PRODUCTION AND PURIFICATION OF T 3 BACTERIOPHAGE CONTAINING P^{32}

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By

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INTRODUCTION

There are seven types of bacteriophage that are active against Escherichia coli strain B. They are designated T and numbered 1 through 7. The T3 bacteriophage is round or possibly of hexagonal shape, and measures approximately 45 mu in diameter. The "tail" of this virus is small, and in air dried specimens is not visible in the electron microscope. (Williams and Fraser 1953). Frozen specimens do show a small tail. T7 bacteriophage is similar morphologically to T3. T2, T4, and T6 are larger, having a diameter of 60 to 80 mu, and possessing a definite tail about 100 mu long. All T bacteriophages are distinguished by their infectivity of the common host, Escherichia coli strain B. (Luria 1953)

The availability of artificially produced radioactive isotopes has made possible a large number of experiments which would have been extremely difficult by other means. The incorporation of a radioactive isotope into the system under study allows the investigator to follow changes or to observe movements which would not be detectable by other chemical or physical means.

Radioactive phosphorus and sulphur have been most commonly used in studying Escherichia coli and its associated T bacteriophages. Kozloff and Putnam (1948) grew E. coli and T6 bacteriophage in a medium containing P^{32} in order to study the origin of virus constituents and the extent to which they were derived from the host. They found that 70% of the virus phosphorus was derived from the medium and the remainder from the host cell. The concentration of P^{32} which they used was 1 uc (micro curie) per ml.

Labaw, Mosley, and Wyckoff (1950) performed a series of experiments to follow the synthesis of nucleic acids in a culture of E. coli growing in a medium labelled with P^{32} to the extent of 10^5 counts per minute per ml. They showed that P^{32} uptake and corresponding cell multiplication continued just as in a non-radioactive culture. Further work by Labaw (1951) showed that the T3 bacteriophage derived 78% of its phosphorus from the host cell and only 17% from the medium. In this experiment, which was similar to that performed by Kozloff and Putnam, he determined that T1, T3, and T7 derived most of their phosphorus from the host cell. This is in contrast to T2 and T6 which derive most of their phosphorus from the medium.

Hershey et al. (1951) investigated the rate of inactivation of T2 bacteriophage containing P^{32}. He grew the virus in a medium containing P^{32} varying in concentration from 0.14 to 2.2 Mc (millicurie) per ml. Stent and Fuerst (1955) also investigated the rate of inactivation of T2 which contained P^{32}. Both these workers used P^{32} of a high specific activity. The specific activity being measured in Mc of P^{32} per total amount of phosphorus present. Stent and Fuerst used P^{32} with a specific activity ranging from 250 to 300 Mc per mg of phosphorus. Both papers reported a gradual inactivation of bacteriophage which depended on the specific activity of the medium in which they were grown. Labaw did not use high concentrations of P^{32}.

Labaw obtained purified virus preparations by means of the ultracentrifuge. Hershey and Stent did not find it necessary to obtain a purified virus preparation.

While most of the work with E. coli and the associated T bacteriophage has been concerned with the origin of virus components, the fate of these components, or with genetics of bacteriophage (Stent 1955, Lesley and Graham 1956, Volkin and Astrachan 1956, Stahl 1956, Hershey 1955) there have been at least two reports concerning the use of radioactive bacteria in aerosols. (Goldberg and Leif 1950, Buckland, Harper, and Morton 1950). These two articles were concerned with the use of bacteria containing P^{32} as a means of determining the amount and extent of retention of the organism by the tissues of the body. Rather than grinding the tissues and plating or examining the ground up mass these workers achieved the same results by counting the radioactivity of ashed portions of tissue. The experiments described in this thesis were done in an attempt to accomplish essentially the same purpose as those of Goldberg and Buckland. That is, to assay bacteriophage labelled with P^{32} by counting its radioactivity rather than by determining biologically the number of viable virus particles.

Work in this laboratory involved the use of the T3 bacteriophage in aerosols. It was found that under conditions of low humidity the recovery of viable virus was much lower than at high humidities; often by a factor of 1000. If, as was assumed, a large fraction of the virus population was inactivated under dry conditions, would it be possible to detect both viable and non viable virus particles by some physical or chemical method? The most obvious means of accomplishing this purpose was to incorporate a radioactive isotope into the virus and use a Geiger-Muller tube to measure the amount of virus in the sample. The work done for this thesis involved:

1) The preparation of T3 bacteriophage suspensions containing a high concentration of P^{32}.

2) The treatment of these suspensions to remove free P^{32}.

3) The estimation of the effectiveness of the removal of free P^{32}.

EXPERIMENTAL METHODS

P^{32} is a radioactive isotope of phosphorus and decays to sulfur 32 upon emission of one electron or beta particle. The average energy of this beta particle is 0.68 Mev and the maximum energy is 1.7 Mev. P^{32} has a half life of 14.3 days.

The P^{32} used was in the form of a Sodium phosphate solution obtained from the Abbott Laboratories, Oak Ridge, Tennessee. The specific activity of the P^{32} varied from 31 to 49 Mc/mg of total phosphorus and averaged about 40 Mc/mg. The P^{32} solution was U.S.P. grade and was obtained already sterilised.

There are numerous methods and techniques used to count samples containing radioactive isotopes. If the isotope is of the type which emits particles of low energy these techniques can become quite complicated. Fortunately P^{32} emits a particle with a maximum energy of 1.7 Mev, and is capable of penetrating a considerable quantity of matter. The method of counting used was chosen because of its simplicity. A quantity of the material to be counted, usually from 0.01 to 0.1 ml was put into a round metal dish or planchet one inch in diameter. Micro pipettes were used to measure the radioactive material and the pipette was washed several times with distilled water which was also put on the planchet. The sample was then dried under an infra-red heat lamp. The samples were counted in a lead chamber with a Geiger Muller tube having a window thickness of 2.4 mg/cm^2 and operating at 900 volts. The counts were registered on a Tracerlab 64 scaler. Samples of low radioactivity were counted long enough so that the standard error was less than 5%. The time each sample

was to be counted was estimated by reference to a chart prepared by W. C. Davidon (1953). All the results are expressed as counts per minute per ml of sample. No attempt was made to convert counts per minute to the amount of radioactivity in terms of millicuries.

The soft agar technique for the assay of bacteriophage was originally developed by Gratia (1936) and described in detail by Adams (1950). The procedure involves placing 0.5 ml of a heavy suspension of growing E. coli cells into a test tube containing 2.5 mls of melted soft agar (7.5g agar in one liter of water) at 45°C. Into this bacteria agar mixture is pipetted 0.1 ml of an appropriate dilution of the virus suspension. This bacteria-virus-agar mixture is poured on a plate of nutrient agar, spread uniformly over the surface of the plate and allowed to harden. After incubation for six hours at 37°C, circular areas or plaques appear as clear spots in the otherwise opaque growing bacteria. By counting the number of plaques, multiplying by 10 to compensate for the 0.1 ml of virus suspension used, and then multiplying by the dilution factor one can obtain the number of viable virus particles per ml of the original suspension. This was the method routinely used for assay of viable bacteriophage. Luria et al. (1951) have shown that this method is extremely accurate and that one plaque corresponds to one virus particle.

There are various chromatographic methods available to the experimenter, all of which are described in detail in Block, Durrum, and Zweig (1955). A descending method of chromatography was used with Whatman #1 chromatographic paper cut into strips three centimeters wide. 0.01 to 0.05 mls of the virus suspension to be tested was placed about six cm from one end of the paper strip. The strip was then suspended from the

solvent trough in an air tight jar. A small amount of solvent was put in the bottom of the jar and the entire assembly left overnight while the atmosphere in the jar became saturated with solvent. The solvent was then added to the trough and allowed to move down the paper strip until it had reached the bottom. The strip was removed, dried, and dipped in a solution of 0.3% ninhydrin in 95% alcohol. After development in the dark for 18 hours a purple color appeared on the paper strip wherever the virus or other nitrogen containing constituents of the medium had been deposited. The migration of virus is reported in terms of the Rf value. The Rf value is calculated by dividing the distance from the center of maximum color development by the distance the solvent front travelled.

RESULTS

Production of T3 Bacteriophage Containing P^{32}

The primary aim of this work was to produce virus suspensions of high concentration; at least 10^{10} virus particles per ml. The second consideration involved putting as much radioactive phosphorus into each virus particle as possible without decreasing the virus concentration. A limiting factor in this respect was that, due to the physical facilities in the laboratory and to reasons of safety, it was decided to have no more than 10 Mc of P^{32} available at any one time.

Non radioactive virus suspensions have been produced which contained as many as 10^{11} virus particles per ml. The method of producing such suspensions is very simple and involves spreading a small quantity of a suspension of E. coli over the surface of an agar plate with a sterile glass rod. After incubation for 18 hours at 37°C, 0.5 mls of a virus suspension is thoroughly mixed with the bacteria with a sterile glass rod. The bacteria-virus mixture is incubated at 37°C. When the bacteria have lysed, five mls of distilled water is added to the plate, and stirred vigorously in order to get all the virus off the agar surface. This water suspension of virus is then centrifuged to remove all bacterial debris and filtered through a Millipore type H A (Hydrosol Assay) membrane filter. (Millipore Filter Company, Watertown, 72, Massachusetts). The resulting filtrate is free of all bacteria and contains only virus in a water suspension. Depending on the volume of virus suspension needed, as many plates as necessary may be used. Because the agar will adsorb some water, only four mls of crude lysate will be recovered for every five mls added. Virus concentrations as high as 3×10^{11} virus

particles per ml have been obtained by this method, with an average concentration of $5x10^{10}$ as the normal.

In the first experiment the above technique of producing a virus suspension was used. The medium on which the bacteria and virus were grown was a modified form of the synthetic, or "F", medium mentioned by Adams (1950), and contained 0.75g KH_2PO_4, 1.75g Na_2HPO_4, 1g NH_4Cl, 0.1g MgCl, 7.2mls lactic acid, and 15g of agar in one liter of distilled water. The pH was adjusted to 7 with NaOH. Five Mc of P^{32} were added to each of two petri dishes, in which 10 mls of agar medium had been allowed to harden. 10 more mls of medium were poured over the P^{32}. Each plate was inoculated with 0.2 ml of an E. coli suspension according to the procedure previously described, incubated for 18 hours, inoculated with 0.5 ml of a virus suspension, and washed with five mls of distilled water. After centrifugation and filtering, the virus suspension was assayed for the number of viable virus particles by the agar layer method, and found to contain $7x10^9$ virus particles per milliliter. A count of the radio activity gave $3.1x10^7$ counts per minute per milliliter. Obviously a great deal of P^{32} had been scraped from the agar surface and was contaminating the virus suspension.

In order to get rid of this contaminating P^{32}, the virus suspension was sedimented in an ultracentrifuge at 110,660 times gravity for one hour, the supernatant fluid drawn off and the sediment resuspended in an equal volume of medium. This was done twice. The second resuspended virus suspension now contained only $1.1x10^9$ viable virus particles per milliliter and had a radioactive count of $2.9x10^4$ counts per minute per milliliter.

Although this method of producing a radioactive virus suspension might, in time, have proved satisfactory, the technical difficulties involved in handling P^{32} were such that it was felt advisable to try a method using a liquid medium. The use of a solid agar medium was wasteful of P^{32} in that the bacteria would utilise only the fraction of P^{32} near the agar surface. A liquid medium would make all the P^{32} theoretically available. A third argument in favor of using a liquid medium was that the T3 bacteriophage derives about 70% of its total phosphorus from the bacterial cell and only some 30% from the medium (Labaw, 1951). By washing the bacterial cells it would be possible to eliminate almost all of the P^{32} in the medium, leaving only that P^{32} which had been incorporated into the bacteria.

The next experiment was performed using "F" medium without the agar into which varying amounts of P^{32} were added. The medium was placed into a Porton Impinger (Ace Glass Inc., Vineland, New Jersey) and P^{32} added to obtain the desired concentration. The medium was inoculated with 0.2 to 0.5 ml of a 24 hour broth culture of E. coli and aerated at 37°C in a water bath for 18 hours. The air was introduced through a glass wool filter. The Porton Impinger was used because it was available and seemed ideal for the purpose. It consists of a flat bottom flask four cm in diameter and 15 cm high. Into the top of the flask is fitted a long glass tube that extends to the bottom of the flask. The air was introduced through the top of the tube and let out through an outlet near the top of the flask.

The 18 hour E. coli suspension was centrifuged at a relative centrifugal force of 1200 times gravity for 15 minutes. The supernatant

fluid was discarded and the cells resuspended in the same volume of fresh non-radioactive medium. This procedure was repeated twice. The final resuspended cells were put into another impinger and 0.5 ml of a virus suspension added. The ratio of the number of virus particles to the number of bacteria was about 1:1. Aeration at 37°C was continued for 10 hours, at which time lysis had taken place. This crude lysate was centrifuged to remove gross particles and the supernatant fluid filtered through a Millipore type H A filter. The results of three experiments are summarised in Table I.

The phosphate buffer in the modified "F" medium undoubtedly prevented utilisation of much of the P^{32} by the E. coli, so the remaining experiments were performed using a glycerol-lactate medium which lacked any inorganic phosphates. The new medium contained 1.5g KCl, 5g NaCl, 1g NH_4Cl, 0.25g $MgSo_4$, 6.3g lactic acid, 2g glycerol, 0.5g Bacto Peptone, 0.5g Bacto Casamino Acids in one liter of distilled water. (Stent and Fuerst 1955). The pH was adjusted to 7 with NaOH. The total amount of phosphorus in this medium was 2% from the peptone and 0.22% from the casamino acids, or 0.011 mg per ml. The utilisation of P^{32} by E. coli grown in this medium could be expected to be much greater than in the "F" medium.

The virus was produced in the manner previously described. The only difference was that the P^{32} concentration was 0.2 MC/ml for all the experiments. The results of these experiments are summarised in Table II.

Purification of the Filtered Virus Suspensions

Growing bacteria in a highly radioactive medium and harvesting the virus from the radioactive cells was a simple task compared to the

TABLE I

RADIOACTIVE AND BIOLOGICAL ASSAY OF BACTERIA AND VIRUS PRODUCED ON "F" MEDIUM

Exp. Number	"F" Medium		Biological titer Infective virus particles/ml		Radioactive titer CPM/ml		
	P^{32} conc.	Bact. conc. viable cells/ml	Filtered virus	Dialysed virus	Bact.	Filtered virus	Dialysed virus
I.	5 Mc/35 ml .145 Mc/ml	6.7×10^{8}	4.4×10^{10}	4.5×10^{10}	309,100	234,000	74,410
II.	10 Mc/35 ml .29 Mc/ml	2.5×10^{8}	1.6×10^{10}	1.3×10^{10}	525,400	276,000	76,700
III.	15 Mc/40 ml .375 Mc/ml	2.5×10^{7}	1.9×10^{9}	9.7×10^{8}	145,100	118,640	33,000

TABLE II

RADIOACTIVE AND BIOLOGICAL ASSAY OF BACTERIA AND VIRUS PRODUCED ON GLYCEROL-LACTATE MEDIUM

Exp. Number	Glycerol-Lactate Medium		Biological titer Infective virus particles/ml		Radioactive titer CPM/ml		
	P^{32} conc.	Bact. conc. viable cells/ml	Filtered virus	Dialysed virus	Bact.	Filtered virus	Dialysed virus
VI.	7 Mc/35 ml .2 Mc/ml		1.6×10^{10}	3.13×10^{9}			1,214,190
VII.	10 Mc/33 ml .3 Mc/ml	1×10^{8}	6.6×10^{9}	7.96×10^{8}	7,762,600	4,142,700	2,706,160
IX.	10 Mc/50 ml .2 Mc/ml	1.5×10^{8}	3.5×10^{10}	5.7×10^{10}	16,100,000	10,667,000	3,793,600
X.	10 Mc/50 ml .2 Mc/ml	4.8×10^{8}	2.4×10^{10}	8.4×10^{9}	16,787,000	10,200,000	2,476,150
XI.	10 Mc/50 ml .2 Mc/ml	4.7×10^{8}	2.1×10^{10}	2.2×10^{10}	21,162,000	14,000,000	4,217,500
XII A	10 Mc/50 ml	2.6×10^{8}	5.3×10^{10}	1×10^{10}	18,590,000	12,235,000	2,405,720
XII B	.2 Mc/ml			2.5×10^{10}	18,590,000	10,150,000	1,357,435

problem of getting rid of contaminating P^{32}. It was assumed that not all the radioactivity present in the filtered virus suspension was confined to virus particles.

Three methods of accomplishing this were tried. The first has been mentioned already. It involved sedimenting the virus particles in the ultracentrifuge and resuspending them in non-radioactive medium. This is the most common method used to obtain a purified virus suspension. In this case the ultracentrifuge did not prove entirely satisfactory for two reasons. First; every time the sediment was resuspended there was a decrease in the number of viable virus particles. Second; it is difficult to fill the ultracentrifuge tubes without spilling sizable quantities of radioactive liquid.

The second method of removing contaminating P^{32} was by filtration through a Millipore virus type membrane filter. These filters have extremely small pores and were reputed to be capable of retaining particles as small as the T3 bacteriophage. The technique involved filtering the virus suspension through one filter, washing that filter with sterile saline solution to remove the virus, filtering the washings through a second filter, and then washing that filter in sterile saline solution. This method, although it sounded promising, proved of little value for one reason. The filters themselves were not of a uniform quality. Most of them would retain the virus, but some would not. Moreover, it was difficult to get more than five or ten milliliters of liquid through any one filter. One 25 ml volume of virus suspension took six hours to pass through the filter, even though considerable vacuum was applied below the filter. The variability in the quality of the filters made them

useless for the purposes of this work.

The third method was to dialyse the filtered virus suspension against distilled water. The virus suspension was placed into a cellophane dialysing bag. The cellophane was obtained from the Visking Corporation. The dialysing bag was put into a beaker containing 500 mls of distilled water. The supporter for the dialysing bag also held a small glass paddle which was inserted into the virus suspension, and kept it continually agitating. The distilled water was changed at intervals of one hour, and samples taken to determine the radioactivity of the water and the amount of virus, if any, passing through the dialysing membrane.

It was hoped that the virus would remain in the dialysing bag, while any P^{32} in the form of phosphates would pass through the membrane.

The counts per minute per milliliter of the dialysing water and the amount of virus passing through the membrane into the dialysing water are given in Table III. Chart I illustrates the relationship between the cumulative radioactivity removed from the virus suspension, and the amount of time which the virus is dialysed.

Chromatography of Filtered and Dialysed Radioactive Virus Suspensions

There is a considerable decrease in the radioactivity of the dialysed virus in contrast to that of the filtered virus. The problem was whether dialysis had removed any of the free P^{32} in the medium and, if so, how much had been removed. Was the dialysed virus purer with respect to contaminating P^{32} than the filtered virus?

TABLE III

AMOUNT OF RADIOACTIVITY PASSING THROUGH

DIALYSING MEMERANE INTO DIALYSING WATER

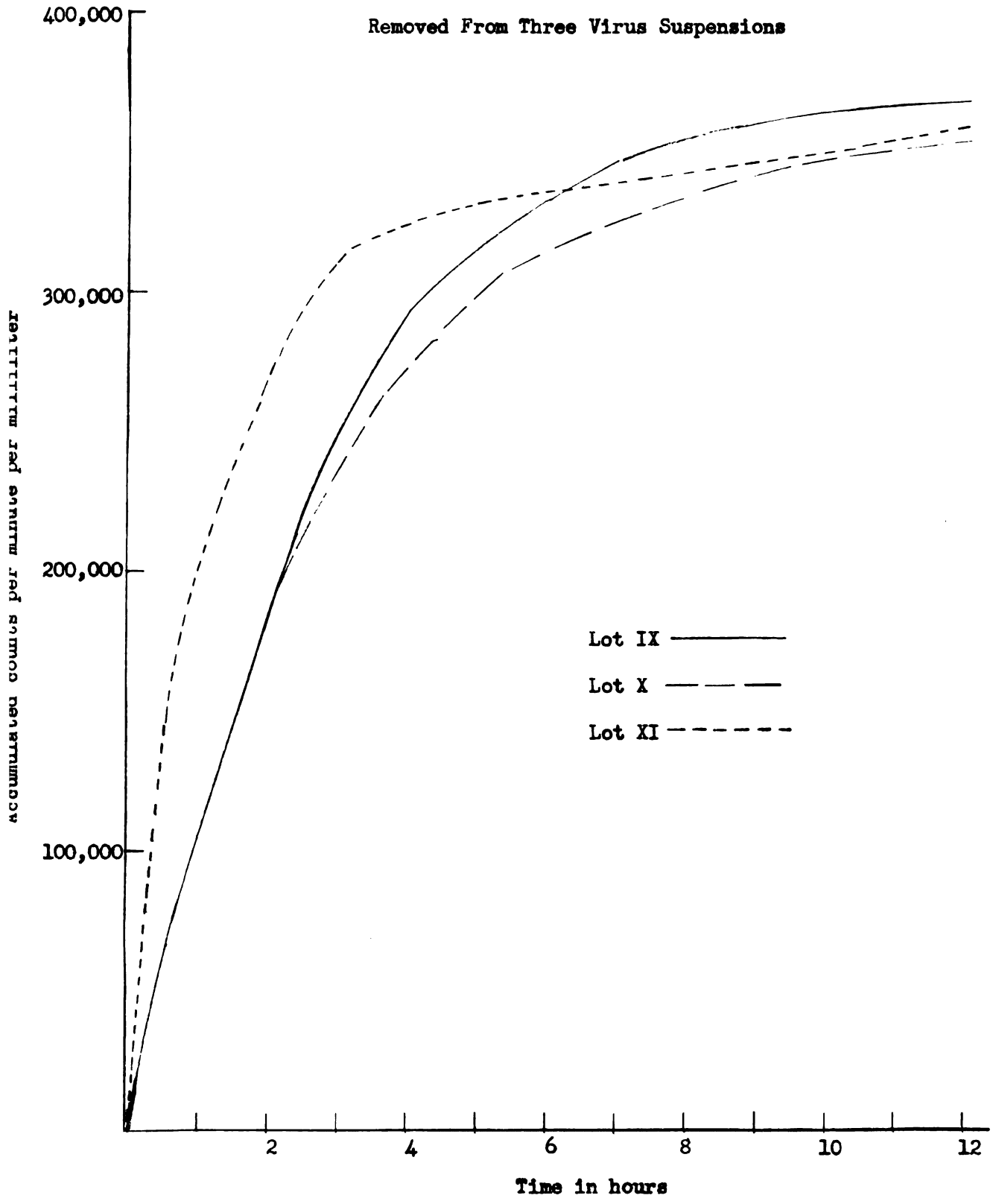
Lot IX*		Lot X*		Lot XI*	
Time in hours	Counts per min./ml.	Time in hours	Counts per min./ml.	Time in hours	Counts per min./ml.
2	183,800	2-1/2	221,000	1/2	146,400
4	112,600	3-1/2	40,500	2	127,000
6	36,600	4-1/2	30,000	3	41,600
7	14,900	5-1/4	15,300	4	9,200
11	19,050	5-3/4	3,100	5	3,300
12	850	9-1/2	37,700	6	7,350
		10	3,050	9-1/2	12,550
		11-1/2	4,130	10-1/4	4,600
		12	200	11	2,530
				12	4,970

* No viable virus particles found in Lot IX, X, or XI.

CHART I

Amount of Radioactive Phosphorus

Removed From Three Virus Suspensions



Precipitation of the virus by acid or salts was first considered. It would then be possible to determine the amount of radioactivity in the supernatant fluid and assume it to be due to contaminating P^{32}. This idea, however, was discarded because it would not be possible to know whether the precipitate contained only virus or whether other P^{32} containing constituents of the suspension had also been precipitated. Sedimentation of the virus in the ultracentrifuge was also considered. Although this is an accepted method of purifying virus suspensions it would have been of dubious value in this case, for there are always a few virus particles that remain in the supernatant fluid.

It was thought that paper chromatography techniques might achieve the desired results. If a solvent could be found which would move the virus at one rate and the contaminating P^{32} at another it would be possible to estimate the amount of contamination removed from a virus suspension by dialysis.

A number of solvents were used:

a) Citrate buffer at pH 5 and pH 6,

b) 50% n-propanol,

c) Water saturated iso-butanol,

d) 50% ethanol,

e) 50% acetone,

f) 50% n-butanol plus 6 mls of glacial acetic acid (the organic layer was used),

g) 10% NaCl, and

h) M/5 sodium acetate plus HCl to pH 6.

All these solvents can be found in Block *et al.* (1955).

Three of the solvents failed to move the virus at all. Therefore results for iso-butanol, 50% acetone, and n-butanol are not reported.

It was established that the T3 bacteriophage migrated down the paper strip and appeared as a purple spot after treatment with ninhydrin by using a virus suspension that had been purified in the ultracentrifuge and resuspended in distilled water. The Rf values of a number of virus suspensions developed in various solvents are given in Table IV.

As mentioned above, a purified virus suspension was used as a control to see how far it would move. Another control, using filtered and dialysed glycerol-lactate medium to which a small amount of P^{32} had been added, was also tested, but in one of the solvents only.

The migration of P^{32} on the paper strip was determined using a Geiger-Muller tube. The strip was inserted in a lead holder which had a slit one cm wide just below the tube. Thus the radioactivity associated only with one cm of the strip was counted. The strip was drawn through the holder and the radioactivity of each centimeter was counted and expressed as counts per minute. These counts are graphically illustrated in Charts II through VI, the counts per minute on one axis and the distance along the strip on the other axis. It must be noted that the height of the curves is not significant, for different volumes of each virus suspension were used depending on the amount available at the time. The important point is the relationship between the amount of radioactivity which migrated and the amount which remained behind in the original position, and the relationship between the point of maximum color development and the radioactive peak. This second relationship, or the Rf values of the maximum color development and the maximum radioactivity are compared in Table V.

TABLE IV

Rf VALUES OF FILTERED AND DIALYSED RADICACTIVE VIRUS SUSPENSION,

AND OF AN ULTRACENTRIFUGE PURIFIED NON-RADIOACTIVE VIRUS SUSPENSION.

Experiment Number	Virus Lot	Solvent	Distance solvent front travelled in cm	Distance to center of visible spot in cm	Rf value
1	II-Filtered	Citrate Buffer pH5	31.6	a) 21 b) 26	a) .7 b) .82
2	"	50% n-propanol	20.8	13.1	.63
3	"	50% Ethanol	23.9	15.1	.63
4	"	10% NaCl	29.5	a) 23.75 b) 27.2	a) .8 b) .9
5	"	M/5 Sodium Acetate	36.4	24.7	.68
6	III-Filtered	Citrate Buffer pH5	23.75	a) 16.75 b) 18.35	a) .7 b) .8
7	IX-Filtered	Citrate Buffer pH6	32.6	25.45	.75
8	XII-Filtered	50% Ethanol	19.6	No visible spot	----
9	II-Dialysed	10% NaCl	26.5	24.8	.93
10	"	M/5 Sodium Acetate	35.6	24.8	.7
11	III-Dialysed	Citrate Buffer pH5	23.9	17.65	.74
12	IX-Dialysed	Citrate Buffer pH6	33.5	24.8	.74

TABLE IV

(cont.)

Experiment Number	Virus Lot	Solvent	Distance solvent front travelled in cm	Distance to center of visible spot in cm	Rf value
13	IX-Dialysed plus purified virus	Citrate Buffer pH6	33.2	24.5	.74
14	XII-Dialysed	50% Ethanol	19.1	No visible spot	----
15	Purified virus	Citrate Buffer pH5	29.3	24.3	.83
16	" "	Citrate Buffer pH6	30.9	22.7	.73
17	Glycerol-lactate medium-filtered	" " "	23.4	19.5	.83
18	Glycerol-lactate medium-dialysed	" " "	24.5	No visible spot	----

Citrate Buffer: .75 mls. .02 M sodium citrate, 50g NaCl, distilled water to one liter, 2N HCl to pH5 or 6.
M/5 sodium acetate: M/5 sodium acetate, HCl to pH6.

CHART II

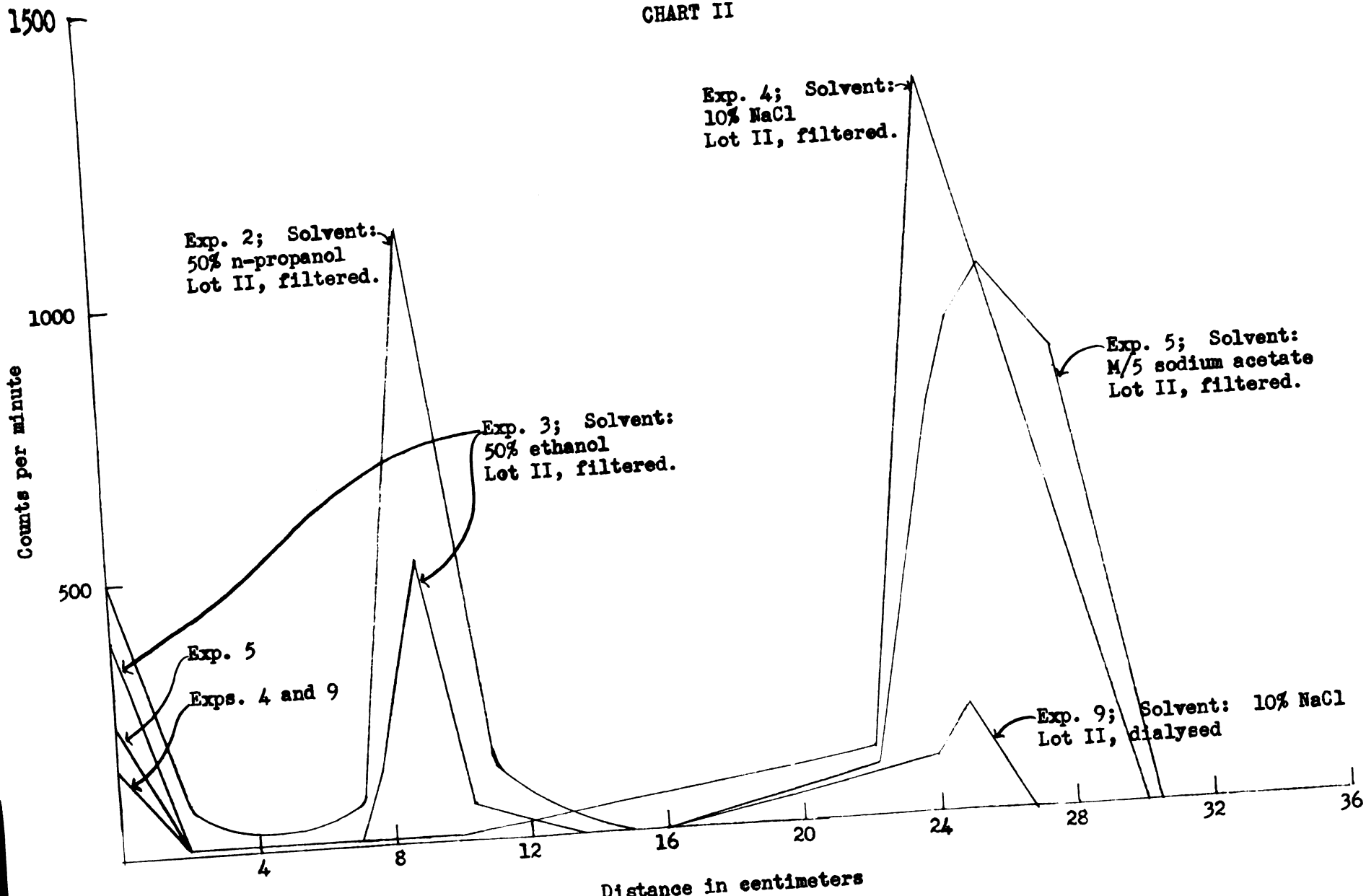

1500
1000
500
Counts per minute
Exp. 4; Solvent:
10% NaCl
Lot II, filtered.
Exp. 2; Solvent:
50% n-propanol
Lot II, filtered.
Exp. 5; Solvent:
M/5 sodium acetate
Lot II, filtered.
Exp. 3; Solvent:
50% ethanol
Lot II, filtered.
Exp. 5
Exps. 4 and 9
Exp. 9; Solvent: 10% NaCl
Lot II, dialysed
4
8
12
16
20
24
28
32
36
Distance in centimeters

CHART III

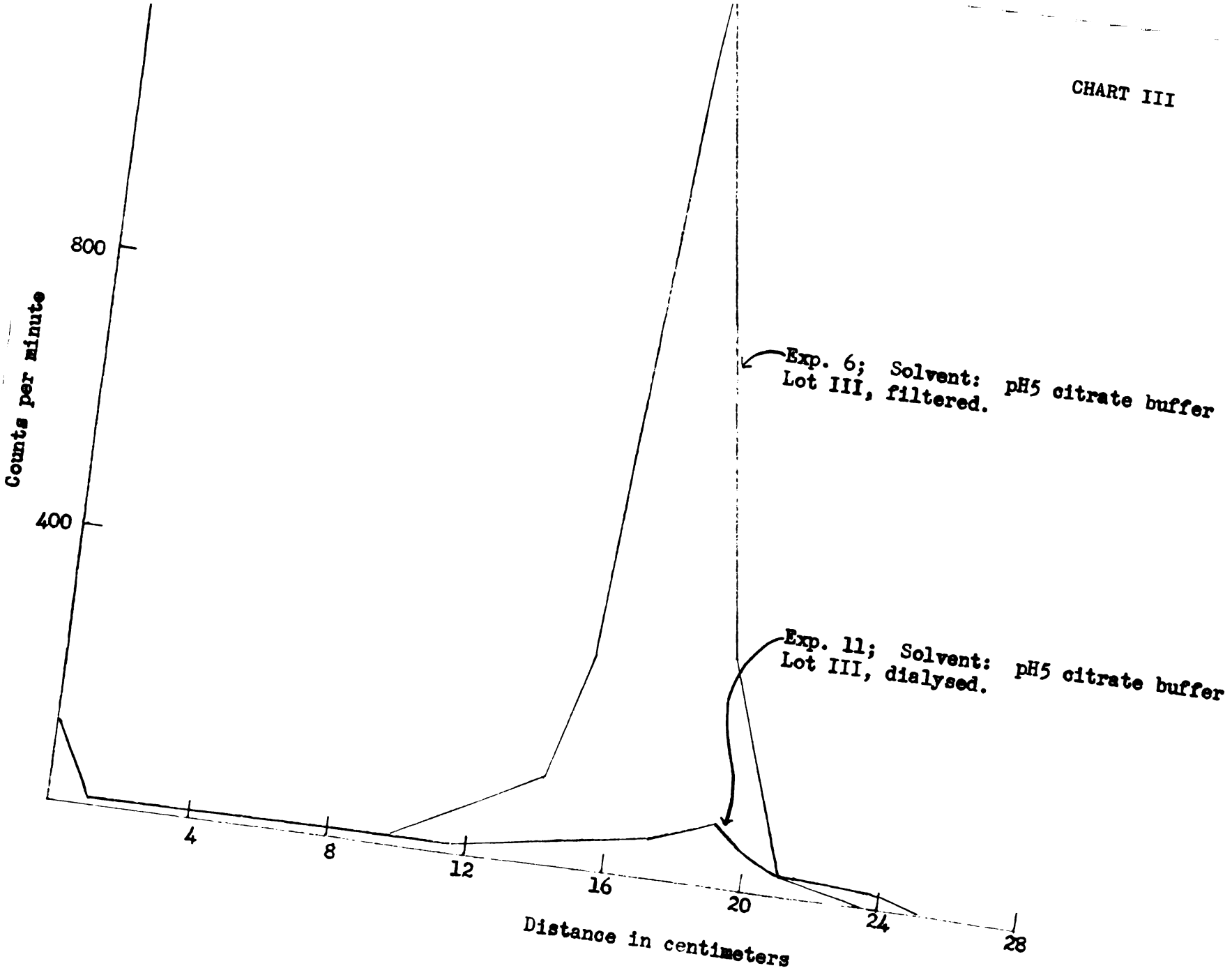

800
400
Counts per minute
Exp. 6; Solvent: pH5 citrate buffer
Lot III, filtered.
Exp. 11; Solvent: pH5 citrate buffer
Lot III, dialysed.
4
8
12
16
20
24
28
Distance in centimeters

CHART IV

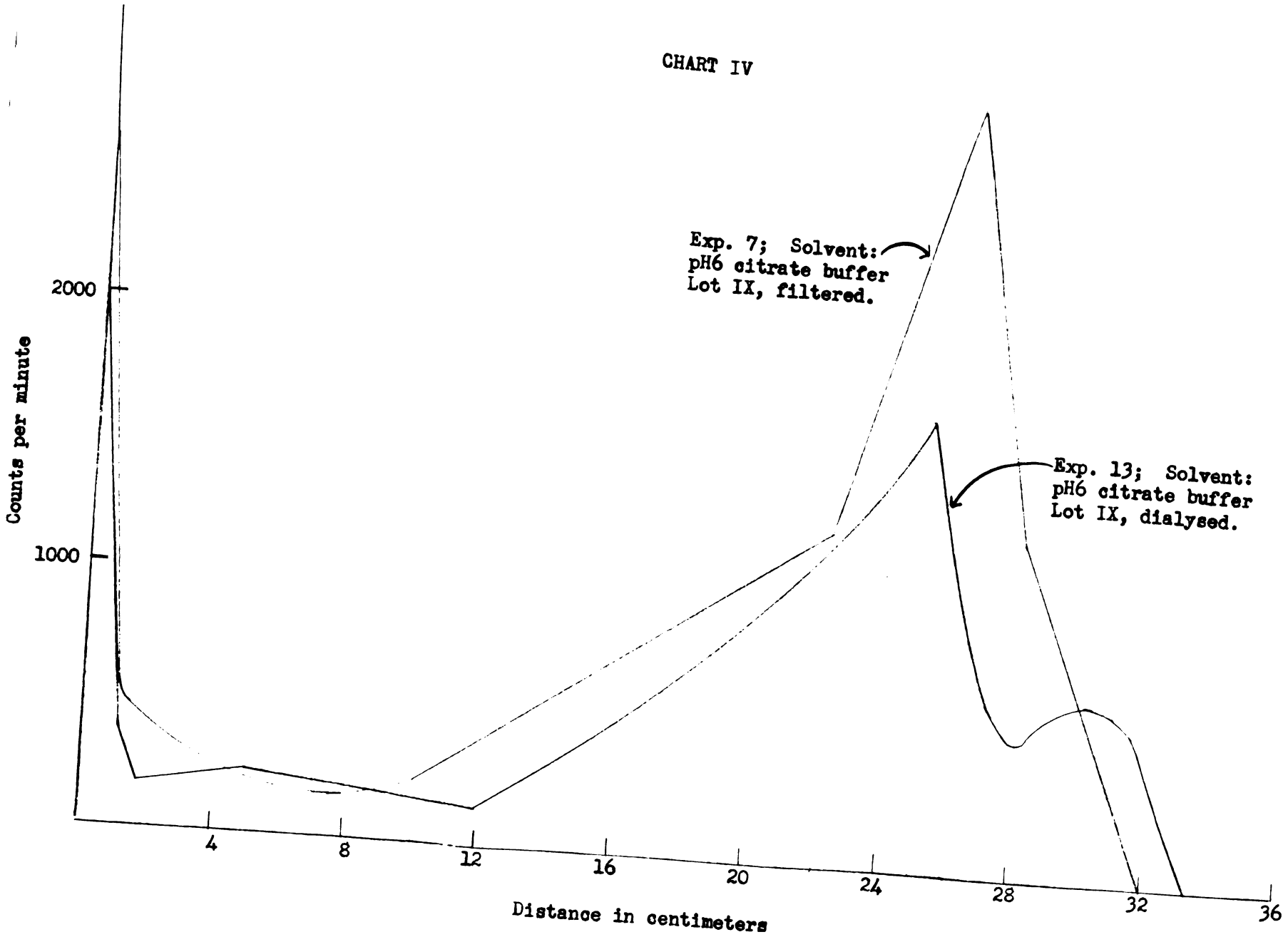

Exp. 7; Solvent: pH6 citrate buffer Lot IX, filtered.
Exp. 13; Solvent: pH6 citrate buffer Lot IX, dialysed.
Counts per minute
2000
1000
4
8
12
16
20
24
28
32
36
Distance in centimeters

CHART V

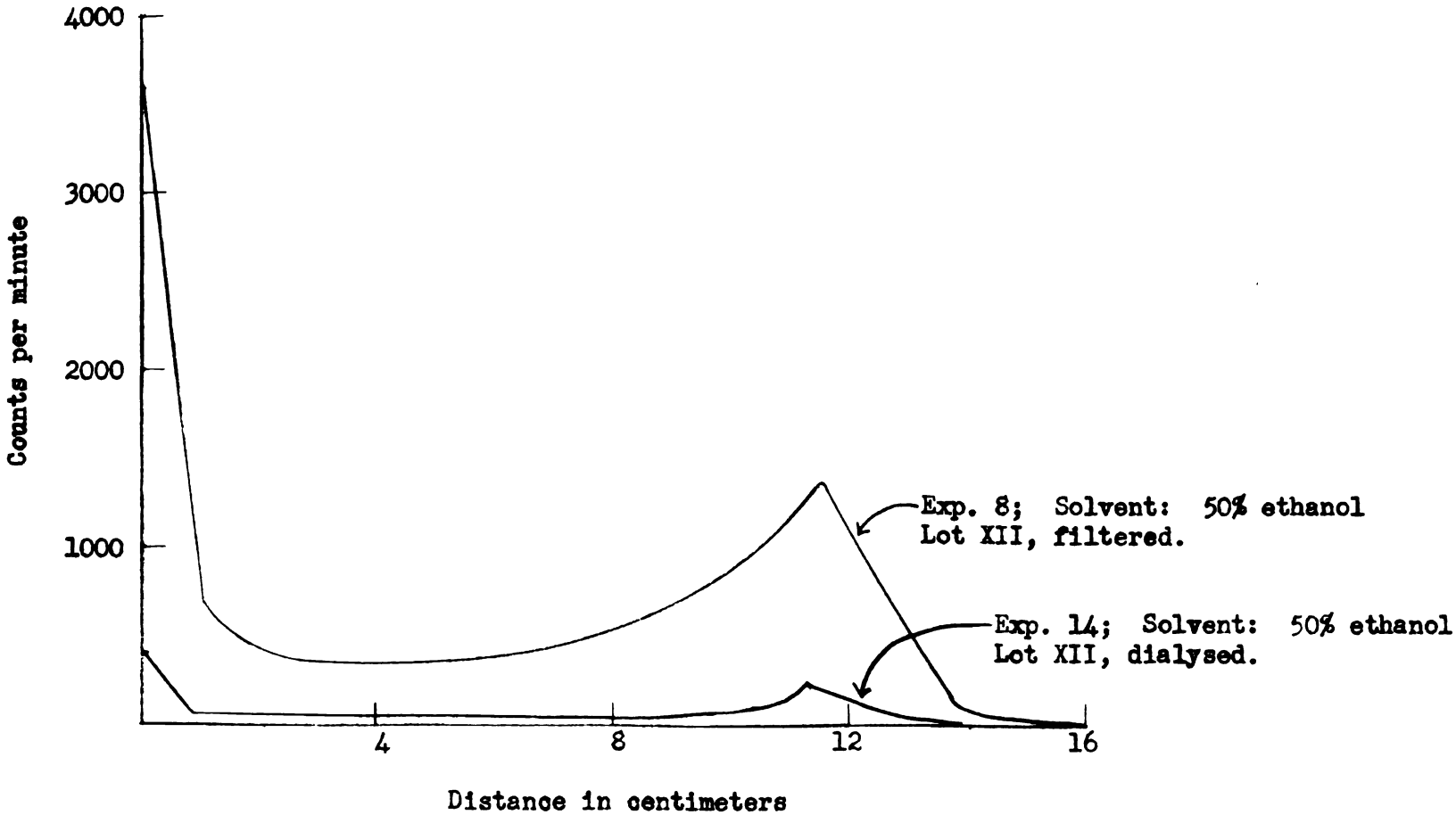

4000
3000
2000
1000
Counts per minute
4
8
12
16
Distance in centimeters
Exp. 8; Solvent: 50% ethanol
Lot XII, filtered.
Exp. 14; Solvent: 50% ethanol
Lot XII, dialysed.

CHART VI

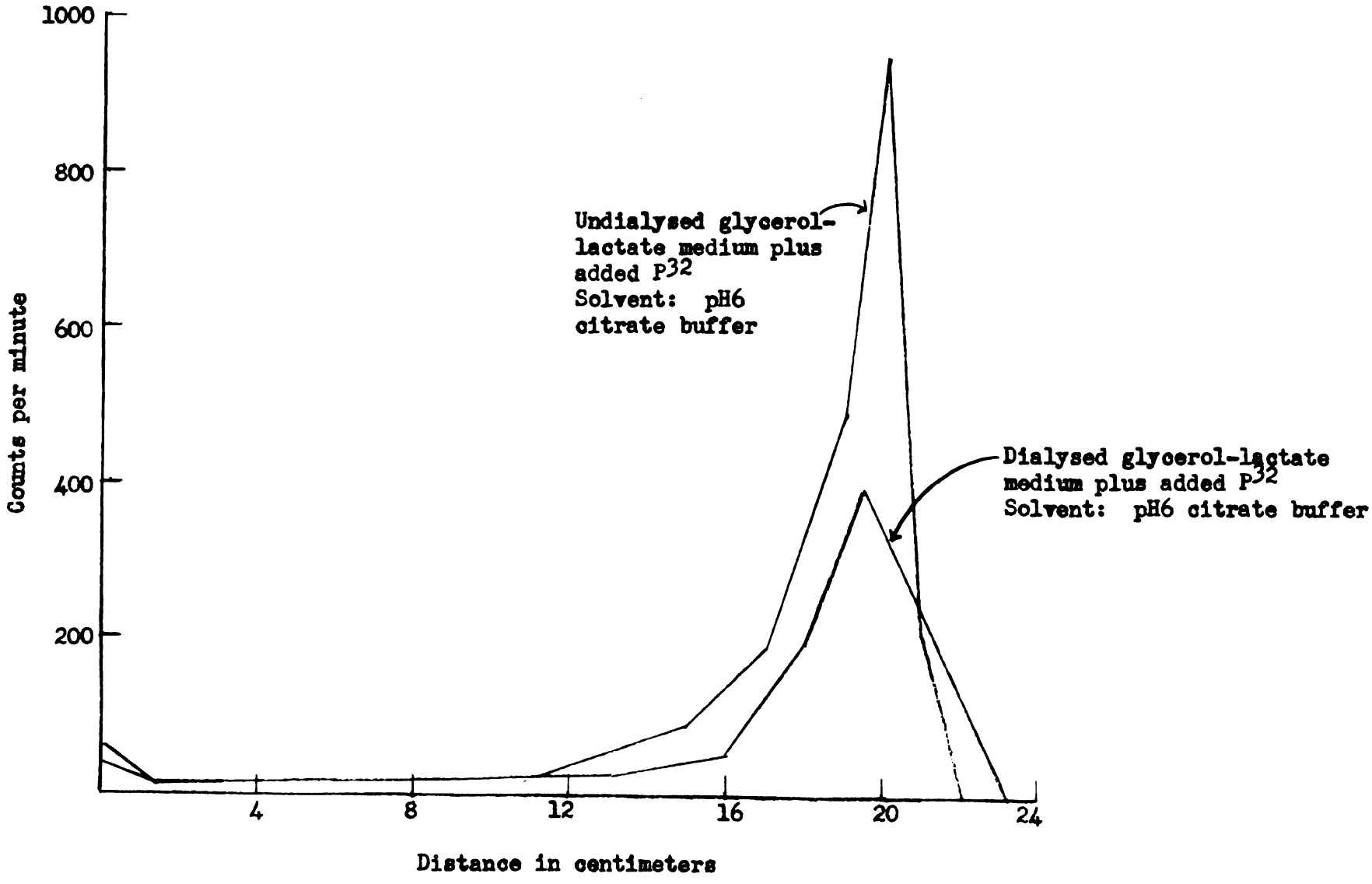

1000
800
600
400
200
Counts per minute
Undialysed glycerol-lactate medium plus added P32
Solvent: pH6 citrate buffer
Dialysed glycerol-lactate medium plus added P32
Solvent: pH6 citrate buffer
4
8
12
16
20
24
Distance in centimeters

TABLE V

Rf VALUES OF MAXIMUM COLOR DEVELOPMENT
AND MAXIMUM RADIOACTIVITY

Experiment Number	Solvent	Rf value of max. color development	Rf value of max. radioactivity
1	citrate buffer pH5	a) .7 b) .82	a) .68 b) .84
2	50% n-propanol	.63	.42
3	50% ethanol	.63	.38
4	10% NaCl	a) .8 b) .9	.83
5	M/5 sodium acetate	.68	.725
6	citrate buffer pH5	a) .7 b) .8	.68
7	citrate buffer pH6	.75	.79
8	50% ethanol		.58
9	10% NaCl	.93	.94
10	M/5 sodium acetate	.7	.89
11	citrate buffer pH5	.74	.79
12	citrate buffer pH6	.74	.8
13	citrate buffer pH6	.74	.75
14	50% ethanol		.59
15	citrate buffer pH5	.83	
16	citrate buffer pH6	.73	
17	" " "	.83	.85
18	" " "		.8

DISCUSSION

The production of T3 bacteriophage on solid "F" medium did not prove to be very satisfactory. Although very high concentrations of the virus were obtained when no P^{32} was present, the addition of P^{32} seemed to prevent the recovery of high virus concentrations. The reasons for this can be ascribed to the technical difficulties of working with P^{32} at such close quarters, and not to any inhibitory effects of the P^{32}. Moreover, the problem of ridding the virus suspension of free P^{32} would have been extremely difficult, for a great deal of P^{32} must have been washed from the agar surface. Another difficulty was that the bacteria could utilise only the P^{32} at or near the surface of the agar, thus, in effect, wasting the P^{32} that was too far beneath the surface. It was for these reasons that production of the virus on solid medium was not continued.

The three experiments performed using liquid "F" medium did, in all but one of the experiments (experiment III), produce satisfactory concentrations of virus. One interesting point to notice is that as the concentration of P^{32} increased, the number of viable bacterial cells decreased. (Table I). Since the bacterial concentration was directly responsible for the virus concentration, the rest of the experiments were performed using a concentration of P^{32} which would not inhibit too greatly the growth of the bacteria. Table II shows the results of using a P^{32} concentration of 0.2 Mc/ml. All the bacterial concentrations are greater than 10^8 viable cells per ml. Although the virus concentrations in experiments I and II were satisfactory, the radioactivity expressed in counts per minute per ml was not as high as had been hoped. After

dialysis, the radioactive count dropped much lower. The count was so low that these virus lots were not considered suitable for use. The reason for this low radioactive count was undoubtedly the fact that the phosphate buffer used in the "F" medium prevented utilisation of the P^{32} by the bacteria. Therefore, the remaining experiments were performed using a liquid glycerol-lactate medium which lacked any inorganic phosphorus.

Production of T3 bacteriophage in liquid glycerol-lactate medium containing P^{32} proved to be very satisfactory. The bacterial concentrations in experiments VI through XII were all above 10^8 cells/ml, and the number of viable virus particles was, with one exception, greater than 10^{10} particles/ml. The effect of dialysis on the virus is less consistent. The number of viable virus particles after dialysis was less in five experiments and greater in two. Neither the increase nor decrease can be accounted for. In only one experiment was any virus recovered from the water in which the suspensions were being dialysed, and the number of virus particles in that instance was not sufficient to account for the decrease in titer. Experiments with non-radioactive virus lots performed previously had indicated that a slight decrease in titer would occur upon dialysis, but that the amount of virus passing through the dialysing membrane was negligible and not sufficient to account for the decrease. The decrease in titer was therefore ascribed either to physical inactivation of the virus while being dialysed, to adsorption of the virus on the membrane and glass stirring paddle, or possibly to both.

At any rate, dialysis appeared to be a relatively simple means of removing dialysable components of the medium which might contain P^{32}

without losing too many virus particles. Dialysis was carried out for 12 hours. After that length of time, the amount of radioactivity being removed became too small to profitably continue dialysis. Samples were taken from each batch of dialysing water before it was changed and assayed for the amount of radioactivity present. The results of this assay are given for three virus suspensions, IX, X, and XI in Table III and in Chart I. The graphs clearly indicate that after eight or nine hours very little radioactivity was being removed compared to the amount removed in the first six hours. Similar graphs for the other virus suspensions show the same results.

Of course, the question of purity of the virus suspension as regards the presence of P^{32} outside the virus particles has still to be answered. This will be taken up in the next section.

An indication of the amount of nitrogen containing compounds removed during dialysis was given by determining the amount of nitrogen present in a virus suspension before and after dialysis. Lot XII (a) was analysed by the method of Folin and Wu using a Bausch and Lomb monochromatic colorimeter with a 505 millimicron wavelength filter to measure the degree of color development with Nessler's reagent. The filtered virus suspension contained 0.316 mg and the dialysed suspension contained 0.03 mg of nitrogen per ml. The total nitrogen of filtered and dialysed glycerol-lactate medium was also determined by the same method. Filtered medium contained 0.366 mg and dialysed medium 0.034 mg of nitrogen per ml. The values for the dialysed virus suspension are as low as values found for virus suspensions purified in the ultracentrifuge by other workers in this laboratory. These results, by themselves, do not prove

that any contaminating P^{32} was removed during dialysis, but they do show that dialysis effectively removes nitrogen containing compounds which may have P^{32} associated with them.

Although paper chromatography has many important applications, its applicability to the separation of proteins has been questioned. (Hall and Wewalka 1951). However, Franklin and Quastel (1949, 1951) have used the technique to separate blood proteins. They claim that paper chromatography can successfully be applied to protein separation when the proteins are sufficiently dissimilar.

Gray (1952) used paper chromatography to detect tobacco mosaic virus in infected tobacco plants. Using 40% and 50% ethanol as the solvent he found that approximately 1/3 of the virus remained at the origin while 2/3 of the virus migrated. The virus did not move as a single compact spot but tended to spread out in a streak. This was the situation in the chromatograms described here. The colored portions, after development with ninhydrin, appeared as streaks from two to four centimeters long. The color was most intense at the center of the streak and less so at the edges. This was true of the filtered, dialysed, and purified virus suspensions. The filtered virus suspensions showed streaks which were a darker purple than those of the dialysed or purified suspensions. This was to be expected, of course, for the filtered virus was suspended in a medium with a total nitrogen content of about 0.4 mg of N/ml, much of which was contained in protein compounds. A great deal of the color development of the filtered virus suspension was due, therefore, to nitrogen containing compounds in the suspending medium, and not to the virus itself. This is illustrated in Table IV (experiments 17 and 18).

The medium which had not been dialysed gave a definite color reaction, while the dialysed medium did not. In none of the experiments was any color seen where the spot had originally been placed.

It was assumed that the color obtained from dialysed virus suspensions after development with ninhydrin was not due to any nitrogen containing compounds in the medium but was due to the virus itself. This assumption was felt to be valid because two virus lots purified in the ultracentrifuge and resuspended in water gave a color reaction which must have been due to the virus. Moreover, the values obtained for the amount of nitrogen remaining in the virus suspensions after dialysis were such that a considerable fraction of the nitrogen must have been virus nitrogen. In a few of the experiments, no color was visible after development with ninhydrin. This was probably caused by the application of too small a volume of the virus suspension. This occurred in at least one chromatogram made with an ultracentrifuge purified virus suspension, (not reported) and in experiments 8 and 14 (Table IV).

It has been suggested by Franklin (1949) that the movement, or lack of movement, of proteins on filter paper is due principally to adsorption of the protein to the paper and not to partition of the protein between the solvent phase in the paper and the moving solvent front. Gray (1952) also noted that under conditions which caused denaturation of protein, such as the use of ethanol as the solvent, certain protein constituents from tobacco leaves would not migrate, while the tobacco mosaic virus, which is less easily denatured by ethanol, did not move with the solvent front.

Any analysis of the chromatography experiments reported in this thesis must be a cautious one. The experiments were originally undertaken because it was felt that chromatography might prove a rapid and simple method of determining the effectiveness of dialysis in removing free or contaminating P^{32} from the medium. Ideally, it was hoped that the virus would migrate to one definite spot which would be easily detectable both visually and by reason of the associated radioactivity, and any contaminating P^{32} would migrate to another definite spot easily distinguishable from the former.

Examination of the experiments performed using citrate buffer at pH 6 (experiments 7, 12, 13, 16, 17, and 18) shows several interesting points. The virus alone has an Rf value of 0.73 (experiment 16), while the medium alone has an Rf value of 0.83 (experiment 17). This would seem to indicate that the color observed in experiments 7, 12, and 13 was due to the presence of virus, since the Rf values of these experiments is 0.74 and 0.75. However, the maximum amount of radioactivity does not always coincide with the visible color spot. P^{32} in association with medium alone (experiments 17 and 18) has an Rf value of .85 and .8 respectively. The radioactivity associated with virus suspensions has Rf values of 0.79, 0.8, and 0.75 (experiments 7, 12, and 13). These values are not as consistent as those computed from visible color spots, and only in experiment 13 do the Rf values coincide. The Rf values for the radioactivity are so inconsistent that it is not possible to say whether the radioactivity is associated with the virus. Experiment 13 seems to indicate that it might be, but experiment 12 seems to indicate the opposite.

The experiments performed using citrate buffer at pH 5 show the same inconsistencies, especially as regards the Rf values of the radioactivity of the virus suspensions. (experiments 1, 6, 11, and 15) Only in experiments 4 and 9 is there any indication that dialysis effectively removes contaminating P^{32}. In experiment 4, using a filtered virus suspension, the radioactivity is not associated with either of the color spots. However, in experiment 9, using a dialysed virus suspension, the radioactivity migrated to the same position as the visible color spot. These two experiments together with experiment 13 are the only ones that indicate that dialysis removes any contaminating P^{32}, and that the radioactivity is associated with the virus after dialysis.

One other observation can be made concerning these experiments. The amount of radioactivity remaining at the origin may be of some significance. The only solvent that moved nearly all the radioactivity from the origin was the citrate buffer at pH 5. (Chart III). All the other solvents left a considerable amount of radioactivity at the origin. However, citrate buffer at pH 6 did move nearly all the P^{32} that had been added to glycerol lactate medium. If then, the movement of P^{32} along the filter paper consists mainly of contaminating P^{32} which might be present in its original form, then the radioactivity remaining at the origin can be ascribed to P^{32} which is bound to some non-movable substance, presumably the virus. However, this neglects the evidence of those experiments performed using a purified virus suspension. In those experiments a definite color spot was visible. This color could only have come from the virus. If both the virus and the contaminating P^{32} migrate down the paper strip, what significance does the radioactivity that remains at the origin have: There is no information at hand to answer this question.

The most likely answer is that the solvents used, with the exception of pH 5 citrate buffer and possibly M/5 sodium acetate, did not move all the virus, and the amount of virus remaining at the origin was insufficient to produce a color reaction when developed with ninhydrin. If this is so, then the radioactivity remaining at the origin would be due to virus, and the radioactivity at the single peak would be due to virus plus any contaminating P^{32}. One reason for the ability of pH 5 citrate buffer to move more of the radioactivity than any of the other solvents might be the fact that it is closer to the iso-electric point of the virus and would tend to neutralise the electrical charge on the virus. Since adsorption to the paper is in part due to the nature of the electrical charge of both the paper and the virus, this might explain the inability of the other solvents which were at or near a pH of 7 to move a large part of the radioactivity of the virus suspension. Unfortunately, no experiments were performed which might have given an indication of the validity of this supposition.

Although it is possible to demonstrate that a considerable quantity of radioactivity is removed from the virus suspension during dialysis without a great loss in the infectivity titer of the virus, the chromatographic means for testing the purity of the dialysed virus did not prove to be entirely successful. It is felt that the reason for this lies primarily in the fact that the virus is adsorbed to the filter paper and does not migrate as a unit. There are other means analogous to paper chromatography which might prove successful in proving the purity of the dialysed virus suspension. For example, paper electrophoresis, using either a single paper strip or a vertically suspended sheet of paper, would overcome to some extent the problem of adsorption

of the virus to the paper. The added "push" from the electric current would result in a better separation. Another piece of apparatus which might prove useful would be the use of a solid block of starch as the stationary phase. Starch is noted for its low adsorptive power, and would minimise the problem of adsorption. An electric current applied through the starch block would provide the motive power for the charged virus particles.

SUMMARY

Three methods of producing T3 bacteriophage in medium containing P^{32} are described. The first, using solid "F" medium, proved to be unsatisfactory because of the technical difficulties involved in working with the P^{32} and because of the difficulty of ridding the virus suspension of free P^{32}. The second, using liquid "F" medium, was also unsatisfactory because the amount of P^{32} taken up by the bacteria and virus was too small. The third method, using liquid glycerol-lactate medium produced both high concentrations of virus and high radioactive counts per milliliter of virus suspension. (Tables I and II).

The virus suspensions were dialysed against distilled water to rid the suspensions of free P^{32}. Tables I and II show the amount of P^{32} removed from the virus suspensions, and Chart I shows the amount of P^{32} removed during the dialysis process.

Paper chromatography techniques were employed to estimate the efficiency of the dialysis procedure in removing free P^{32} from the virus suspensions. It was concluded that chromatographic methods were not satisfactory for this purpose because adsorption of the virus to the paper prevented free migration of the virus.

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