

**THE VIRUCIDAL ACTION OF THE MEDITERRANEAN
SEA WATER**

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VIRUSES AND SEA WATER

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ABSTRACT

The inactivation rate of poliovirus type 1 was determined in untreated sea water, and in sea water samples that were Seitz-filtered, autoclaved, boiled, heated at 60°C., and stored at room temperature. All types of treatment, except filtration, increased the survival time of the virus over that of untreated sea water.

The major chemicals in solution in sea water showed only slight virucidal properties when acting singly, however, with various combinations a significant degree of inactivation of the virus was observed at low virus concentrations, but not at high virus concentrations.

The effect of reconstituted sea water on inactivation rate of poliovirus type 1 approached that of natural sea water. Autoclaving of the reconstituted samples in this case prolonged the survival time of the virus, when virus was exposed to it while boiling had no such effect.

Lysates of marine bacteria when added to reconstituted sea water showed increased virucidal properties as compared to reconstituted sea water receiving no such treatment.

From these results it was postulated that the virucidal property of natural sea water is filtrable. This property of the sea water was partly due to the chemicals in solution and partly to a heat labile substance of marine origin. Further work should be carried on to characterize this substance.

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INTRODUCTION

Sewage pollution of sea water has been a subject of increasing concern during the past few years. The contamination of sea-food with enteric organisms and the apprehension of contracting disease through sea or river bathing are the main problems under consideration.

Two epidemics of infectious hepatitis, the first in Sweden (Rous, 1956), and the second in Mississippi and Alabama (Mason *et al.*, 1962), have been found to be associated with consumption of raw oysters. However, evidence relating water-borne epidemic diseases, like typhoid fever or poliomyelitis, to sea bathing is still lacking.

In a review on poliomyelitis in relation to river bathing, Boyer and Tissier (1950) investigated a total of 767 poliomyelitis cases occurring within a period of seven years, and were struck by the rarity of a history suggesting any likelihood of infection resulting from bathing in polluted rivers. Thomson (1950) in studying a poliomyelitis outbreak in Auckland, New Zealand, during the years 1947 to 1949, noticed that disproportionately few cases of this disease had occurred at coastal residential areas near the main sewer outfalls, where bathing was common in the summer months.

A controlled epidemiological study was carried out during the years 1953 to 1959 by a committee of the Medical Research Council in London, to determine the number of poliomyelitis cases among children who were permanent residents of various sea-side towns. Data supplied by this Committee provided no evidence relating bathing with causation of poliomyelitis.

It would be reasonable to assume that as a result of accumulation in the coastal areas of infectious material brought in by sewage or by other means, there would be a great danger for swimmers in these areas to contract disease. Nevertheless, the relative paucity of disease among swimmers in polluted areas indicates that inactivation of infective matter takes place in the sea by some means. Factors which bring about death of bacteria in sewage have been extensively studied, but little work has yet been carried out to determine the fate of infectious viral particles which enter the bathing areas through sewage or other routes.

The present study is aimed at an assay of the various factors existing in natural sea waters which may inactivate viruses. In view of the prevalence of poliomyelitis in Lebanon and its increased incidence in the country during the past 10 years (Garabedian et al., 1963), poliovirus was selected for carrying on these studies. Furthermore, the

ability of this virus to remain active when subjected to various adverse environmental conditions, makes it a suitable infectious agent for experimentation.

GENERAL CHARACTERISTICS OF SEA WATER

The marine habitat, as opposed to other major habitats, is characterized by its vast area and large volume, its low content of organic matter, relatively high salinity, low temperature and high pressure at great depths.

Chemical and Physical Properties

The major constituents, exclusive of gases, which constitute more than 99% of the salts in solution in sea water are the following: (Lyman and Fleming, 1940)

<u>Constituent</u>	<u>Grams/Kg.</u>
NaCl (Sodium chloride)	23.476
MgCl ₂ ·6H ₂ O (Magnesium chloride)	10.635
Na ₂ SO ₄ (Sodium sulfate)	3.917
CaCl ₂ (Calcium chloride)	1.102
KCl (Potassium chloride)	0.664
NaHCO ₃ (Sodium bicarbonate)	0.192
KBr (Potassium bromide)	0.096
H ₃ BO ₃ (Boric acid)	0.026
SrCl ₂ ·6H ₂ O (Strontium chloride)	0.040
NaF (Sodium fluoride)	0.003

A large number of trace elements, some in concentrations less than 1×10^{-10} mg/liter, make a total of 0.01% of the solids in sea water. Many of these minor constituents are concentrated several hundred-fold or thousand-fold in certain marine plants or animals, thus their concentration varies in

Chemical Abundance in the Marine Hydrosphere*

	mg/L		mg/L
H	108,000.	Ag	0.0003
He	0.00005	Cd	0.00011
Li	0.2	In	0.02
Be		Sn	0.003
B	4.8	Sb	0.0005
C	28.	Te	
N	0.5	I	0.05
O	857,000.	Xe	
F	1.3	Cs	0.0005
Ne	0.0003	Ba	0.0062
Na	10,500.	La	0.0003
Mg	1,300.	Ce	0.0004
Al	0.01	Pr	
Si	3.	Nd	
P	0.07	Pm	
S	900.	Sm	
Cl	19,000.	Eu	
A		Gd	
K	380.	Tb	
Ca	400.	Dy	
Sa	0.00004	Ho	
Ti	0.001	Er	
V	0.002	Tm	
Cr	0.00005	Yb	
Mn	0.002	Lu	
Fe	0.01	Hf	
Co	0.0005	Ta	
Ni	0.0005	W	0.0001
Cu	0.003	Re	
Zn	0.01	Os	
Ga	0.0005	Ir	
Ge	0.0001	Pt	
As	0.003	Au	0.000004
Se	0.004	Hg	0.00003
Br	65.	Tl	0.00001
Kr		Pb	0.003
Rb	0.12	Bi	0.0002
Sr	8.	Po	
Y	0.0003	At	
Zr		Rn	9.0×10^{-15}
Nb		Fr	
Mo	0.01	Ra	3.0×10^{-11}
Tc		Ac	
Ru		Th	0.0007
Rh		Pa	
Pd		U	0.003

*Oppenheimer, C.H. (ed.), Symposium on Marine Microbiology
(Thomas, Illinois, 1963), p. 7

different water masses according to the abundance of such factors (Harvey, 1955).

Although the salinity of sea water, remote from external influences, such as melting ice, large rivers, heavy rainfall or excessive evaporation, varies within the range of 33% to 38%, the ratios between the major ions are virtually constant (ZoBell, 1963). The salinity values for Lebanese coastal waters measured for the year 1964 varied between 38.96% and 39.79% (George, 1965).

The seasonal variation in temperature of the surface waters is least in tropical waters and greatest in temperate zones. Unpublished data on the seasonal variation in temperature of the Lebanese coastal waters indicate that during the years 1962 and 1963 the minimum temperature was 15°C. and the maximum 33°C.; the average temperatures for the winter and summer months were 17°C. and 29°C., respectively (Acra, 1965). Below 200 meters, ocean water has a nearly constant temperature at any specified depth (Sverdrup et al., 1942).

The average pH value for sea water given by Goldberg (1963) is 8.1±0.2, and the average redox equilibrium 12.5±0.2.

Organic Constituents

There is considerable variation in the quantity as well as in the kind of organic matter present in sea water.

The concentration of organic matter ranges roughly from 0.4 to 10 mg/liter in the open sea and up to 100 mg/liter in shallow productive bays. From 60 to 85% of organic matter exists in a colloidal or other finely particulate state and less than 25% in a dissolved state (Fox et al., 1953).

Living cells, including plankton, make up a small fraction (less than 10%) of the total organic matter. A considerable part of the organic matter consists of relatively inert substances such as humus, chitin, lignin, cellulose, complex proteins and some partially decomposed cellular material. Organic substances dissolved in sea water include traces of polypeptides, various amino-acids, organic acids, certain sugars, biotin, carotenoids, enzymes, niacin, thiamine and cobalamine. The organic content of sea water is in a constant state of flux due to the metabolic activities of the biota (ZoBell, 1963).

Normal Bacterial Flora

Despite its low concentration of organic matter, sea water supports a considerable bacterial biomass. In the littoral zone where biological activity is usually most intense colony counts commonly indicate the presence of from 10^2 to 10^3 bacteria per ml. of water. Bacterial populations of less than 1 and up to 10^8 per ml. have also been observed; the lowest counts are encountered in the open ocean particularly at depths greater than 1000 meters (ZoBell, 1963).

According to Bergey's Manual of Determinative Bacteriology, (1957) microorganisms obtained from the marine habitat belong to the class Schizomycetes of von Naegeli and are distributed in the orders: Pseudomonales, Eubacteriales, Actinomycetales, Beggiotoales, Myxobacteriales, and Spirochetales (Breed et al., 1957). The number of bacterial species of marine origin given in Bergey's Manual is 183, constituting 12% of a total number of 1550 species belonging to the class Schizomycetes. Of 188 genera in the class Schizomycetes, 59 contain species living both in marine environment and on land.

The bacterial flora of pure sea water is predominantly gram-negative with a varying proportion of motile and non-motile types. Liefson et al., (1964), during a study of motile marine bacteria isolated from the waters of the Long Island Sound, Narragansett Bay and Atlantic Ocean, observed that the great majority of the bacteria isolated had polar flagella, fermentative abilities, were non-pigmented, and gram-negative. The non-motile isolates numbered the same as the motile isolates, but on storing the water the percentage of motile isolates increased. Motile gram-positive bacteria, including spore-formers were found to be rare in sea water and intestines of marine animals but very prevalent in bottom mud. Among the total isolates, yellow-pigmented, rosette-forming and luminescent bacteria were also identified.

Conditions in the sea are relatively unfavorable for growth and multiplication of many forms of microorganisms. This is apparent from the fact that in the open areas of the seas and oceans there is comparatively little diversity of microbial species than on land (ZoBell, 1963; Waksman et al., 1937).

Bacteriophage Content of Sea Water

There have been a number of investigations concerning the existence in sea water of bacterial viruses. Several investigators have isolated bacteriophages active against Escherichia coli, Pseudomonas pyocyanea, Shigella dysenteriae and the typhoid group of bacteria mainly from harbors, and coastal areas (D'Herelle, 1926; Guelin, 1952a,b; Carlucci et al., 1960). Bacteriophages of the luminescent bacteria were isolated by Spencer (1955) from sea water, collected 10 miles off Aberdeen, Scotland. This bacteriophage had a lytic action on bacteria at 0°C. It is of interest to note that lysis of the bacteria did not take place when the salt concentration in agar was below 2-3%. Kriss (1963) showed the presence of bacteriophages at great depths, in the hydrogen sulphide zone of the Black Sea and in the bottom deposits.

Bactericidal Properties of Sea Water

Sea water has been found to be distinctly bactericidal to sewage organisms (Greenberg, 1956). Studies on the

survival of freshwater and sewage bacteria in the sea have concluded that wide variety of factors are responsible for the rapid decline of these bacteria in sea water (Greenberg, 1956; Orlob, 1956; Waksman, 1937). The physical, chemical and biological factors postulated to explain this phenomenon include: the destructive action of the ultraviolet rays of sunlight (Gaarder and Sparck, 1931); the adsorption and sedimentation of microorganisms (Rubentschik et al., 1936); the lack of essential nutrients (Korinek, 1927); the presence of destructive bacteriophage (Arloing et al., 1925); the utilization of microorganisms as food by protozoa (Waksman et al., 1937); the presence of toxic substances (ZoBell, 1936), and the osmotic shock caused by transfer of the cells from an environment of relatively low salinity to one of high salinity (Pramer and Carlucci, 1963).

Sea water treated by boiling, autoclaving, pasteurizing and chlorinating has been found to suffer a loss in its bactericidal activity (Vaccaro et al., 1950). Pramer (1963), studying the survival of E. coli in untreated filtered and autoclaved portions of sea water observed that, in most of the samples, the organisms died more rapidly in untreated than in autoclaved or filtered water. This property of the untreated water was possibly due to the presence in water of competitors and predators. In two of six samples the survival time of E. coli was the same in filtered and autoclaved waters.

In four of the samples E. coli survived considerably longer in autoclaved water than in filtered water. The beneficial effect of autoclaving on the survival time of bacteria in sea water is possibly due in part to the influence of high temperatures attained during autoclaving, on the physico-chemical characteristics of sea water, including the redox-potential, concentration of dissolved gases and ionic equilibria (Framer et al., 1963). Jones (1964) and Scarpino (1962) showed that heavy metals in sea water restrict the development of E. coli. By adding a variety of chelating agents to sea water this toxicity was reversed.

That biological factors play a major role in the enhancement of bactericidal properties of the sea water, has been demonstrated by Aubert et al., (1964). For example, while studying the antibiotic capacity of phytoplankton, they observed that in autoclaved sea water sewage bacteria proliferated rapidly, while in the presence of added phytoplankton the survival time of the bacteria was reduced to about 144 hours. The authors have also shown that the antibiotic capacity of phytoplankton was totally abolished by heating at 110°C. for one hour.

Antibiotic production in the sea by marine bacteria and algae, has also been reported (Provasoli, 1963). The activity of various species of sea weeds was found to vary in

different months. Most of the species tested were more inhibitory to Gram-positive bacteria than to Gram-negative bacteria.

The mechanism of bactericidal action of sea water still stands as a complex phenomenon. The results obtained by various investigators are inconclusive and the toxic factor, which presumably exists in the sea water (ZoBell, 1936), has not yet been identified.

SURVIVAL OF POLIOVIRUS IN VARIOUS WATERS

The survival of poliomyelitis virus in different kinds of natural waters has been investigated as early as 1911 when Landsteiner et al. observed that poliomyelitis virus in the form of a suspension of monkey cord remained infective for at least a month when dispersed in tap water and stored at room temperature.

Kling et al. (1929) reported that a suspension of spinal cord infected with poliomyelitis virus in tap water survived for at least 114 days at room temperature in the dark. No mention is made by these workers about the concentration of the virus suspension or the characteristics of the water used.

Studies on the survival of poliovirus in river water were carried out by Rhodes et al. (1950). A dilution of the stool of a paralytic child was made in river water to a final concentration of 1:200, and the preparation stored at 4°C. The virus was assayed by cerebral inoculation into monkeys. Infectivity was lost in the sample between 6 and 7 months after the start of experiment.

Survival of poliovirus in distilled water, spring water and well water was studied by Squeri et al. (1961). The

original concentration of virus was 10,000 infective doses (ID) per liter as tested by tissue culture technics. The survival times for the three types of waters containing poliovirus type 1 (Brunhilde strain), were 140 days in distilled water, 100 days in spring water and 48 days in well water.

The effect of living bacteria and bacteria killed by heat or by repeated freeze thawing on poliovirus type 1 (Brunhilde strain) was studied by Squeri et al. (1963). They observed that living bacteria such as E. coli and Klebsiella sp. did not affect the survival of the virus in distilled, spring or well waters. However, the addition of heat killed E. coli to low concentrations of virus reduced the survival time of the virus. Further studies on the effect of distilled, spring and well water on different concentrations of virus indicated that survival periods in all three types of waters were positively related to the concentration of the virus. With 1×10^7 infective doses per liter, the virus survived for 133, 84 and 56 days respectively in different types of water; while with 1×10^4 infective doses per liter the survival time was 56, 28 and 21 days. Virus survived longer in well and spring waters that were sterilized.

Sea water was found to exert an unfavorable effect on enteroviruses. Cioglia et al. (1962), observed that the self purification effect in sea water was greater than in river

water. Thus the survival time of enteroviruses at 25°C. dropped to a minimum of 8 to 15 days in sea water and 15 to 30 days in river water. It was also observed that generally the viruses resisted inactivation better at lower temperatures (5-15°C.) than at higher temperatures (20-30°C.).

Plissier and Therre (1961) reported that the inactivation of poliovirus type I Mahoney in sea water and drinking water depended on the original concentration of virus. In sea water an original concentration of 10^3 TCD₅₀ of virus per ml. was totally inactivated in 30 days, while a concentration of 10^5 TCD₅₀ per ml. lost 1.7 log units, during the same period. Samples were kept at 17°C. in a water bath. The inactivation of poliovirus in Melnick's lactalbumin medium, however, did not depend on the initial concentration of virus. Each concentration after 30 days lost a titer of one log unit.

Plissier (1963) reported that poliovirus type I (Mahoney) at an initial concentration of 10^5 LD₅₀ per ml. was found to survive for 9 months in Melnick's lactalbumin medium, 5 months in tap water and 3 months in sea water at a temperature of 17°C. At a concentration of 10^3 LD₅₀ per ml. the corresponding survival times were 3 months in Melnick's lactalbumin medium and one month for both tap water and sea water. Tap water contained chlorine and ozone.

EFFECTS OF VARIOUS CHEMICAL AND PHYSICAL AGENTS
ON POLIOVIRUSES

pH Resistance

The poliovirus group is known to be among the hardiest of all known animal viruses, the stability range in acid and alkaline media being from pH 3 to pH 10 (Gard, 1955). The virus rapidly loses infectivity in more acid or alkaline media.

Inactivation by Heat

Younger (1957) carried out thermal inactivation experiments on different types of poliovirus at 50°C. and 36.5°C. He noticed strain differences in regard to inactivation at 50°C. The mean percent infectivity remaining after 1 hour of heating was 24% for type 1, 14% for type 2 and 0.1% for type 3. For type 1, infectivity was reduced to 0.0001% in 12 days at 36.5°C. The inactivation rate at this temperature was slower for the other two types.

Oxidation-Reduction Potential

Lund and Lycke (1961) investigated the effect of alterations in the oxidation-reduction potential on the inactivation of type 3 poliovirus. They observed that at redox potentials of -250 to +200 millivolts the rate of virus inactivation was the same as in phosphate buffer. The inactivation rate was enhanced at potentials above 300 millivolts. The

oxidizing agent used was potassium-permanganate.

Irradiation and Light

Poliovirus was found to be inactivated by ionizing irradiation and ultraviolet light the maximum effect being obtained with wavelengths of about 2760 Å°. (Fogh, 1955; Taylor, 1957a). Wallis (1963) studying the photodynamic inactivation of poliovirus reported that virus free of heterocyclic dyes is light resistant.

Chemicals

The inactivation of poliovirus by chlorine has been widely investigated (Weidenkope, 1958; Emerson et al., 1940; Clarke et al., 1964). Kelly et al. (1958) reported that at pH 7 and 25°C. a minimum free residual chlorine concentration of 0.3 ppm and a contact period of at least 30 minutes were required to inactivate enteric viruses.

Lund (1961) reported that chlorine compounds like hypochlorite and chloramine owe their virucidal activity to their oxidizing effect. Chloride ion which is the major form of the element in sea water has no virucidal effect (Kelly et al., 1958). Likewise, elementary iodine is a rapid devitalizing agent. However, I⁻ and IO₃⁻ have little or no demonstrable effect on polioviruses (Berg et al., 1964).

The in vitro resistance of poliovirus to various chemical agents has been studied by Edwin et al. (1955). Within 2 hours at 37°C. the following minimal doses of chemicals were found to inactivate polioviruses:

Copper sulfate	0.03 gm/100 ml.	inactivated 20 ID (IC monkey)
Mercuric chloride	0.005 gm/100 ml.	inactivated 20 ID
Stannic chloride	0.17 gm/100 ml.	inactivated 20 ID
Zink sulfate	0.28 gm/100 ml.	inactivated 15 ID
Ferrous iodide	0.3 gm/100 ml.	inactivated 20 ID
Ferrous sulfate	0.28 gm/100 ml.	inactivated 15 ID
Uranium acetate	0.42 gm/100 ml.	inactivated 15 ID

The concentrations of these chemicals by far exceed those observed in natural sea waters.

MATERIALS AND METHODS

Sea Water

Sea water samples were obtained in sterile flasks from a selected spot along a rocky coast in Beirut, far from any sewer outfall. Samples were collected on calm mornings near the surface of the sea and all determinations were initiated within 1 hour of collection.

Artificial Sea Water

Attempts were made to constitute the sea water artificially following the data presented by Constan (1956) for Beirut sea water.

Composition of Beirut Sea Water (Constan, 1956)

Water	H ₂ O	96.38%
Sodium chloride	NaCl	2.8%
Potassium chloride	KCl	0.08%
Magnesium chloride	MgCl ₂	0.37%
Magnesium sulfate	MgSO ₄	0.23%
Calcium sulfate	CaSO ₄	0.14%
Calcium carbonate	CaCO ₃	0.013%

The sea water was also constituted artificially using values reported by Lyman and Fleming (1940), (page 4), but utilizing a W/V basis.

Standard analytic reagents were used for both preparations. NaCl, KCl, MgCl₂, MgSO₄, NaF, Na₂SO₄, H₃BO₃, NaHCO₃ were obtained from the Merck Chemical Laboratories, Germany; CaSO₄ from Hopkin and Williams Ltd., England; CaCO₃ from the British Drug House Ltd., Poole, England. SrCl₂, KBr from Merck Laboratory Chemicals, New Jersey, U.S.A.

The solutions were sterilized by filtration through Seitz pad type EK or autoclaved at 15 lbs. pressure for 15 minutes.

Solutions of Chemicals

- a. Single Chemicals: Solutions of the major chemicals present in sea water were prepared in distilled water using the same amount of constituent as determined by Constán (1956) for Beirut sea water. A 4% solution of NaCl was also prepared in distilled water. Sterilization of solutions was carried out by autoclaving at 15 lbs. pressure for 15 minutes.
- b. Combined Chemicals: Groupings of the various major chemicals in sea water using the data of Constan (1956), were prepared as follows:

Group 1. NaCl	2.8 gm.
KCl	0.08 gm.
MgCl ₂	0.37 gm.
Distilled water up to 100 ml.	

Group 2. MgSO_4 0.23 gm.
 CaSO_4 0.14 gm.
 Distilled water up to 100 ml.

Group 3. NaCl 2.8 gm.
 KCl 0.08 gm.
 MgSO_4 0.23 gm.
 CaSO_4 0.14 gm.
 Distilled water up to 100 ml.

Solutions were sterilized by autoclaving at 15 lbs. pressure for 15 minutes. If not used immediately they were stored at 4°C.

Filtrates from Sea Water Bacteria

A 100 ml. amount of freshly obtained sea water was passed through a millipore membrane filter to collect all the bacteria. The filter pad was placed in a nutrient medium containing various salts, amino acids, thiamine and pyrimidines (Wrights, 1954). Turbidity was observed in the medium after an incubation period of 1 day at 30°C. A gram-stained smear of the culture indicated the presence of gram-negative rods. Incubation was continued for another 7 days after which the culture was filtered through a sintered glass filter. The filtrate, assumed to contain bacterial autolysates and other products was used immediately after preparation.

Virus Strain

The poliovirus type 1, attenuated Sabin strain, kindly supplied by Dr. T. Frothingham, Tulane University School of Medicine, New Orleans, was used throughout these experiments.

For stock virus preparation 0.5 ml. of virus suspension was inoculated into bottles of monkey kidney monolayer cell cultures. The bottles were incubated at 37°C. for $\frac{1}{2}$ hour to allow virus adsorption on cells, 10 ml. of maintenance medium was then added and the bottles re-incubated. After complete CPE had occurred (commonly within 24 hours), the virus suspension was harvested, centrifuged at 3000 revolutions per minute for 5 minutes to remove debris, tested for sterility and stored at -70°C. until use.

Monkey Kidney Cell Cultures

The continuous monkey kidney cell line available in the Department of Bacteriology and Virology, American University of Beirut was kindly supplied by Dr. Chu of the Lebanese Government Laboratories at Fanar.

Nutrient Medium

Lactalbumin hydrolysate (0.5%) in BSS	80 ml.
Eagles solution 10X	10 ml.
Glucose (10%)	5 ml.
Calf serum	15 ml.

Penicillin 100 units/ml.
 Streptomycin 100 ugm/ml.
 Sodium bicarbonate (2.8%), a sufficient quantity added
 to produce a pH of 7.4.

The medium was filtered through Seitz filter, and incubated for 72 hours at 37°C. to test for sterility. Antibiotics were added just before use.

Maintenance Medium

Lactalbumin hydrolysate (0.5%) in BSS 90 ml.
 Glucose (10%) 5 ml.
 Calf serum 5 ml.
 Penicillin 100 units/ml.
 Streptomycin 100 ugm/ml.
 Sodium bicarbonate (2.8%), a sufficient quantity added
 to produce a pH of 7.4.

Agar-overlay Medium

Lactalbumin hydrolysate (0.5%) in BSS 85 ml.
 Glucose (10%) 5 ml.
 Calf serum 10 ml.
 Agar* (6%) 10 ml.

*Purified agar for tissue culture use was obtained from Difco Laboratories, Detroit, Michigan. Agar, 6 gm., was dissolved in 100 ml. distilled water by boiling dispensed in 10 ml. amounts in screw-capped tubes and autoclaved at 15 lbs. pressure for 15 minutes and stored at 4°C. Just before use the agar was melted by heating in a water bath.

Penicillin	100 units/ml.
Streptomycin	100 ugm/ml.
Sodium bicarbonate (2.8%), a sufficient quantity added to produce a pH of 7.4.	

Hank's Balanced Salt Solution (BSS) (Merchant et al., 1960)

Stock Solution 10X

Solution A.-

NaCl	80.0 gm.
KCl	4.0 gm.
MgSO ₄ .7H ₂ O	2.0 gm.
Na ₂ HPO ₄ .2H ₂ O	0.6 gm.
Glucose	10.0 gm.
KH ₂ PO ₄	0.6 gm.
Distilled water up to 800 ml.	

Solution B.-

CaCl ₂	1.4 gm.
Distilled water up to 100 ml.	

Solution C.-

Phenol Red	0.2 gm.
Distilled water up to 100 ml.	

The three solutions are mixed and preserved with 3-4 ml. of chloroform at 4°C.

Working Balanced Salt Solution

The working BSS was prepared by diluting 10X stock BSS with distilled water, dispensed in screw-capped bottles, and autoclaved at 120°C. and 15 lbs. pressure for 15 minutes. The pH was adjusted to 7.4 with 2.8% sodium bicarbonate.

Lactalbumin Hydrolysate - Balanced Salt Solution

Lactalbumin hydrolysate, 0.5 gm., was dissolved in 100 ml. of BSS, dispensed in screw-capped bottles and autoclaved at 15 lbs. pressure for 15 minutes. The pH was adjusted to 7.4 with 2.8% sodium bicarbonate.

Eagle's TC Medium

Constituents for Eagle's medium were obtained in powder form in vials, as TC vitamins, TC amino acids and TC glutamine, 5%, from Difco Laboratories Detroit, Michigan, U.S.A.

10X Eagle TC Medium

TC Vitamins	0.10 gm.
TC Amino acids	0.22 gm.
Glutamine 5%	6.00 ml.

Make up to 100 ml. with BSS and stored at -20°C. until used.

Glucose Solution

Dextrose	10 gm.
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Distilled water up to 100 ml.

Autoclaved at 15 lbs. pressure for 15 minutes and stored at 4°C.

Antibiotics

Penicillin 10,000 units and 10,000 ugms of streptomycin per ml. in sterile bi-distilled water were distributed in 2 ml. amounts in screw-capped tubes and stored at -20°C.

GKN Solution

a. Formula for 10X

Glucose	20 gm.
Sodium chloride	160 gm.
Potassium chloride	4 gm.

Dissolved in 2 liters of bi-distilled water. Phenol red 1%, 40 ml. and 6-8 ml. of chloroform were added and the mixture stored at 4°C.

b. Working Solution

The stock solution was diluted 10-fold with distilled water, autoclaved at 15 lbs. pressure for 15 minutes and stored at 4°C.

Trypsin Solution

Trypsin stock solution (1:250) 2.5% in isotonic saline, was obtained in vials from Microbiological Associates Inc.,

Bethesda, Maryland. Working solution for dispersing monkey kidney cells was prepared by diluting the stock solution to 0.25%, using GKN, pH 7.8, as diluent. It was stored at -20°C .

Calf Serum

Calf blood was obtained from a slaughter-house near Beirut city. After 2-3 hours of storage at room temperature the clot was broken and the flasks containing the blood were stored overnight at 4°C . Next day the serum was separated from the clot by centrifugation, filtered through Seitz filter, inactivated for 45 minutes at 60°C . and stored at -20°C . until use.

Neutralization tests with poliovirus type 1, were carried out on the serum lots according to the method described by Merchant et al. (1960) and Godtfredsen and Von Magnus (1959), with some modification. Equal amounts, 0.3 ml., of diluted (1:5) and inactivated serum and 100 TCD₅₀ virus were mixed and kept for 1 hour at room temperature for neutralization to occur. The mixture was then inoculated into 2 cell culture tubes using 0.2 ml. amounts for each tube. The tubes were incubated for 30 minutes at 37°C ., after which 0.8 ml. of maintenance medium was added to each tube. They were then re-incubated in an inclined position. Uninoculated cell cultures, virus inoculated cell cultures and cell cultures containing serum alone were kept as controls. Only those serum lots which

were free of neutralizing antibodies were used in the preparation of maintenance media.

Glassware

The tissue culture tubes and bottles were obtained from Kimble Glass Company, U.S.A. Sterile screw-capped glass bottles of 200 ml. capacity were used in all experiments unless stated otherwise.

EXPERIMENTAL PROCEDURES

Titration Methods

- a. Tube Method: Stock virus suspensions and virus in test solutions were titrated by inoculation of 0.1 ml. amounts from each of 10-fold dilutions of virus, 10^{-1} - 10^{-8} , prepared in Hank's balanced salt solution at pH 7, into monolayer tubes of continuous monkey kidney cell cultures. Virus was allowed to adsorb on cells for 30 minutes at 37°C ., after which 0.9 ml. of maintenance medium was added and the tubes re-incubated in an inclined position. Uninoculated cell cultures and virus inoculated cell cultures were kept as controls. Readings were done on the 6th day and the titers calculated according to the method described by Reed and Muench (1938).
- b. Plaque Method: The methodology of the plaque technique employed in these experiments was essentially that of Holland and McLaren (1959) with some modification. Monolayers of continuous monkey kidney cells in rectangular screw-capped glass bottles of about 50 ml. capacity were washed with Hank's BSS, pH 7, and inoculated with various dilutions, 10^{-1} - 10^{-8} , of virus suspension. The bottles were incubated for 30 minutes at 37°C . after which they were washed with Hank's BSS, pH 7, to remove unattached virus, and overlaid with 5 ml. of maintenance medium containing

0.6% agar. The cultures were re-incubated until development of plaques occurred, generally within 48-72 hours. At this stage the medium was poured off the bottles. Monolayers were then washed with 0.9% saline and fixed and stained by exposure for 2 minutes to dye solution (1% crystal violet and 20% ethanol in distilled water). Excess dye was rinsed off with tap water and the plaques counted.

Filtered and Unfiltered Sea Water and Poliovirus

To determine the effect of microorganisms in sea water on the inactivation rate of poliovirus, 150 ml. amounts of sea water samples were filtered through Seitz pad type EK to remove bacteria, other microorganisms and any particulate matter. The inactivation rate of 10^3 TCD₅₀/ml. of poliovirus contained in 100 ml. amounts of the filtrate was determined at 25°C. Unfiltered sea water, 100 ml. amounts, containing the same amount of virus were used as controls. Determinations of the virus titres, using 1 ml. of each specimen, were carried out once every day by the tube titration method.

The bacteria in fresh samples of sea water were counted by making 10-fold dilutions of sea water in sterile distilled water and incorporating 1 ml. of each dilution with nutrient agar in Petri plates. The colonies were counted after an incubation period of 48 hours at room temperature. The number of counts varied from 180-250 bacteria per ml. of sea water.

Sea Water Chemicals and Poliovirus

- a. Single Chemicals: To test the effect of sea water chemicals on poliovirus, the inactivation rate of 10^4 TCD₅₀/ml. of poliovirus contained in 100 ml. amounts of each solution was determined at 25°C. Virus in each sample was titrated, using 1 ml. amounts, once every 2 to 3 days for the first month and once every week for the following month by the tube method. The inactivation time of 10^4 TCD₅₀/ml. of virus in 100 ml. of distilled water was taken as control.
- b. Combined Chemicals: The effect of combination of the major chemicals in sea water on two concentrations of poliovirus was tested. The inactivation rate of 10^4 TCD₅₀/ml. and 10^3 TCD₅₀/ml. (or 10^6 PFU/ml.), contained in 100 ml. amounts of solutions of each group of chemicals was determined at 25°C. Virus titrations using 1 ml. amounts of each sample were carried out by the tube or plaque method. Determinations of the inactivation rate of both concentrations of virus in sea water and distilled water were carried on simultaneously with the tests.

Artificial Sea Water and Poliovirus

A comparison of the virucidal effect of the filter sterilized, artificially constituted sea water preparations according to the data of Constan (1956) and Lyman and Fleming (1940), was carried out. The inactivation rate of 10^6 PFU/ml. of poliovirus

contained in 100 ml. amounts of each solution was determined at 25°C. Virus titrations using 1 ml. of each sample were carried out once every 2-3 days by the plaque method.

The inactivation rate of 10^6 PFU/ml. of poliovirus in 100 ml. amounts of sea water and 2.8% and 4% solutions of NaCl, was determined simultaneously.

Treated Sea Water Preparations and Poliovirus

To test the effect of heat or aging on the virucidal efficiency of sea water, freshly obtained sea water samples were treated as follows:

- a. Boiled for 5 minutes to destroy, if possible, the toxic substances assumed to be present in sea water.
- b. Heated at 60°C. for 30 minutes to determine the resistance to heat of toxic substances.
- c. Autoclaved for 15 minutes at 15 lbs. pressure.
- d. Allow to stand at room temperature for 2 weeks to determine loss, if any, of virucidal activity. Sea water from Denmark* collected in 1963 was also used for the same purpose.

The rate of inactivation at 25°C. of 10^6 PFU/ml. of poliovirus in 100 ml. amounts of these differently treated samples was determined. Virus titrations using 1 ml. of each sample were carried out every 2-3 days by the plaque method.

*Obtained in vials from the Standard Sea-Water Service, Denmark, and kindly supplied by Dr. Carl George of the Department of Biology, American University of Beirut.

Untreated sea water and artificially constituted sea water subjected to similar treatments were used as controls.

Filtrates from Sea Water Bacteria and Poliovirus

To test the effect of bacterial autolysates on poliovirus, equal amounts of artificially constituted sea water and bacterial filtrate, prepared as described previously were mixed and the inactivation rate of 10^6 PFU/ml. of poliovirus in 100 ml. of this mixture was determined at 25°C. The control consisted of equal amounts of culture medium subjected to the same treatments and artificially constituted sea water. Virus titrations were carried out every 2-3 days by the plaque method.

RESULTS AND DISCUSSION

Results of in vitro studies of the effect of sea water on poliovirus indicated that sea water has a definite virucidal property. Poliovirus 10^4 TCD₅₀/ml. was totally inactivated in about 32 days in sea water at 25°C., while in distilled water the infectivity was reduced to about 10^3 TCD₅₀/ml. during the same period (Fig. 2).

The inactivation time of poliovirus in sea water was found to be dependent on the virus concentration, 10^3 TCD₅₀/ml. being inactivated totally in about 9 days while 10^4 TCD₅₀/ml. of virus lost about 1.25 log units during the same period (Figs. 1 and 2). This corroborates the results of Plissier and Therre (1961), who reported that an original concentration of 10^3 TCD₅₀/ml. of virus was totally inactivated in 30 days in sea water at 17°C., while a concentration of 10^5 TCD₅₀/ml. lost only 1.7 log units during the same period.

The results presented in Fig. 1 indicate that the virucidal property of sea water is filtrable; the time required for inactivation of 10^3 TCD₅₀/ml. of virus at 25°C. in Seitz-filtered sea water was one day longer when compared with that of untreated sea water. This difference is not significant since the inactivation time of 10^3 TCD₅₀/ml. of virus in samples of sea water collected at different times

varied from 6-9 days (Fig. 1, 4, 5 and 6).

After establishing that sea water had definite virucidal properties, the effect on survival time of poliovirus of various constituents in sea water was next tested. The virucidal effect of various chemicals, at concentrations determined for Lebanese coastal waters, were tested on 10^4 TCD₅₀/ml. of poliovirus. NaCl, 2.8%, had a greater virucidal effect than KCl, MgCl₂, MgSO₄, CaSO₄ and CaCO₃ when used singly in concentrations indicated in Fig. 2. In 2.8% NaCl there was a loss in titer of 3 log units in 50 days, however, for inactivation of all the virus present, a period longer than 72 days was required (Fig. 2).

The effect of various combinations of chemicals upon the inactivation of poliovirus was determined at two virus concentrations 10^4 TCD₅₀/ml. and 10^3 TCD₅₀/ml. As indicated in Fig. 3, 10^4 TCD₅₀/ml. of virus in an artificially constituted sample of sea water (Constan, 1956) and in a mixture of chemicals, NaCl, KCl and MgCl₂ in concentrations described for sea water, lost equally titers of 3 log units in 50 days. This could be attributed to the effect of NaCl (2.8%) alone (Fig. 2). The same concentration of virus lost a titer of 2 log units in a solution containing KCl, MgCl₂, CaSO₄, MgSO₄ and in another containing CaSO₄ and MgSO₄ in concentrations described for sea water.

It could be concluded from Fig. 2 and 3 that there are factors in sea water which have virucidal efficiency, other than the chemicals so far tested; also KCl and MgCl₂ have no demonstrable virucidal effect.

The data from Fig. 4 and 5 suggest that the chemicals in artificially constituted sea water have a cumulative effect upon the inactivation of poliovirus. The survival time of 10⁶ PFU/ml. (or 10³ TCD₅₀/ml.) of virus in 3 groups of chemicals NaCl, KCl, MgCl₂; KCl, MgCl₂, CaSO₄, MgSO₄, and CaSO₄, MgSO₄, in concentrations described for sea water by Constan (1956) were 32, 33 and 34 days respectively. The survival time of 10⁶ PFU/ml. of virus in artificially constituted, autoclaved sea water was 26 days (Fig. 4). It was further observed that in Seitz-filtered waters constituted artificially according to Constan (1956), and Lyman and Fleming (1940), the survival times of 10⁶ PFU/ml. of poliovirus were 15 and 11 days respectively (Fig. 5). This difference of 4 days could be attributed to the presence of Br⁻, F⁻, HCO₃⁻, Sr⁺⁺ and H₃BO₃ in the sea water artificially constituted according to the data of Lyman and Fleming (1940). As indicated in Fig. 5, the survival time of 10⁶ PFU/ml. of poliovirus in two solutions of NaCl, containing 2.8% and 4% of salt respectively, was about 28 days.

The effect of various treatments on the virucidal effect of sea water was also studied. The survival time of

10^6 PFU/ml. poliovirus in untreated sea water was 8 days; in sea water stored for 2 weeks, 12 days; in sea water heated for 30 minutes at 60°C ., 18 days; in boiled sea water, 20 days, and in sea water received from Denmark (1963), 24 days (Fig. 6). The increase in survival time of poliovirus in all these treated samples of sea water indicates the presence in untreated sea water of some inhibitory factor which loses potency on storage and is heat labile. This assumption was further substantiated by the fact that boiling was found not to effect the virucidal efficiency of artificially constituted sea water. The increase in survival time of the virus in autoclaved, artificially constituted sea water could be due to some alteration in the chemical composition of the artificially constituted sample due to the effect of heat (Fig. 7).

The addition of bacterial lysates to artificially constituted sea water reduced the survival time of poliovirus by 3 days as compared to the control sample (Fig. 8). This does not imply however, that the inhibitory factor in the sea is necessarily of bacterial origin. For example, Moewus (1963) reported that poliovirus inoculated on fish cell monolayer cultures did not produce cytopathic changes and lost infectivity within a shorter span of time than could be accounted for by incubating at 20°C . This "factor" released by fish cells into the medium lost its effect gradually by duration of incubation.

In conclusion, the virucidal property of sea water seems to be due to the combined effect of its chemical composition and an unknown inhibitory factor. Slight shifts in the potency and/or concentration of this inhibitory factor may be the cause of the variation in survival times of poliovirus in different samples of sea water.

The variation in survival time in sea water of sewage bacteria was attributed by Jones (1964) to be due to slight changes in salinity and redox potential. However, in the case of poliovirus, Lund and Lycke (1961) reported that the inactivation rate was the same in a wide range of redox potentials, -200 to +200 millivolts. In results indicated in this thesis the inactivation rate of poliovirus was not different in NaCl concentrations of 2.8% and 4% (Fig. 5).

- x—x Sea water - untreated
- Sea water - Seitz-filtered
- Saline 0.85 %

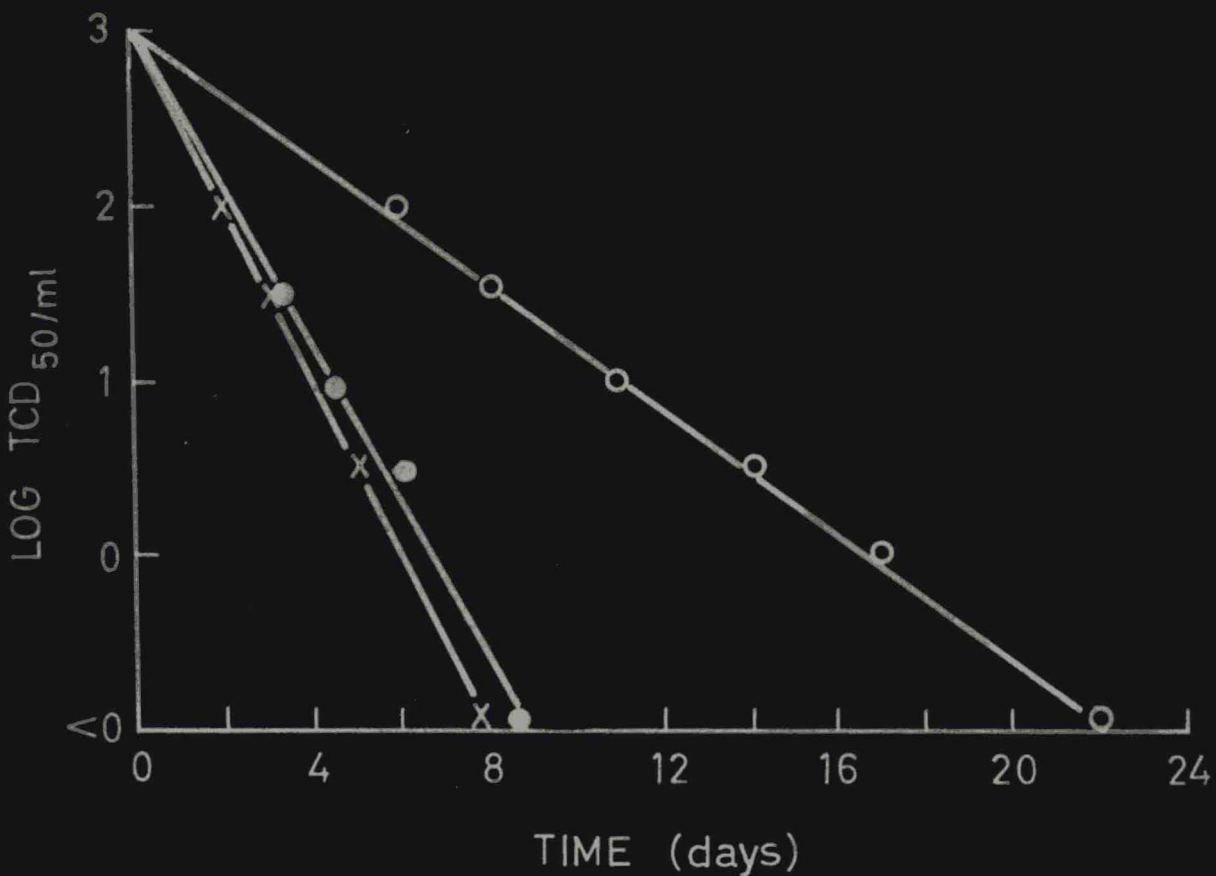


Fig. 1. The inactivation rate of poliovirus in Seitz-filtered and untreated sea waters. (Virus concentration 10³ TCD₅₀/ml., Temperature 25°C.).

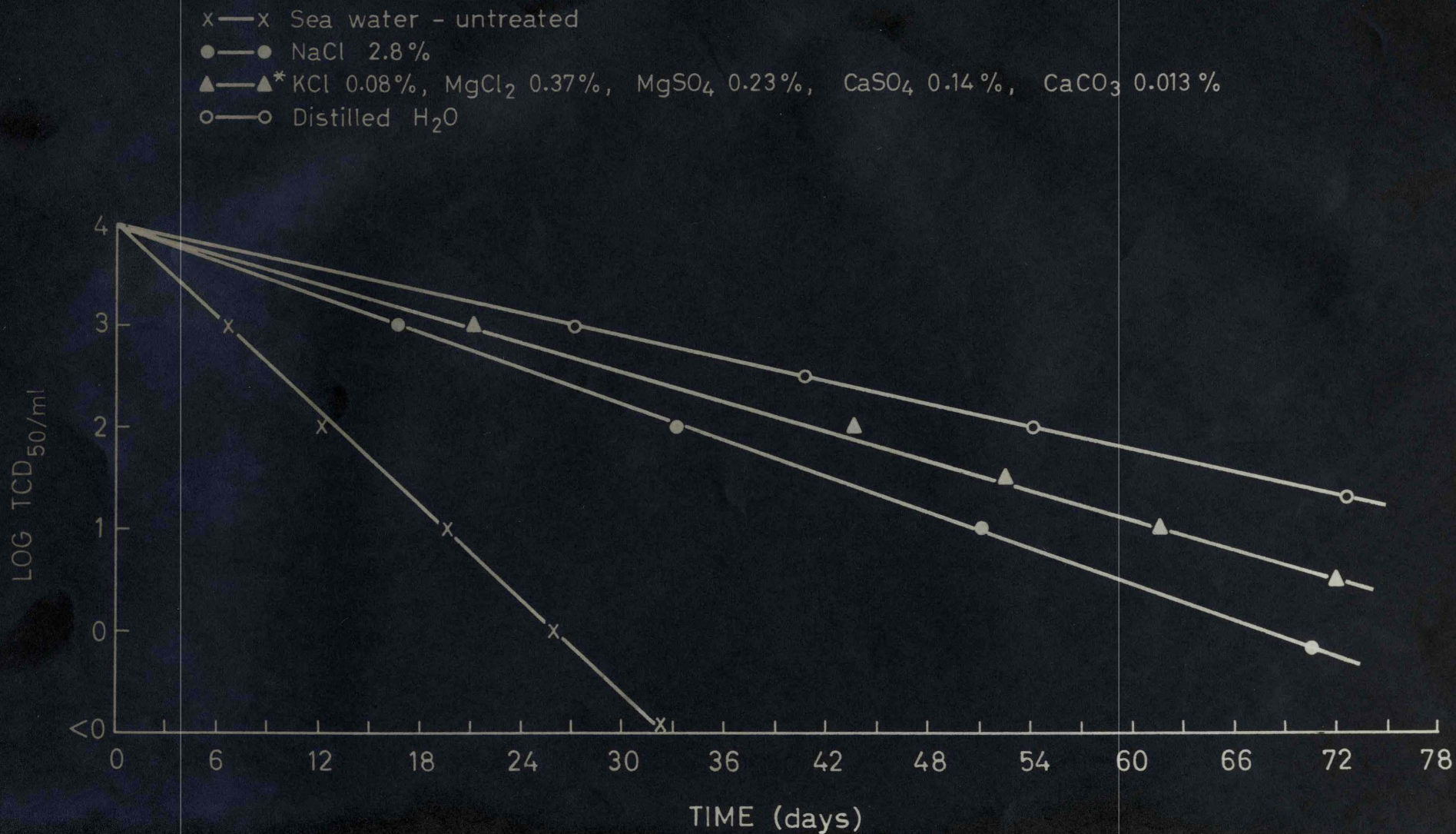


Fig. 2. The inactivation rate of poliovirus in presence of various chemicals applied singly. (Virus concentration 10^4 TCD₅₀/ml., Temperature 25°C.).

*(▲—▲) Applies to any one of the chemicals tested singly.

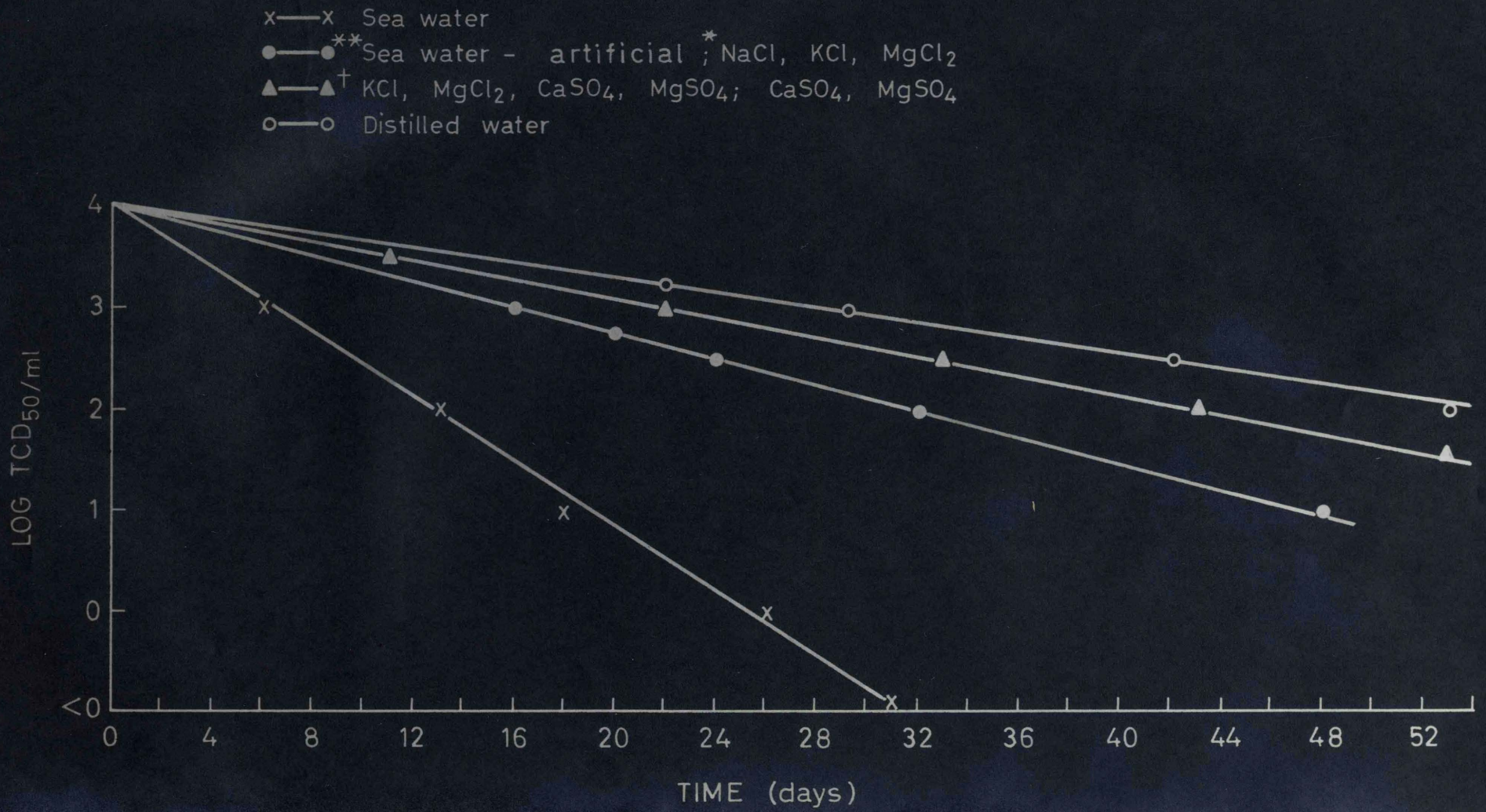


Fig. 3. The inactivation rate of poliovirus in presence of various chemicals applied in combination. (Virus concentration 10^4 TCD₅₀/ml., Temperature 25°C.).

*Data from Constan (1956)
 ** (●—●) Applies to sea water - artificial or the combination of NaCl, KCl and MgCl₂
 † (▲—▲) Applies to the mixture of KCl, MgCl₂, CaSO₄ and MgSO₄ or CaSO₄ and MgSO₄

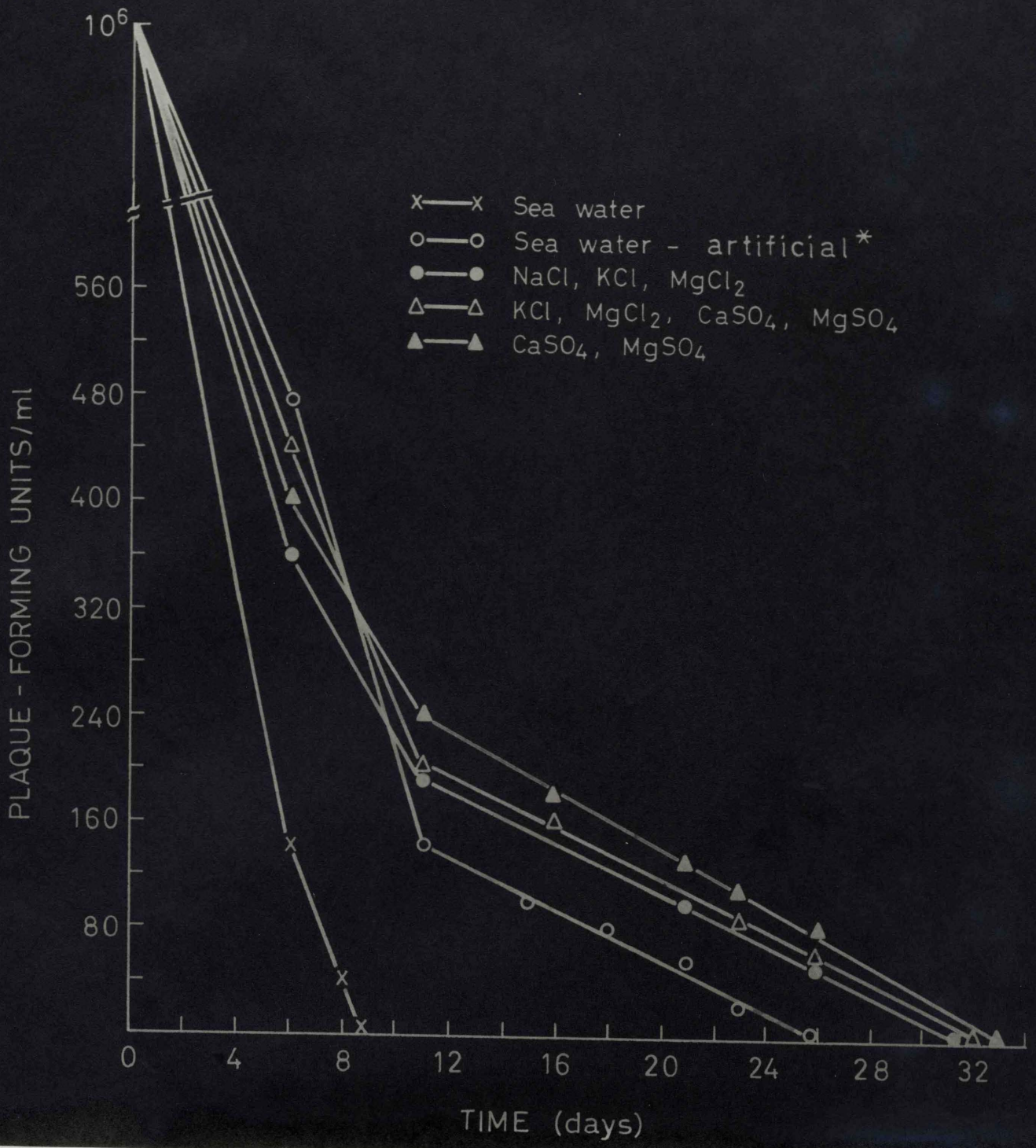


Fig. 4. The inactivation rate of poliovirus in presence of various chemicals applied in combination. (Virus concentration 10^6 PFU/ml., or 10^5 TCD₅₀/ml., Temperature 25°C.).

*Data from Constan (1956)

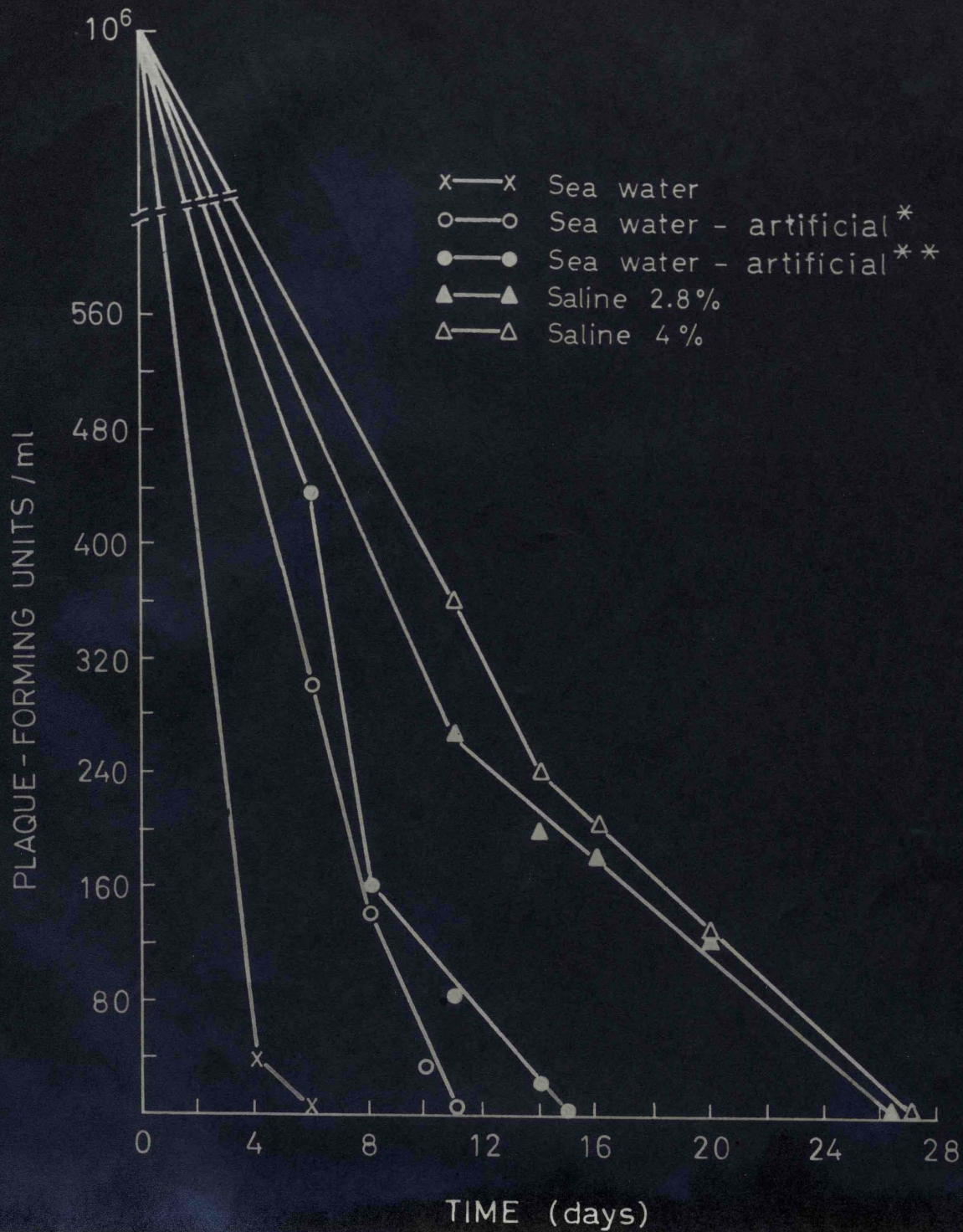


Fig. 5. The inactivation rate of poliovirus in presence of two types of artificial sea waters and different concentrations of NaCl. (Virus concentration 10⁶ PFU/ml., Temperature 25°C.).

*Data from Lyman and Fleming (1940)
 **Data from Constan (1956)

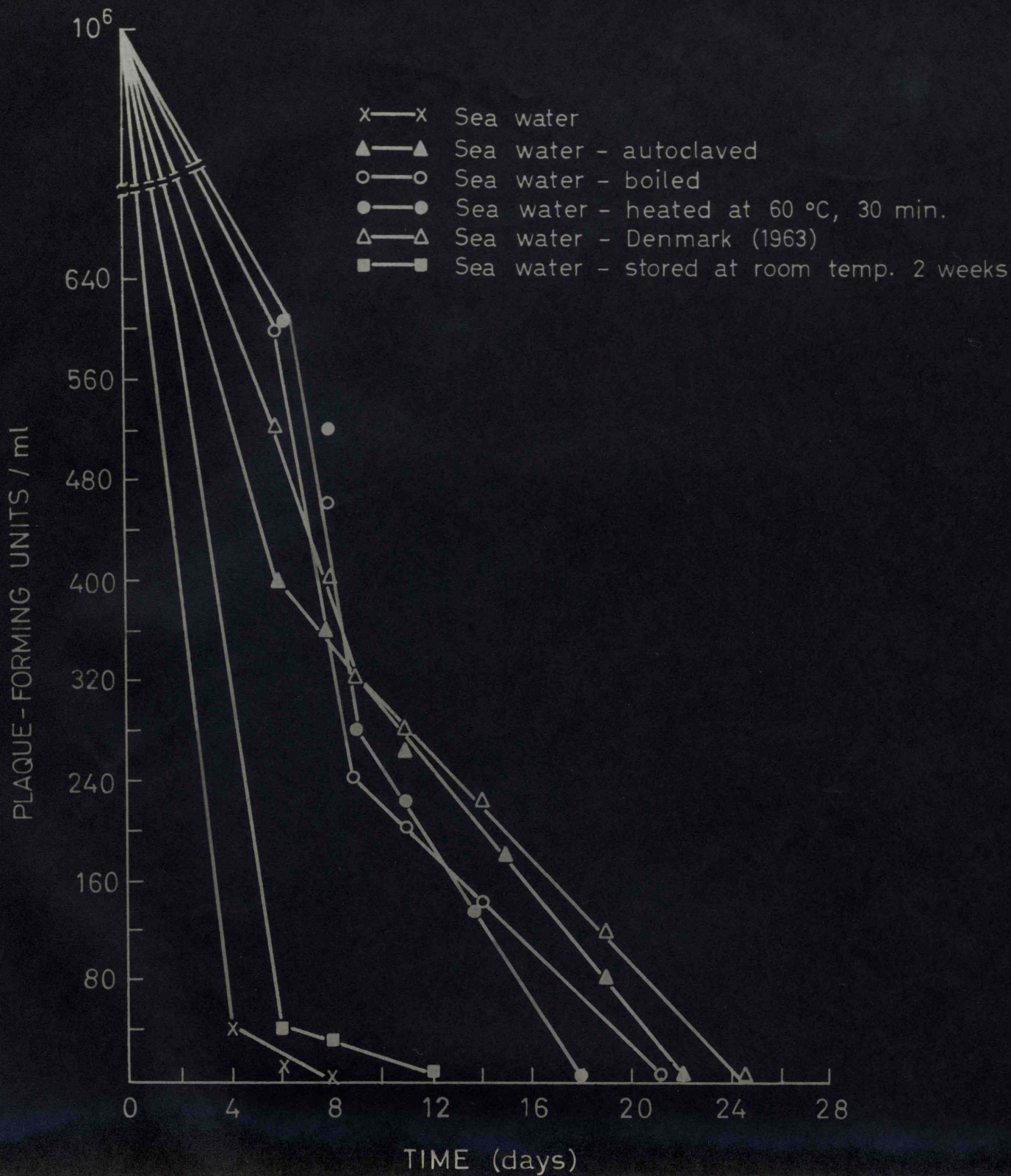


Fig. 6. The inactivation rate of poliovirus in sea water samples subjected to various treatment. (Virus concentration 10⁶ PFU/ml., Temperature 25°C.).

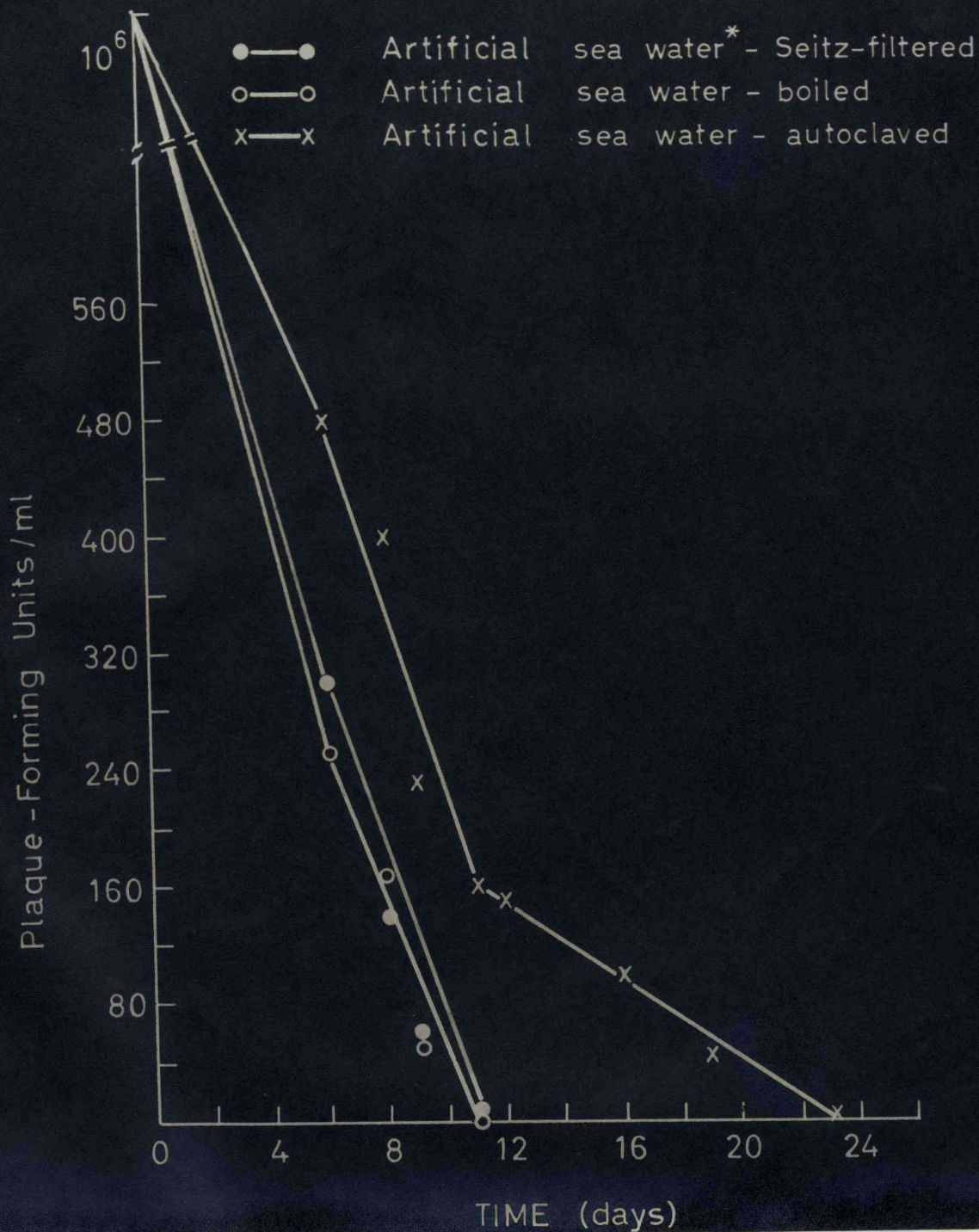


Fig. 7. The inactivation rate of poliovirus in artificial sea water subjected to various treatment. (Virus concentration 10^6 PFU/ml., Temperature $25^{\circ}\text{C}.$).

*Data of Lyman and Fleming (1940)

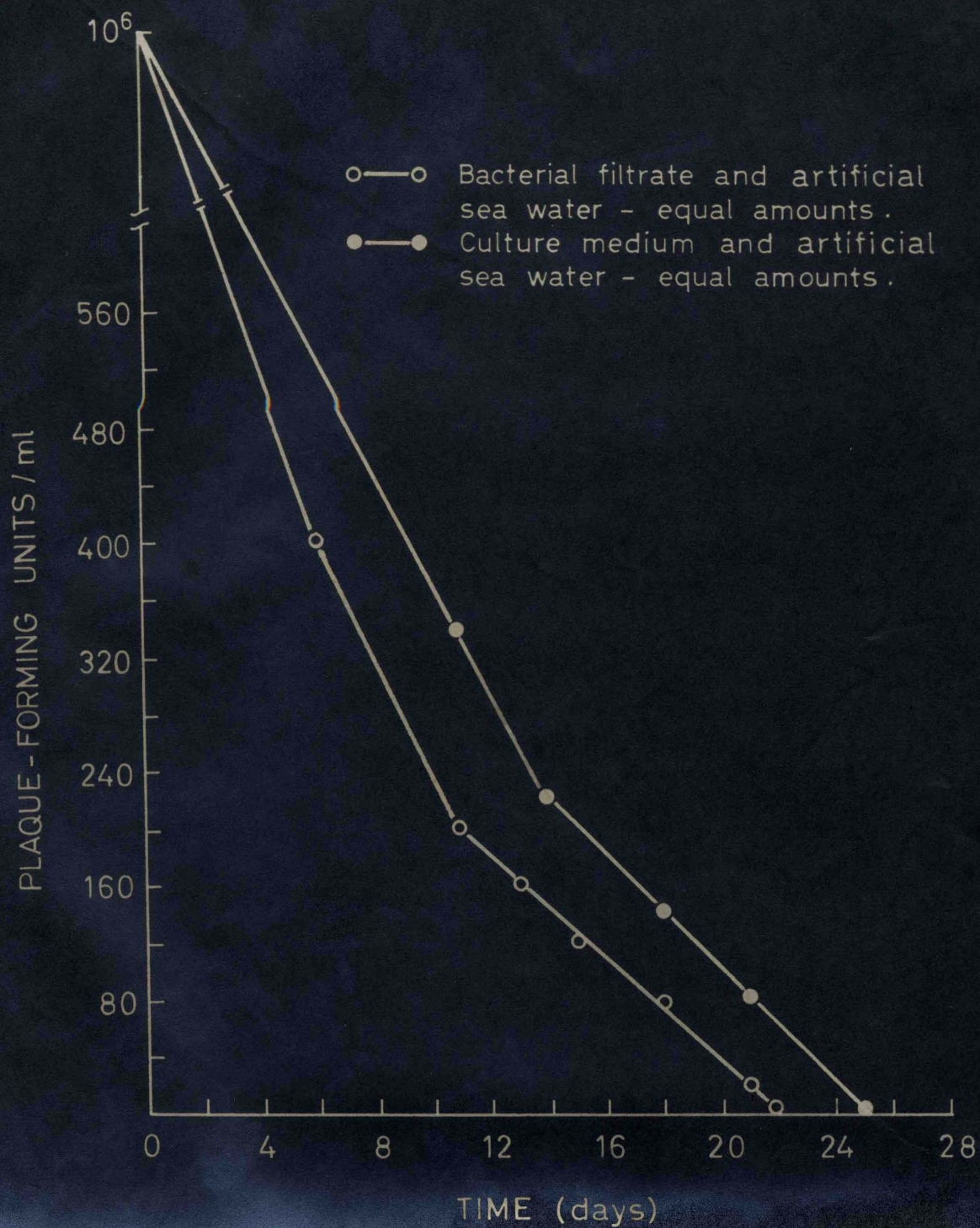


Fig. 8. The inactivation rate of poliovirus in presence of bacterial lysates. (Virus concentration 10^6 PFU/ml., Temperature 25°C .).

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