challenged 4 weeks later with a large dose of virus, and all proved susceptible except for the one survivor at dilution 10^{-7}. Inapparent infection therefore seems rare in ICR mice.

Growth of WSA virus in tumor-bearing A2G mice. A2G mice carrying Ehrlich ascites tumors of 4 days' duration were inoculated intraperitoneally with 100 EID_{50} of WSA virus. At intervals, 2 mice were killed, their peritoneal exudates were pooled, frozen-thawed, homogenized and centrifuged; the supernatants were titrated in eggs. The following infectivity titres were obtained: at 24 hours, $10^{4.2}$ EID_{50}; at 48 hours, $10^{6.0}$ EID_{50}; at 96 hours, $10^{7.1}$ EID_{50}. No virus could be recovered 10 days after infection.

Oncolysis of Ehrlich ascites tumor by WSA virus. Groups of ICR and A2G mice were inoculated with 10^6 Ehrlich ascites tumor cells. Each mouse was weighed daily. On the sixth day after tumor implantation, when all mice were showing considerable weight increases, 10^4 EID_{50} of WSA virus were inoculated intraperitoneally. Fig. 4 and 5 show the dramatic effect this treatment had on the weight curves of 5 representative mice of each group.

Coinciding with the rapid weight loss, ICR mice became ruffled, developed diarrhea and invariably died between the 2nd and 7th day after viral infection. Virus was present in low titres in the brains and frequently also in the lungs of dead ICR mice. A2G mice also looked sick during the period of acute weight loss, and some of them, usually around 20%, died between the 5th and 15th day after infection. Virus could never be recovered from the brains of such mice. A larger proportion died when the time interval between tumor implantation and viral treatment was delayed beyond 7 days.

When animals were sacrificed at intervals after viral challenge, it was found that within 24 hours the peritoneal cavity no longer contained fluid ascites, but a gelatinous mass of aggregated necrotic cells. Sometimes, and particularly in ICR mice, the intestines seemed glued together by this solidified exudate, and parts of the gut were vastly distended.

Fate of A2G mice surviving oncolysis. Three different patterns were observed in mice surviving oncolysis: a) Mice recovered their normal appearance and remained healthy throughout the observation period, 1 to 4 months. b) After disappearance of the ascites, mice developed along the needle track of the original tumor implantation a palpable subcutaneous growth which eventually regressed. Sometimes regression was accompanied by exulceration of the subcutaneous tumor, leaving a small scar in the skin. Thereafter the animals remained healthy. c) Again, as in b), a subcutaneous nodule developed which increased progressively, exulcerated and killed the mouse within 1 to 2 months from the time of the primary tumor inoculation.

The frequency of this last outcome could be influenced by manipulating the amount and timing of viral challenge. When ascites tumors were treated very early, on the second or third day, with large doses of virus (10^7

EID_{50}) a high proportion of mice died eventually of progressive subcutaneous tumors. The same was true when advanced tumors, of 6 to 8 days' duration, were treated with very small doses (10^0-10^1 EID_{50}) of the virus. The treatment of 4-day-old tumors with intermediate doses of virus (10^3 to 10^5 EID_{50}) gave the largest proportion of overall cures, and no progressive subcutaneous tumors were observed at these dosages. The titration endpoint of WSA virus as estimated from oncolysis in A2G mice was very close to the egg infectivity endpoint.

Absence of oncolysis in vaccinated mice. It had been shown previously that A2G mice inoculated intracerebrally with NWS virus went through an inapparent infection resulting in serological immunity against related strains (7) and that cross-immunity existed between NWS and WSA(11). The following experiment was performed to determine whether immunization with NWS would prevent oncolysis by WSA: A2G mice were inoculated intracerebrally with 10^4 EID_{50} of live NWS; 3 weeks later, these mice received 10^6 Ehrlich ascites cells intraperitoneally. After 6 days, the tumors were treated with 10^4 EID_{50} of WSA virus; A2G mice pretreated with saline instead of NWS served as controls. In the NWS-pretreated mice the tumor grew unchecked and killed the mice, while the saline-treated controls showed the usual behavior with oncolysis and long-term survival of 13 out of 16 mice. When 10^4 to 10^5 EID_{50} of live WSA virus given intracerebrally were similarly used to "vaccinate" A2G mice, there was again full inhibition of oncolysis; smaller doses were ineffective.

Resistance of cured mice to challenge with Ehrlich ascites tumor. A2G mice which had survived viral oncolysis for 1 to 4 months were challenged by intraperitoneal inoculation of 10^6 Ehrlich ascites cells. They showed no weight increase during the subsequent 3 weeks and remained healthy; control mice inoculated with the same cell suspension developed ascites and died within the usual time interval. Five mice were challenged twice: After a first challenge with 10^6 Ehrlich ascites cells 4 months after oncolysis, a second dose of 10^7 Ehrlich ascites cells was given 3 weeks later; these mice likewise failed to develop ascites and stayed healthy.

Discussion. Myxoviruses have not generally been regarded as potent oncolytic agents. Exceptions to this rule are Newcastle disease virus and the neurotropic derivatives of mouse-adapted influenza A virus. A pronounced oncolytic effect of tumor-adapted Newcastle disease virus was found by Flanagan *et al.*(12); unfortunately, this strain has been lost (Cox, personal communication). Neurotropic influenza A virus, both the Stuart-Harris NWS and the Francis-Moore WSN strains, showed a limited ability to grow in tumor tissue(8,11). It has been pointed out that these strains should be more properly called pantropic(13). Ackermann and Kurtz (8) succeeded in growing WSN serially in Ehrlich ascites tumors; this is the strain we have called WSA. In the experiments of Ackermann and Kurtz most treated mice died of viral infection, but some survived. No survivors were observed by Cassel(14) working with the same strain. The reason for this difference is not known.

In our own experiments virus-susceptible ICR mice carrying Ehrlich ascites tumors invariably died when infected intraperitoneally with WSA. The cause of death remained obscure. Evidently the massive destruction of large numbers of tumor cells within a short time could upset the host organism sufficiently to kill it, but this should have applied equally to A2G mice. Possibly the deaths seen in A2G mice were due to this cause, or to the mere mechanical disturbance of peristalsis brought about by the sudden solidification of the ascites. There must have been another and more important cause of death in ICR mice. The finding of virus in brains and lungs of dead ICR mice, but not of A2G mice, suggested generalized viral infection as the cause of death. However, virus-sensitive mice not carrying ascites tumors and infected with WSA intraperitoneally also showed spread of virus to brains and lungs in similar titres to those found in tumor-bearing mice and nevertheless survived(14). Tumor-bearing mice were perhaps killed by the toxic effect of the

large amount of virus produced in the peritoneal cavity(14). This seemed difficult to reconcile with the observation that A2G mice were as sensitive to the toxic action of influenza viruses as virus-susceptible mice(7). Possibly in ICR mice the virus multiplied in the cells lining the peritoneal cavity, in inflammatory cells or in the tissue of some key organ as well as in tumor cells, while in A2G mice the bulk of viral replication was confined to the neoplastic cells.

The experiments carried out with mice immunized to the virus established two points: First, antiviral immunity was effective in preventing viral oncolysis. It therefore seemed probable that in animals not previously immunized viral oncolysis would be quenched after some time by the emergence of antiviral antibodies. Second, the cross-immunity existing between NWS and WSA supported the idea that oncolysis was indeed caused by the influenza virus itself and not by some "passenger" virus picked up during passages in mice and ascites tumors.

A2G mice surviving oncolysis proved resistant to challenge with the same tumor, even when the challenge dose was quite large. Tumor immunity most probably played a part in the recovery of those mice in which palpable subcutaneous tumors developed that later regressed. Since in the non-immune host 100 tumor cells were sufficient to initiate malignant growth, it seemed likely that even in the mice showing no gross subcutaneous tumors some tumor cells escaped primary destruction by the virus and had to be eliminated by an immune mechanism.

During undisturbed tumor growth the immune response of the host was either not sufficiently stimulated or was outgrown by rapid tumor proliferation. The latter explanation seemed less probable since primary subcutaneous implants grew much more slowly, yet killed their host. Apparently the destruction of large numbers of tumor cells by the virus liberated enough antigenic material to stimulate the immune response efficiently. The magnitude of this antigenic stimulus might have been the decisive factor in final recovery: When 2-day old ascites tumors were infected with large doses of virus, a majority of mice developed subcutaneous tumors to which they later succumbed; the amount of tumor destroyed was too small to immunize efficiently. Conversely, when advanced tumors were treated with small doses of virus, again some mice died of slow subcutaneous growths; perhaps in these cases virus proliferation was halted by antiviral immunity before a sufficient mass of tumor tissue had been destroyed. Thus the cure of A2G mice bearing Ehrlich ascites tumors, a system in which there is probably a considerable genetic gap between tumor and host, would seem to depend on the complex interplay of viral oncolysis, antiviral immunity and antitumor immunity.

Most findings in the A2G-WSA system so far parallel those in the PRI-West Nile system as observed by Koprowski *et al.*(5). It remains to be seen whether mice surviving Ehrlich ascites tumors prove immune to other malignancies.

Summary. Ehrlich ascites tumors grew equally well in ICR and inbred A2G mice. ICR mice were sensitive, A2G mice were fully resistant to WSA influenza virus given intracerebrally. When mice bearing Ehrlich ascites tumors were inoculated intraperitoneally with WSA virus, rapid oncolysis ensued. In ICR mice this invariably led to death of the animals, while around 80% of A2G mice survived. Of these, some later succumbed to slowly growing subcutaneous tumors developing at the site of primary tumor implantation. This occurred frequently when tumors were either treated early with large doses of virus, or late with small doses. With other combinations of timing and dosage, some mice developed subcutaneous tumors which later regressed. Mice surviving the primary tumor inoculation for 1 to 4 months were resistant to challenge with the same tumor.

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Quantitative Estimation of Some Phosphatides and Their Hydrolysis Products by Thin Layer Chromatography. (28285)

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Many improvements and simplifications have been made in the analysis of phosphatides and water soluble phosphate esters of glycerol. Silicic acid impregnated paper techniques have greatly speeded up the process of identifying and quantitating phosphatides (1,2,3). Dawson and coworkers(4,5) have carried out extensive studies on paper of the hydrolysis products of phosphatides. With the introduction of TLC,* qualitative separation of lipids and phospholipids was speeded up remarkably. The precision of the separations offered the possibility of quantitating the compounds so separated on thin layer plates. Two groups have analyzed some phosphatides quantitatively by TLC(6,7). The separation of the hydrolysis products of phosphatides on TLC has also been reported recently(8,9); however the separations achieved were not suitable for quantitation.

In this report we describe a procedure for quantitative analysis of phosphatides and of the water soluble choline and ethanolamine containing phosphate esters, and apply it to the analysis of serum phosphatides.

Methods. The plates were prepared as previously described(10). Choline chloride and ethanolamine (redistilled) were obtained from Eastman Organic Chemicals, phosphorylcholine (chloride form) and α-glycerophosphate (DL) from Sigma Chemical Co., and o-phosphoethanolamine from California Foundation for Biochemical Research. Lecithin and phosphatidylethanolamine were prepared from egg yolk essentially by the method of Rhodes and Lea(11). Lysolecithin was prepared from egg lecithin as described by Hanahan and coworkers(12). GPC and GPE were prepared from lecithin and PTE respectively according to Dawson(4).

Separation of the choline and ethanolamine derivatives of phosphatides was readily accomplished by the use of 2 solvent systems differing in the amount of acid present. The choline derivatives were separated in a solvent system containing chloroform:methanol: 3M TCA:water, 40:60:20:12.5 (v/v), the solvent run taking approximately 1-1½ hours. The ethanolamine derivatives required less acidic conditions in which the solvent system contained chloroform:methanol:10% TCA:water, 45:55:8:4 (v/v). The phosphatides were separated and identified as previ-

* The following abbreviations are used: TLC, thin layer chromatogram or chromatography; C, choline; PC, phosphorylcholine; GPC, glycerylphosphorylcholine; E, ethanolamine; PE, phosphorylethanolamine; GPE, glycerylphosphorylethanolamine; GP, glycerophosphate; P, phosphate standard; Le, lecithin; LLe, lysolecithin; PTE, phosphatidylethanolamine; Sph, sphingomyelin.