COMPARATIVE BIOCHEMICAL STUDIES ON NORMAL AND ON POLIOMYELITIS VIRUS-INFECTED TISSUE CULTURES

V. Profound Alteration of Acid and Alkaline Phosphatase Activity in Infected Rhesus Kidney Cells*

By ERNEST KOVACS, M.D.

(From the Department of Hygiene and Preventive Medicine, University of Toronto, Toronto)

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A survey of the literature of tissue cultivation does not disclose much information on enzymes in this particular branch of experimental biology (1). The majority of writers dealing with phosphomonoesterases in cultivated cells (2, 3), with the exception of Fell and Robinson (4, 5), applied histochemical tests (6), which do not permit quantitative evaluation. We followed the activities of various enzyme systems in normal tissue cultures (TC) by biochemical methods (7, 8). Using similar techniques striking changes were observed in nine enzyme systems of cells infected with various strains of poliomyelitis virus and the findings published in a preliminary report (9). The present publication deals exclusively with various aspects of phosphatase activities in virus-inoculated TC and in normal controls. Previous works in this field were made mostly on organ homogenates of experimental animals (10), containing all the products of inflammatory reactions of the host. Our contribution on relatively simple systems concerns the mechanism of drastic modifications in cell physiology during poliomyelitis infection, *in vitro*.

Methods

Only the variations in the handling of TC will be mentioned here; for more information and the general methods the reader is referred to the previous papers of this series (7–9).

Explants.—Tissue cultivation, virus production, and titration were made by the Poliomyelitis Assay Department.[1] Most of the experiments were carried out on roller tubes of trypsinized *rhesus* monkey kidney cortex, grown about 14 days with synthetic nutrient 199 (11) and later with a modified form of it, termed medium 597 (7). The roller tube cultures were inoculated with various dilutions of virus, generally 24 hours after the second fluid change, and were incubated in roller drums at 37°C. for 6 days (12). For calculation of the titre Kärber's method was used (13) and virus concentration was expressed in negative logarithms (14). Specially designed experiments were run with large inocula (10^{-5}, or 10^{-3}

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dilutions of seed virus of titres $10^{-7.2}$ to $10^{-7.5}$). Pools of TC or its fractions were used in the great majority of experiments, each new assay being carried out on different batches of cultivated cells. Similar work on suspended tissue fragments will be published separately (15).

Phosphatase Determinations.—Shinowara's technique was used with 0.5 per cent glycerophosphate as substrate (16, 7, 8), followed by colorimetric estimation of inorganic phosphate (IP) by Fiske and Subba Row's procedure (17). The King-Armstrong method (18) was also employed as a check on the standard techniques. As enzyme material, 1 to 2 ml. of infected or uninfected TC pools were mixed with 6 to 9 ml. buffered acid or alkaline substrate, preheated for 30 minutes. Enzyme:substrate ratios of 2:6 for acid and alkaline phosphatase were used as a routine. The assays were always run at least in duplicate. The splitting of ribonucleotides (another substrate for phosphomonoesterases) at pH 8.5, will be described separately, together with 5-nucleotidases (15).

Incubation.—Incubation was carried out for ½ to 4 hours at 37°C., the 4 hour period being used the most frequently. Effect of incubation for 16 to 24 hours and of various temperatures, up to 56°C., was also explored.

Hydrogen Ion Concentration.—As a routine this was about pH 5 (acetate or veronal-acetate buffer) for the acid and about pH 10.9 for the alkaline substrate (borate-borax or veronal-NaOH buffer). Other pH and other types of buffers were also tested. The above mixture (16) although not at the optimal pH, was the most suitable, because it gave the greatest contrast between infected and normal TC.

Particular TC Fractions.—Various enzyme activities can be detected depending on the fraction of the TC used (8). For this reason pooled and centrifuged fluids were assayed, or TC homogenates ground with the original, or occasionally with fresh medium (7, 8). This method was easy to carry out, large pools could be made, exactly measured portions could be pipetted off, and the tissue residue could be used for other purposes. In other instances the homogenates were separated by centrifugation (10 minutes at 2000 R.P.M.) and the clear supernatant "extract" and the sediment were assayed in parallel for phosphatases.

Experiments with Living Cells.—The use of whole TC in the original roller tubes was found the most simple and practical approach. The fluid medium was usually about 2 ml. in these cultures and 6 ml. of the acid or alkaline substrate was added before incubation. After the period of time required at 37°C., 2 ml. ice cold 30 per cent trichloroacetic acid was pipetted into the tubes chilled in ice, and ½ of the filtrate used for phosphate determinations. The values obtained were summed and the mean calculated. TC with buffer, substrate with buffer, and occasionally synthetic medium plus substrate served as controls, treated in exactly the same way as the test system. The measure of phosphatase activity was the increase in IP concentration, micrograms per milliliter of system, calculated from the difference between the values of the controls and the reaction mixture.

A further refinement of these techniques consisted in replacing the old nutrient with unused medium, or adding the substrate directly to the tissue residue growing on the inner surface of test tubes, after decantation of the nutrient fluid. In this case the deficiency in volume is replaced by buffered substrate. After incubation, the system and controls were handled individually and the average values calculated; or the tubes were chilled and the fluids pooled in a large dish standing in ice. Portions of these pools were taken for 2 or 3 parallel determinations of IP. Controls were identically handled.

Estimation of Tissue Mass.—The turbidimetric method of Looney and Welsh (19) was used (7–9) in order to have an estimate of the tissue mass of homogenates, or of substances released into the TC fluids In some experiments the normal and infected material was brought to "isoturbidity" by dilution with medium, assuring about the same tissue concentrations per milliliter. The spectrophotometric method as recommended by Kalckar (20) was used with the TC fluid. A quick estimate of protein and NA was made from the extinction

values at 2600 and 2800 A. These methods could be supplemented by more time-consuming nitrogen determinations (7).

Finally, the effect of various temperatures such as freezing, boiling, and storage at 4°C., and the influence of ionic ($MgSO_4$, $ZnSO_4$, NaF, KCN) activators or inhibitors was also explored. Strict asepsis was observed throughout in the handling of all the assay material.

Statistical analysis of various groups was made by the methods recommended by Fisher (21). Mean experimental error was under 2 per cent in the phosphate determinations. Mean values ± standard error were tabulated for the fully randomized groups and Student's test carried out for significance (22).

RESULTS

Introductory Remarks.—Several thousand TC of *rhesus* kidney cortex were examined individually or in large pools. In preliminary assays it was found that the type and age of culture or of nutrients influenced the enzyme levels, especially with regard to their distribution between nutrient fluid and cells (7, 8). Whole cultures in roller tubes were found to be the most suitable tool, because the effect of virus on the enzyme systems could be demonstrated most clearly. Although these unmanipulated "living" cells gave the most significant results, phosphatase activities of supernatant fluids and of homogenates will be also presented, always in infected-uninfected pairs. In this way a more detailed picture could be traced as to the behaviour of virus infected cells accompanied by normal TC of the same age, of the same nutrient, explanted from the same large pools of trypsinized kidney, and grown at the same temperature. The results are grouped in 7 tables and illustrated by 5 diagrams.

Description of Findings.—Examples of typical experiments are shown in Table I. The great difference in enzyme activities between the normal and the infected cultures is striking. Uniformly high values for normal and low values for TC tubes exhibiting virus growth were observed in whole explants, in which the substrate was added to the original tubes (group 1). There was some variation with the second technique by which centrifuged, cell-free TC fluids were used (group 2). The rare exception to the pattern in acid phosphatase assays is also illustrated in this group. The results give only indirect information on the cells. The presence of biocatalyst in the fluid phase is a function of the physiological state of the explant, thus too high activities in the supernatant may be due to unusual permeability or to cell disintegration (7, 8). This method complements the former, as a check on the integrity of the cells. The consequences of the late cytopathogenic effect (CPE) of the virus could lead to great increase of enzyme levels in the supernatant, compared to normal TC fluid, or to its own residual cells.

The third group, namely the TC homogenates gave comparable results to the first two groups. The slight increase in acid phosphatase observed in some infected homogenates, emphasizes that breaking up of the cells may

TABLE I

Phosphatases in Normal and in Virus-Infected TC

TC + medium	Age	Interval of infection	Virus titre in negative logarithms	Acid phosphatase, as increase in IP μg./ml. of system	Alkaline phosphatase as increase in IP μg/.ml. of system
	days	*days*			
1. Whole explant in original roller tubes					
Normal TC, in medium 597,* pool of 10 samples, mean of duplicate determinations	14	—	—	1.13	1.20
Same, inoculated with Mahoney strain	14	6	$10^{-6.8}$	0.23	0.60
Normal TC, in medium 597, pool of 10 samples, mean of duplicate determinations	14	—	—	0.63	0.82
Same, inoculated with MEF1 strain	14	6	$10^{-7.1}$	0.30	0.43
Normal TC, in medium 597, pool of 10 samples, mean of duplicate determinations	14	—	—	0.73	1.10
Same, inoculated with Saukett strain	14	6	$10^{-7.0}$	0.53	0.29
2. Centrifuged supernatant of TC fluids					
Normal TC, in medium 199,‡ pool of 100 samples, mean of duplicate determinations	14	—	—	0.57	1.62
Same, inoculated with Mahoney strain	14	6	$10^{-6.9}$	0.57	1.19
Normal TC, in medium 597, pool of 100 samples, mean of duplicate determinations	14	—	—	0.40	0.56
Same, infected with Saukett strains	14	6	$10^{-7.3}$	0.06	No activity
3. Whole homogenate of TC					
Normal TC in medium 199, seventy samples pooled after grinding, mean of duplicate determinations	14	—	—	0.46	0.74
Same, infected with Mahoney strain	14	6	$10^{-7.2}$	0.26	0.10
Normal TC, in medium 597	14	—	—	1.13	2.07
Same infected with Saukett strain	14	6	$10^{-6.8}$	1.26	1.76
4. Homogenates and/or extracts					
Normal TC in medium 597, homogenates, pool of 10 samples, mean of duplicate determination	14	—	—	0.43	0.43
Same, extracted by centrifugation	14	—	—	0.26	0.46
Infected TC homogenates, Mahoney strain	14	6	$10^{-7.0}$	0.10	0.15
Same, extracted by centrifugation	14	6	$10^{-7.0}$	0.10	0.30

* Modified form of medium 199 (7, 8)

‡ Complex synthetic nutrient of Morgan *et al.* (11).

TABLE I—*Continued*

TC + medium	Age	Interval of infection	Virus titre in negative logarithms	Acid phosphatase, as increase in IP μg./ml. of system	Alkaline phosphatase as increase in IP μg./ml. of system
	days	*days*			
5. Cell residue (direct determinations, substrate added to cells)					
Normal TC, in medium 597, original tubes	14	—	—	0.80	4.69
Same TC, cells only + buffered substrate	14	—	—	0.83	0.10
Same TC, infected with Saukett strain	14	6	10^{-7}	0.33	3.66
Same, cells only, infected with Saukett strain	14	6	10^{-7}	0.10	0
6. Effect of refreshment of medium					
Normal TC, in medium 597	15			0.35	1.10
Same infected with MEF1 strain	15	7	$10^{-6.8}$	0.14	0.77
Normal TC + fresh medium 597	15	—	—	0.43	1.16
Same infected with MEF1 strain	15	7	$10^{-6.8}$	No activity	0.33
Normal TC fluid, supernatant of above	15	—	—	0.10	0.20
Same, infected with MEF1 strain	15	7	$10^{-6.8}$	No activity	0.03

alter the general pattern, especially with this sensitive enzyme (7, 8, 23). Thus preference was given whenever possible to unmanipulated, whole, "living" TC, and grinding was used only for special studies.

Differences between normal and infected TC homogenates in extractability of phosphates may be explained in group 4. In the normal TC homogenate 40 per cent of acid phosphatase activity remains with the sediment after centrifugation, and about 100 per cent of alkaline phosphatase is carried by the supernatant. Infected TC behaved differently; the markedly diminished acid phosphatase exhibited the same activity in extract as in whole homogenates; alkaline phosphatase was 100 per cent higher in the extract than in the residue. These findings may be explained in part by the increased fragility of infected cells, but other factors also are involved, modifying the kinetics, as will be discussed.

Finally, a few examples are given, in which the tissue explant was assayed with the substrate added to the cells directly (group 5). The activity of these cells is comparable with that of the original whole culture in respect to acid phosphatase although these cultures were deprived of nutrients for about 2 hours. A great part of alkaline phosphatase was carried away with the medium. This fact is interpreted as a sign of beginning degeneration in morphologically slightly altered normal controls. Otherwise the partition of enzyme activities

between the two phases should be more proportionate. (For normal behaviour see group 6 and reference 8.) The drop in activity in group 5 was also due to the loss of Mg^{++} (2.2 mg. per cent in Hank's solution used for preparation of

TABLE II

Statistical Analysis of Results

Assays	No. of experiments on different batches	Acid phosphatase as increase in IP µg./ml. of system	Alkaline phosphatase as increase in IP µg./ml. of system
		mean S.E.	*mean* S.E.
1. Whole living TC in original roller tubes			
Normal TC in medium 597, pool of 10 samples for each experiment, duplicate determinations	10	0.90 ± 0.05	1.00 ± 0.12
Same inoculated with various strains of poliomyelitis virus, average titre $10^{-6.8}$. 6 days' incubation	10	0.29 ± 0.02	0.42 ± 0.05
2. Supernatant of TC fluids			
Normal TC in medium 597, pool of 50 to 200 samples for each experiment, duplicate determinations	10	0.38 ± 0.04	0.61 ± 0.04
Same inoculated with various virus strains, average titre $10^{-6.8}$	10	0.22 ± 0.04	0.26 ± 0.07
3. Whole homogenates and extracts: (mixed group)			
Normal TC in medium 597, pool of 10–20 samples of each experiment, duplicate determinations	10	0.48 ± 0.04	0.55 ± 0.07
Same, infected with various strains of poliomyelitis virus, average titre 10^{-7}	10	0.18 ± 0.05	0.27 ± 0.07
4. Homogenates			
Normal TC in medium 597, pool of 10 to 20 samples from various batches	5	0.61 ± 0.004	0.71 ± 0.12
Same, infected with various strains of poliomyelitis virus, average titre $10^{-6.8}$	5	0.34 ± 0.004	0.39 ± 0.12

medium 597) and other activators, as well as to the effective loss in enzyme (23–27). This assumption is supported by the findings of other assays (group 6) which illustrate a further biodynamic aspect, namely the effect of replacement of the used nutrient fluid with a fresh one. In this way the activity of alkaline phosphatase can be restored in normal TC, but not in explants in-

fected with virus. The findings with acid phosphatase are very much to the point. This enzyme system exhibited about the same activity in normal TC residue after removal of the old nutrient (group 5) and about 22 per cent higher value with fresh medium (group 6). The decrease caused by the virus in the activity of this biocatalyst however cannot be restored by fresh nutrient, in agreement with the observations on alkaline phosphatase. The kinetic aspects of magnesium activation and inhibition by cyanides and other salts will be described in a separate publication (15).

Table II presents a statistical survey of the results. The same number of experiments was carried out in the majority of groups, all of different lots and assays on large pools at least in duplicate. The most significant results were obtained with the intact TC, the differences between infected and uninfected pairs being the highest here and smallest in the homogenates group. Supernatant and the mixed homogenate series (5 lots separated from group 3) exhibited the smallest standard error for the acid phosphatase assays. Relatively high variations were encountered in normal TC, which fact may be inherent in tissue cultivation (28, 29, 8). Random samples were used throughout; the only selection was the use of 14 day old TC, after 6 days' infection, when the virus growth was at its height, as revealed by destruction of the host cells. No separation of virus types and dilutions ($10^{-6.5}$ to $10^{-7.5}$) was made. The titres ranged from $10^{-6.0}$ to $10^{-7.5}$, which may account for some degree of variation. Furthermore the time of harvesting was not always exactly 6 days; differences of several hours in some experiments were unavoidable, which also may add to the inhomogeneity of the groups. The results are significant under the conditions of the experiments, as assessed by Student's test (22). More selective studies with one type of virus, large inocula, and rigorously observed time factors were characterized by lower standard errors (see Table III).

Assays with High Concentrations of Virus

The effect of large inoculum is strikingly illustrated in Table III. One strain, the Type I (Mahoney) was used in massive doses, (10^{-3} dilution of the seed exhibiting titers of $10^{-7.5}$). The assays were generally run on supernatant fluids of 20 tubes, pooled after exactly 24, 48, and 72 hours' infection and incubation in roller drum at 37°C. Thus 3 to 6 day younger cells were assayed in this group compared to the previously tabulated experiments. Time for the enzyme tests was exactly 4 hours at similar temperature, with preheated ingredients. Means of duplicate values were calculated and average ±standard errors of the results are presented. Assays on whole TC represent single experiments with less than 1 per cent experimental error. One type and high concentrations of virus reduced the variations considerably. The general pattern is similar to those observed previously (Tables I and II),

TABLE III

Effect of Large Inocula

Explant and medium	Age of TC	Interval of infection	Inoculum	Acid phosphatase increase in IP μg./ml. of system		Alkaline phosphatase increase in IP μg./ml. of system	
	days	*days*		*mean*	S.E.*	*mean*	S. E.
1. Supernatant of roller tube TC							
Normal TC fluid in medium 597, mean of 5 experiments duplicate determinations on pool of 20 tubes	9	—	—	0.32	± 0.03	0.36	± 0.05
Same, infected	9	1	Mahoney strain; titre: $10^{-7.5}$; dilution: 10^{-3}	0.08	± 0.03	0.16	± 0.004
Normal TC fluid in medium 597, mean of 2 experiments on various batches, pool of 20 tubes, duplicate determination	10	—	—	0.35	± 0.07	0.30	± 0.0001
Same, infected	10	2	Mahoney strain; titre: $10^{-7.5}$; dilution: 10^{-3}	0.16	± 0.08	0.06	± 0.03
Normal TC fluid in medium 597, mean of 2 experiments, pool of 20 tubes, duplicate determinations	11	—	—	0.60	± 0.10	0.30	± 0.06
Same, infected	11	3	Mahoney strain; titre: $10^{-7.5}$; dilution: 10^{-3}	0.10	± 0.05	0.02	± 0.0005
2. Homogenates							
Normal TC mean of 3 experiments, etc.	9–11	—	—	0.45	± 0.02	0.42	± 0.03
Same, infected	9–13	3	Mahoney strain; titre: $10^{-7.5}$; dilution: 10^{-3}	0.14	± 0.01	0.17	± 0.05

TABLE III—*Continued*

Explant and medium	Age of TC	Interval of infection	Inoculum	Acid phosphatase increase in IP µg./ml. of system		Alkaline phosphatase increase in IP µg./ml. of system	
	days	*days*		*mean*	S. E.	*mean*	S. E.
3. Living whole tissue culture							
Normal TC, in medium 597, one experiment, mean of duplicate assays	9	—	—	1.12		1.00	
Same, infected with Mahoney strain	9	1	Mahoney strain; titre: $10^{-7.5}$; dilution: 10^{-3}	0.43		0.56	
Normal TC, as above	10	—	—	0.53		1.59	
Same, infected with Mahoney strain	10	2	Mahoney strain; titre: $10^{-7.5}$; dilution: 10^{-3}	0.07		1.29	
Normal TC, as above	11	—	—	1.23		3.92	
Same, infected with Mahoney strain	11	3	Mahoney strain; titre: $10^{-7.5}$; dilution: 10^{-3}	0.20		1.23	

* The following formula was used to evaluate the significance of difference between normal and infected means $= \frac{m_1 - m_2.}{\sqrt{s^2_{m_1} + s^2_{m_2}}}$.

m_1, mean of normal.

m_2, mean of infected.

s_1, standard error of normal mean.

s_2, standard error of infected mean.

but the early quantitative change in enzyme activities is striking. Since CPE was not evident after 24 hours by microscopic inspection the result was termed negative or doubtful (±). However, enzyme assays at this stage already revealed a marked quantitative difference between normal and inoculated explants. Infected cultures exhibited about 5 times lower acid phosphatase values on the 3rd day of inoculation. Homogenates and whole cultures behaved similarly. The standard error was generally lower than in the previous tables, and the number of lots assayed was also smaller.

Correlation of Virus Growth with CPE and Enzyme Activity

The effect of multiplication of virus on the activity of phosphatases of the TC was estimated from 24 hours after infection until the destruction of the host cells. Fig. 1 and 2 present the results of such experiments. 140 tubes, all of the same lot of TC, were inoculated with moderately high titre of Mahoney

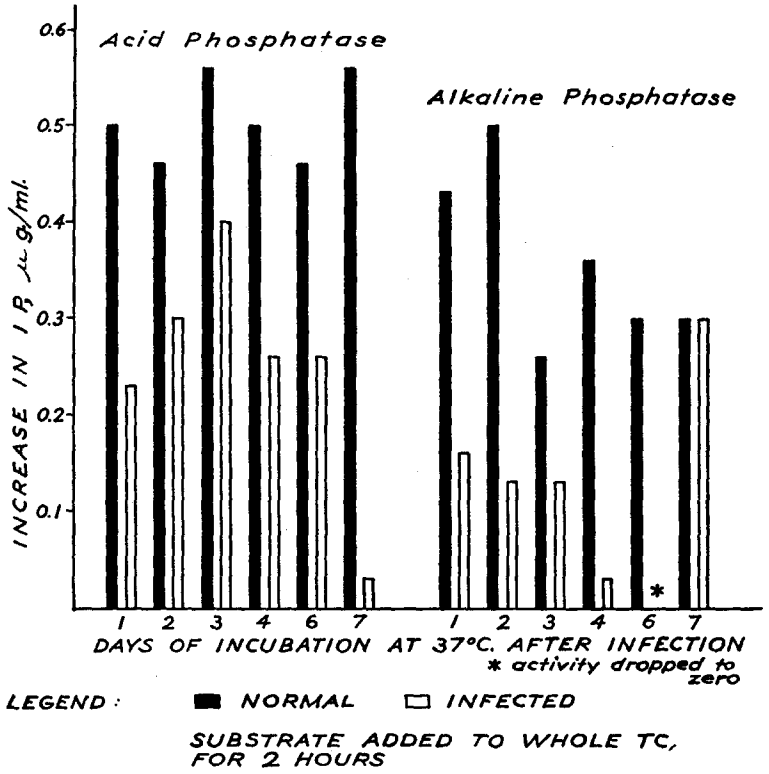

FIG. 1. Phosphomonoesterase activities in normal and in virus-infected explants

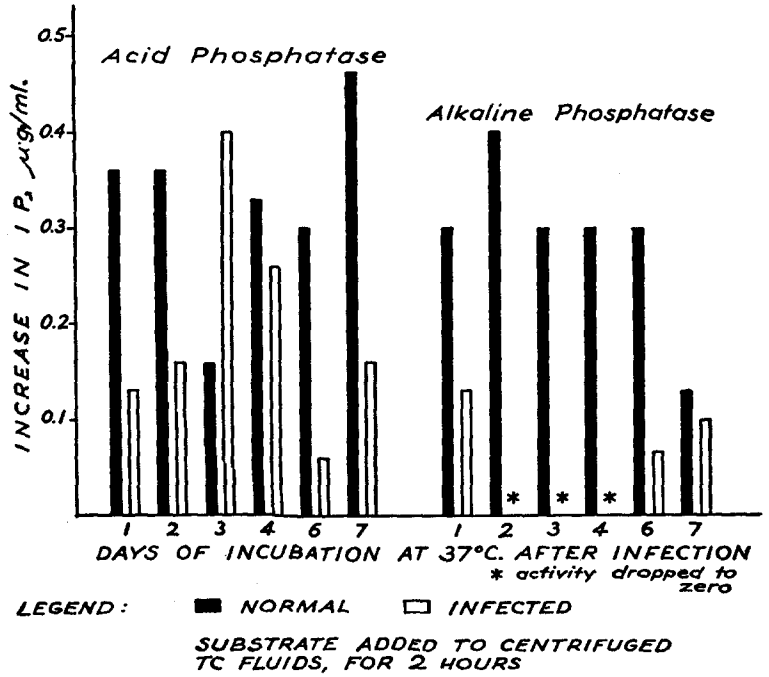

FIG. 2. Phosphomonoesterase activities in supernatant of normal and infected explants

strain (10^{-5} dilution of the seed of $10^{-7.5}$, 0.5 ml. in 4 ml. medium 597). These were paralleled by 140 normal controls. Twenty samples were tested daily from the two main groups. The TC fluids were pooled, 2 ml. pipetted back to each tube and the whole explant incubated at 37°C., with 6 ml. substrate added. Simultaneously similar assays were run from the remaining centrifuged cell-free pool, always in duplicate.

The activity of acid and alkaline phosphatases in whole TC is presented in the first histogram (Fig. 1) measured daily for 7 days. Acid phosphatase activity of normal living cultures reached peak values at the 3rd and 7th days. The decrease in activity after 1 day of infection with virus is strikingly illustrated. By the 2nd day some increase in infected TC and some decrease in normal controls was observed. The 3rd day was critical for acid phosphatase, reaching peak values in both uninfected and infected explants, followed by a sharp and progressive drop in the presence of virus and high activities in normal cultures. The 7th day the normals exhibited peak values and the infected declined almost to zero.

Alkaline phosphatase during the same period behaved somewhat differently. The highest level was present in normal TC on the 1st and 2nd day, lowest on the 3rd day, followed by some rise till the end of the 7 day period. This could be considered a good normal activity. The infected TC shows a marked and progressive decrease to zero level on the 6th day. This minimum is followed by an abrupt rise, attaining the value of the control on the 7th day.

The behaviour of the enzymes in supernatant fluids is illustrated in Fig. 2. Acid phosphatase of the fluid phase of normals during the first days is of much lower activity than that of the whole normal TC. This is especially true in the 3rd day assays and is a good sign of the integrity of the cells (7). In the older cultures a higher enzyme level is observed, culminating on the 7th day and due to the effect of aging of TC. The lower activities in infected fluids with some daily increase till the 3rd day is significant, when a similar peak is attained as in whole infected TC. This latter finding suggests that most of the activity is in the supernatant at this particular time, as compared to whole TC. The following days bring gradual reduction of acid phosphatase in infected fluids, with constant normal values. The marked decrease is followed by a rise on the 7th day, reaching about the 2nd day levels of the infected TC fluids, but only $\frac{1}{3}$ of the activity of controls.

The alkaline phosphatase values for normal are almost constant, except for a peak at the start and a marked drop at the end. In infected explants the changes are very instructive; the lowered activity on the 1st day is followed by complete disappearance of the enzyme from the fluid phase till the 6th day, when some activity is detected, and which 1 day later attains almost the value of the normal controls.

The CPE in these cultures is absent till the 3rd day of infection, $\pm$ on the

4th day, positive on the 5th and 6th and strongly positive on the 7th day. Both phosphatase activities are markedly reduced 24 hours after infection in the presence of virus, although not uniformly. The very low level during the disintegration of cells (6th to 7th day) in whole TC (Fig. 1) and the only moderate increase in the fluid phase may mean that a fraction of the acid phosphatase persisted and entered into the supernatant. The increase of virus titres at first reduces the enzyme activity in the TC fluid and later the disruption of cells caused the opposite effect. The drop of activities precedes the visible CPE for days. Only a small amount of alkaline phosphatase undergoes destruction because eventually the uninfected and the infected explants exhibit equal activity. The findings were verified in many instances under various conditions. Unconcluded assays on TC homogenates suggest that the alkaline phosphatase may go through an early maximum. The time factor is of great importance, because the rapidity of virus effect and because the early biochemical changes may be covered by secondary alterations (see Table IV). Results of experiments from 0 time of inoculation up to 24 hours will be reported among other observations not presented in this report (15). The changes in ultraviolet light absorption of the TC fluids are in agreement with the above findings (Fig. 5).

Unapparent or Latent Infection and Enzyme Activities

In the previous illustrations the effect of large inocula and of growing virus was shown. In Fig. 3 assay results are presented with high dilutions of the seed virus, without increase of titre after 6 days' infection and without destruction of the cells of the explant. These TC are termed "negative tubes" compared with uninfected controls, or with the "positive tubes" which do show CPE and increase of virus titre. It was interesting to see that certain enzymes of these three categories showed a distinct pattern. Highest activity was demonstrated in normals, lowest in the positives, and intermediate in negatives. Twenty explants were used for each group—14 days of age, of the same batch, the 7th day after inoculation. The normal control TC exhibited good growth, the positive tubes a high titre and CPE; the negatives were indistinguishable from the normals by microscopic inspection. Phosphatase determination was carried out with substrate added to whole cultures, incubated for 2 hours at 37°C. and tested as usual. Upon longer incubation with the substrate the differences between the three groups were even more marked (not illustrated). These are important, reproducible findings and will be described in detail together with assays on other enzymes (15). Some possible explanation will be put forward during the discussion of results.

The Enzyme Activities of TC on Storage at 4°C., or at 37°C

The effect of standing on acid and alkaline phosphatases was examined in normal and inoculated TC, without addition of fresh nutrient. Time of storage

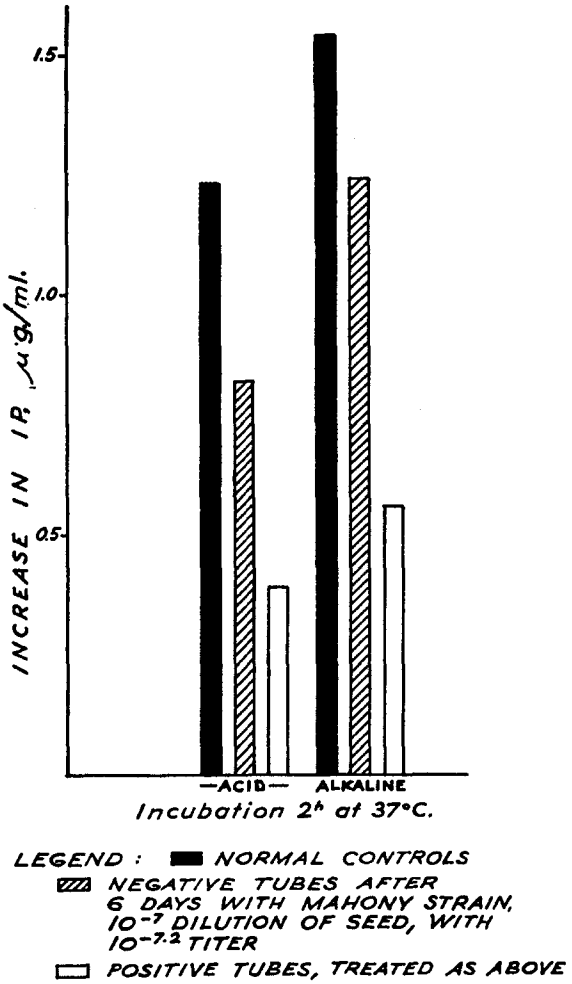

FIG. 3. Effect of unapparent virus infection on phosphatases

at 4°C. or at 37°C. was counted from 14 days of age and 6 days' infection respectively. Good enzyme activities were observed in some lots even after 60 days' storage at 4°C. (Table IV). The size of inocula was not excessive, average titre 10^{-7}, dilution $10^{-6.5}$ to $10^{-7.5}$. The phosphatases pattern, however, was different from the one observed in "fresh" cultures; the difference between normal and inoculated tubes decreased, or was reversed. Evidently the disintegration of cells caused a rise of intracellular enzymes liberated into the supernatant, which persisted in the presence of virus because the autolysis may have been inhibited (30). The decrease observed in normal controls under these conditions is due to degeneration of the cells (7, 8), in

agreement with others (31). Similarly the effect of long storage at 37°C. resulted in a great or complete loss of phosphatase activities of normal and

TABLE IV

Effect of Storage after 14 Days' Incubation at 37°C.

Explant and medium	Storage at 4°C. in days	Acid phosphatase as increase in IP μg./ml. of system	Alkaline phosphatase as increase in IP μg./ml. of system
1. Original whole explant			
Normal TC in medium 597, 10 tubes pooled	7	0.05	0.33
Same infected with Mahoney strain, titre $10^{-6.5}$	7	0.43	1.26
2. Supernatant of TC fluids			
Normal TC in medium 597, pool of 20 tubes	60	0.40	0.56
Same infected with Saukett strain, titre 10^{-7}	60	0.86	1.16

infected cultures, after 2 to 4 weeks at this temperature. These findings emphasize the protective influence of lower temperatures (32, 28) and may be of practical significance.

The Effect of Freezing or Boiling

Table V illustrates the effect of some physical factors on the enzymes. Deepfreezing at −25°C. affected the phosphatase activities of normal TC or TC fluids only slightly, causing some increase as a rule. On the other hand TC infected with poliomyelitis viruses were very sensitive in this respect and they generally showed a marked reduction of enzyme activities. Acid phosphatase suffered greater loss than alkaline phosphatase (Table V). Similarly, when whole TC or supernatant fluids were heated for 10 minutes at 100°C. before incubation with substrate, the following observations were made. In the normal TC the acid phosphatase activity was depressed 63 per cent, as

TABLE V

Effect of Low Temperature

Explant and nutrient	Age of TC	Acid phosphatase as increase in IP, μg./ml. of system		Decrease in activity	Alkaline phosphatase as increase in IP, μg./ml. of system		Decrease in activity
		Before freezing	After freezing		Before freezing	After freezing	
	days			*per cent*			*per cent*
Normal whole TC, in medium 597, original tubes	14	0.80	0.69	14	4.69	3.56	25
Infected, 6 days with Saukett strain, titre $10^{-6.8}$	14	0.33	0.13	70	3.66	1.89	46

tested upon 2 hours' incubation at 37°C. In TC inoculated with Saukett strain the activity was reduced by 55 per cent in fresh and only by 43 per cent in 50 day old tubes, stored at 4°C. Explants infected with Mahoney strain in this series of assays were similar to normal explants, with respect to acid phosphatase. Normal supernatant fluid exhibited 75 per cent decline in alkaline phosphatase activity and in TC infected with Mahoney strain 100 per cent reduction occurred. All these experiments were made with moderate titres.

Effect of Time and Temperature of Incubation

The results presented were for short incubation assays. The optimal time factor was from ½ to 4 hours, both in normal and virus-containing TC; on the average, 2 hours gave excellent results. Infected whole TC showed increasing activities only after ½ hour at 37°C., the 30 value being lower than that observed in cell-free supernatant. Phosphatase of infected TC fluids reached peak activity in 2 hours, and of whole explants of the same lot in 3 to 4 hours. The decrease in rate of IP formation after 4 hours was found

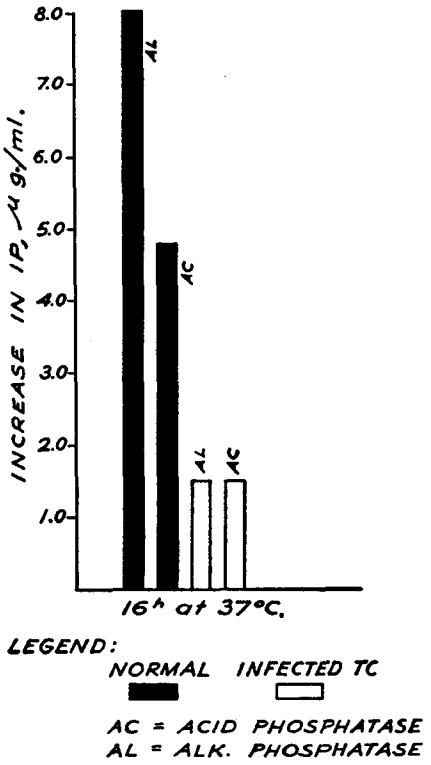

FIG. 4. Effect of long incubation on phosphomonoesterase in TC

with both enzymes (at pH 5 and 10.9 respectively) and may be due to the inactivation of the phosphatase as effect of pH (33). Assays of long term incubation also gave comparable results but the rate of alkaline phosphatase activity was markedly reduced in normal 14 day old cultures. Fig. 4 presents some typical findings.

Temperature of incubation was tested from 22 to 56°C. As one would expect, differences were found in optimal temperature of phosphatases in infected and uninfected TC. The highest activity for acid phosphatase during 1 hour incubation was at 30°C. in normal, and about 56°C. in infected explants. Optimal for alkaline phosphatase was 37°C. in uninfected cells and about 30°C. in the presence of virus. An increase in temperature from 37 to 45°C. showed about 10 per cent increase in normal explants. The infected tissues displayed significant decrease in alkaline phosphatase activity with increase of temperature over 30°C.; in other words there was a 76 per cent decrease at 37°C., about the same value at 45°C. and a further decline (83 per cent) at 56°C. The decrease in the normal TC at this elevated temperature was only 28 per cent. The divergencies between normal and infected TC are striking and may be of a different nature for acid and alkaline monoesterase.

Effect of pH, Buffers, Media, and Substrates

In a series of experiments different pH optima were found for alkaline phosphatase in infected and uninfected whole TC, during 1 hour incubation in veronal-acetate and in borate-borax buffers. Optimum activity for normal TC was at pH 9 and pH 10.9 for infected TC. At both pH's the normal explant exhibited higher values. Acid phosphatase displayed more activity at pH 3 in both normal and infected TC, the former being significantly greater. Veronal-acetate buffer at pH 5 was more suitable for testing the enzyme activity than acetate buffer. Na-β-glycerophosphate (16), Na-diphenylphosphate (18, 34), ribonucleotides (15), and dinitrophenylphosphate (35) were hydrolyzed at about the same rate under identical conditions. The various substrates may penetrate the cells or may be hydrolyzed on the cell surface, but extracellular splitting was also assumed (8). The specific effect of the synthetic nutrient media on these enzymes was thoroughly investigated (7, 8, 23).

Various Types of Poliomyelitis Virus

Special studies are needed to decide the differences with regard to the effect on enzymes of the various immunological types of poliomyelitis virus. However, many of our observations suggest that using about the same cell population, differences were in fact found in the enzyme activities. Typical examples are given in Table VI. It seems that these variations are connected with the various degrees of CPE and the titres characteristic for each strain.

TABLE VI

Effect of Various Types of Poliomyelitis Virus

Explant medium Type of virus	Age of TC	Infection	Titre* and dilution	Acid phosphatase μg./ml. ½ hr.	Alkaline phosphatase μg./ml. at 37°C.
	days	*days*			
Pool of TC fluids, medium 597, infected with Mahoney strain	14	7	T-10^{-7}; dilution $10^{-7.5}$	0.06	0.24
Same, infected with MEF1 strain	14	7	" "	0.06	0
Same, infected with Saukett strain	14	7	" "	0.20	0.03
Pool of TC fluids, infected with Mahoney strain	44 (30 days at 4°C.)	36	T-10^{-7}; dilution $10^{-7.5}$	0.56	0.45
Same, infected with MEF1 strain	" "	36	" "	0.11	0.26
Same, infected with Saukett strain	" "	36	" "	0.16	0.26

* Titration of virus on the 7th day.

Control of Tissue Mass

Changes in optical density and turbidity of TC have been reported (9) and still are the subject of special studies (15). A few examples are given to illustrate the decrease of tissue mass during poliomyelitis infection (Table VII). The depression of phosphatase activity is more marked than that expected from the reduction of turbidity values. In assays when normal and infected homogenates were brought to "isoturbidity" by dilution with medium, the difference in phosphatase activities remained unchanged. This fact indicates that the

TABLE VII

Turbidity Measurements of TC Homogenates

Explant and medium	Age of TC	Infection	Turbidity readings
	days	*days*	*Klett units/ml.*
Normal TC homogenates, pool of 20 tubes, medium 597	14	—	650
Same, infected with Mahoney strain, titre $10^{-7.3}$ dilution $10^{-7.5}$	14	6	535
Normal TC homogenates, pool of 20 tubes, medium 597	9	—	600
Same, infected with Mahoney strain, titre $10^{-7.0}$ dilution $10^{-8.0}$	9	1	535

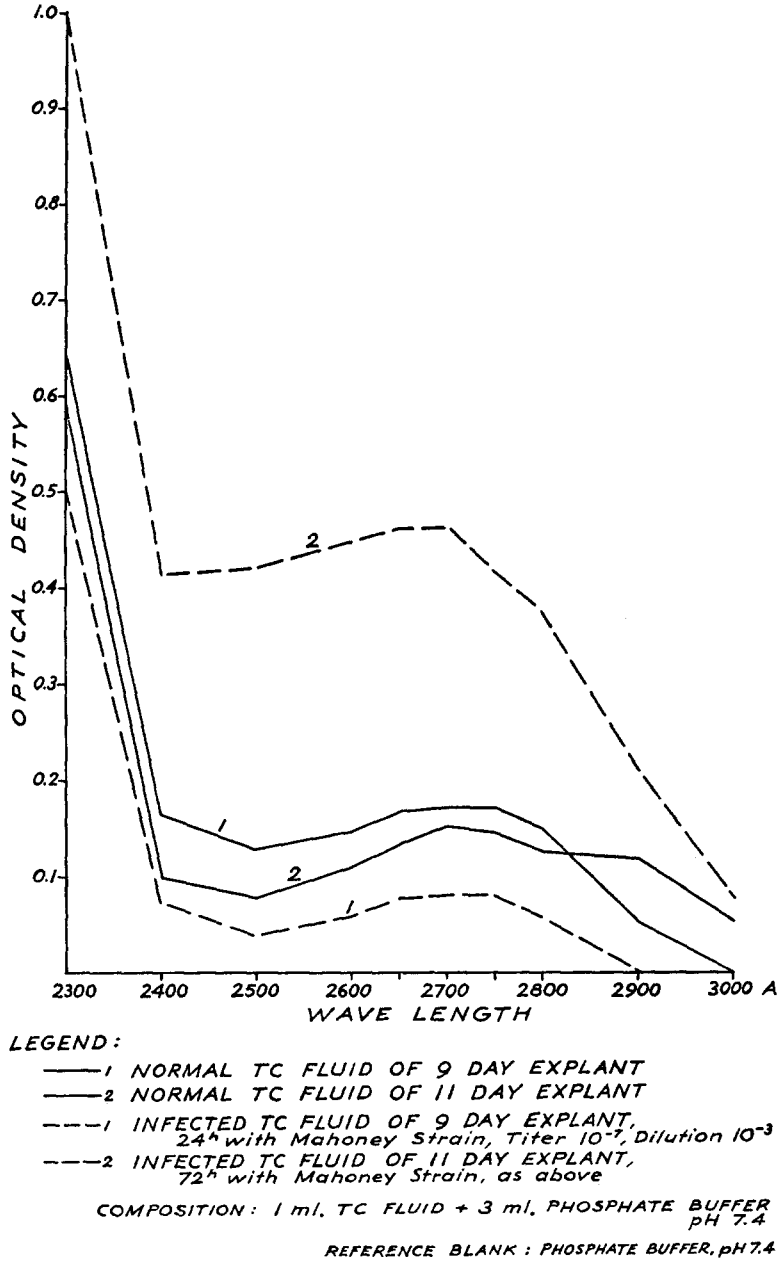

FIG. 5. Ultraviolet light absorption curves in normal and virus-infected TC fluids

cause is not simply the decrease of tissue mass, but the inhibition of the biocatalysts. The ultraviolet light absorption as checked by spectrophotometry on daily portions of TC fluids of trypsinized kidney cells, growing in large bottles (15) shows interesting qualitative and quantitative differences between normal and virus-infected explants (Fig. 5). After 1 day incubation with a large seed of virus (Mahoney strain $10^{-7.0}$, dilution 10^{-3}) there is marked decrease in the extinction of the infected TC, when the visible CPE is still doubtful. Qualitatively there is much similarity between the two spectra. The 2nd day there is no essential difference between the optical density of the infected and normal fluids, the former giving slightly higher readings (not illustrated). At this time the CPE is positive. The true nature of the reversion of the changes is explained by the 3rd day's findings, when the infected fluid shows a sudden rise in extinction values (about twofold of normal), due to the disintegration and entrance of cellular material into the TC fluid. Some quantitative estimate for protein and nucleic acids can be made from the absorption curves using the Warburg-Christian formula described by Kalckar (20). Similarly the estimation of mass of homogenates allows some quantitation, using purified albumin, or fresh kidney homogenates for standard, as has been recommended (7, 8). The absorption curves of TC fluids illustrate the minute amount of substances involved (9), of which only a part is enzyme protein.

DISCUSSION

The detailed illustrations presented confirm our preliminary report on the profound alteration of phosphomonoesterase activities during poliomyelitis infection *in vitro* (9). When the first observations were made it was felt that the uniform decrease of enzyme activities at the height of poliomyelitis infection was characteristic. Although this was verified as a general rule, it was found that these changes are of a more complex nature; that is, the acid phosphatase activity went through an early maximum before a complete drop. The time coincides with the exponential multiplication of the virus, as shown on growth curves in explants of monkey testis by Scherer *et al.* (36). Several possible explanations lend themselves to the interpretation of the results.

Firstly, the virus may be adsorbed to the cells at the sites of acid and alkaline phosphatase activity (37) causing first inhibition and later destruction, or release of the enzymes into the TC fluid. The difference in localization, chemical structure, and susceptibility to virus would be the cause of the different behaviour of the two enzymes.

The second possibility would be the inhibition of enzyme synthesis as observed with bacteriophage (38); thus the difference between normal and infected TC would come from the difference in synthetic activity, checked by the adsorbed particles or some soluble toxic principle (48, 15). At the time of inoculation the enzyme activities

of fluid-changed TC are lowered (8) but increase greatly in normal and only slightly, or decrease, in infected TC. With the multiplication of virus progressive deterioration or drop in activity occurs.

A third possibility is the early destruction of the more susceptible cells invaded, with release of virus and of the acid phosphatase into the TC fluid. This phase corresponds to the first cycle of virus reproduction (36). Change in spatial relationships causing cessation of the inhibition by virus and an inbalance in the activator-inhibitor enzyme complex, abnormal metabolites and other yet unknown factors may be responsible for the enhanced activity of alkaline phosphatase following the end stage (15). The liberation of intracellular enzymes, especially nuclear, also has to be considered. There is little evidence of cell multiplication in the presence of large inocula; therefore the initial increase of enzyme activity is hardly explained by growth (39). The deterioration of acid phosphatase in normal TC (8) and inhibition by synthetic media (23) adds to the significance of this increase.

The lower sensitivity of this biocatalyst towards inhibition by the inoculum suggests that its role in cellular metabolism (40) and its synthetic activity (41–43) may go on for some time. Thus acid phosphatase may participate in virus synthesis. Later with increasing virus titres and positive CPE only a fraction of it can be detected in the supernatant or in whole cultures. The complete disappearance of alkaline phosphatase activity from the supernatant at a certain time is striking; it may be explained by strong inhibition of enzyme synthesis and activity in a phase in which perhaps all available building stones are required for virus synthesis. When the cells disintegrated the same level was attained as in the controls, due to the liberation of the enzyme molecules from the virus. The drop to zero level before the terminal rise of activity in whole TC indicates that all cellular functions cease at this point and disruption of the cells follows with subsequent return of enzyme activities in the supernatant. This is an important point, meaning that the physiological role of alkaline phosphatase was checked *in situ* from the beginning of infection. Its part played in mitosis, growth and synthetic processes (3, 40, 42, 44) may explain the cessation of multiplication and replacement of enzyme losses, when inhibited (7, 8). The findings of Bauer on the decrease of magnesium in nerve tissue of virus infected animals (10), if verified in TC, would be a further explanation, since alkaline phosphatase is activated by this ion (24, 25, 15).

Most authorities agree that the great majority of viruses do not contain enzyme systems (10). For instance in rather extensive studies no evidence was found that phosphomonoesterases are part of the Rous sarcoma virus (45). On the other hand through the work of the Duke University group at least one virus seems to be identified, as an enzyme, adenosinetriphosphatase (46, 47). Our studies point to the virus as an inhibitor of essential enzyme systems (9) of the host cells. The findings may explain the mode of action of poliomyelitis viruses in the destruction of the host cell. There may be various possible mechanisms. Firstly, the virus would act as a particle of molecular size, affecting elementary cell constituents. Secondly, a toxic substance may develop during the host-virus interaction, which acts as a general inhibitor (48, 15). The differentially inhibited enzyme systems lead to a complete disorganization of cell metabolism, some metabolic pathways being hindered or obstructed (49);

this is followed by accumulation of metabolites, virus particles, and "toxic substances" during the disfunction of biological processes. Death and ultimately disruption of the infected cells are the terminal stage.

The difficulty in this type of study lies in the fact that it is impossible to set a standard value for the normal control. The individual variations were checked by the use of large pools of the same batch, but the criteria of normality have to be carefully weighed. The following observations helped to assess the physiological state of the explants: (*a*) absence of, or very small inorganic phosphate increase, when the TC was incubated with buffer alone, (*b*) considerably lower enzyme activities in the supernatant of TC fluid compared to the whole explant (7, 8), (*c*) degeneration, in agreement with Fell and Robinson (31) causes a rapid irreversible loss in enzyme. In contrast with this, growth results in a linear increase of enzymes (8). Good growth *in vitro* is a function of various factors (28). Although the synthetic media used are very suitable for enzyme work because they do not contain enzymes (7–9) the growth of tissues is not so great with the majority of these mixtures (11, 50). Suspended kidney fragments with complete and incomplete media, and human amniotic cells grown in complex natural nutrients are under study, to correlate the findings on different materials (15). The obvious advantage of the human amniotic cells is the absence of orphan viruses. The difficulties are in the high enzyme content of embryonic extracts (51, 8) and of other natural nutrients used. The effect of cultivation was thought to alter the physiological state of the explant, which change renders the cell susceptible to virus infection (7, 8). The presence of virus is the cause of further progressive alterations, modifying radically the cell physiology and rendering the cells very vulnerable to external and internal factors, such as heat, cold, more complete disruption by grinding, changes in optima for kinetics.

Both phases, the early decrease and the late increase of enzyme activity, are characteristic, and could be used as a quantitative measure of the CPE of virus, compared with uninfected normal controls. The inhibition may be the primary change and precedes the visible CPE for days, depending on the size of inoculum. Any phosphatase method (16, 18, 34, 35) could be adopted for the purpose of measuring enzyme activities in roller tubes or large production bottles (15).

Through the present work an interesting approach to the problem of unapparent infection is suggested. We might consider this as a localized virus attack, by which, by some as yet unknown mechanism, only one part of the cell population becomes infected, and only a few cells injured or even killed. The virus is arrested or consumed after inoculation, but modifies the biochemical and physiological aspect of the explant, without appreciable morphological changes. This process is somewhat similar to that described by Morgan as latent

infection in TC inoculated with psittacosis virus (52). The effect of medium 199 in his experiments may be interpreted by our previous work with this nutrient (8, 23).

As far as we know no phosphatase assays were made in connection with poliomyelitis virus grown in TC; mostly organ homogenates were investigated (10). It is interesting to see the effect of ectromelia infection *in vivo* (53) on phosphomonoesterases of the liver. It was claimed that the appearance of alkaline phosphatase in the cytoplasm was secondary to the failure of glycogen synthesis. One cannot directly compare those results with ours, but it is of interest to note the agreement of findings on acid phosphatase in TC of kidney with the results on liver cells in entirely different experimental conditions.

The decrease of tissue mass in infected explants is in agreement with our previous observations (9) and with the findings of Soraunder (54) on rabies-infected neurons *in vivo*. This may mean increased nucleoprotein catabolism or a decreased synthesis of protein compounds. The observations on infection with phage (38, 55) also point to this latter possibility.

No special effort was made to separate the various immunological types of poliomyelitis virus. However, it seemed that Mahoney, MEF1, and Saukett strains behaved differently with respect to enzyme changes. The dissimilarities observed may be due to their biological differences (56, 57).

The influence of the infecting dose was illustrated repeatedly; it seems that the dilution of the inoculum and the time of occurrence of enzyme changes are the most directly connected. Namely, with high titres accelerated, with lower titres prolonged changes occur. This might be expected; when more particles are present and/or more toxin, there is a more prompt interference with the function of various enzyme systems. It is of interest that even after the sudden increase in activity following the manifestation of CPE the phosphatase activities are inversely proportional to the virus increase (15). Except for this, the size of enzyme alterations in whole TC seems to be the same in the long run with lower or higher dilutions, only the time of onset varies with the amount of inoculum. Further studies are needed to clarify many details, possibly with highly purified virus. Thus primary changes could be separated from the secondary, supposedly toxic effects. These latter may be additional factors aggravating the disturbances in cell physiology, as first demonstrated in connection with poliomyelitis with inactivated seed virus (48, 15). Previously in other diseases such as influenza (58), lymphogranuloma venereum (59), and Newcastle disease (60) the generation of some toxic principle was assumed or demonstrated.

CONCLUSIONS AND SUMMARY

Experimental evidence is presented for drastic changes in phosphomonoesterase activities of tissue cultures, brought about by infection with polio-

myelitis viruses. Acid phosphatase activity went through a maximum before decreasing almost to zero level. Alkaline phosphatase activity diminished progressively to zero, then with disruption of the cells attained normal levels. Various aspects of the kinetics were investigated and illustrated. The initial increase of acid phosphatase, in contrast with the alkaline, may mean that the reactions catalyzed by this enzyme continue during the early phase. This period is the time of intense virus production and therefore it was supposed that this enzyme may play some role in virus synthesis. It was assumed that the virus acts as a particle of molecular size and becomes associated with the enzyme complex physicochemically or chemically. This association ends with the disintegration of the host cells. During the cell-virus interaction a toxin may develop which is a strong and general enzyme inhibitor. Various enzyme systems differ in sensitivity toward these virus effects; for instance, acid phosphatase is irreversibly inhibited or may be destroyed. The visible CPE of virus is preceded by a drastic reduction of enzyme activities in whole TC and in its various fractions, which may suggest causal relationship in the mechanism of cell destruction. In arrested or latent infection these processes are operative, but on a smaller scale. The drop in activities cannot be explained by the reduction of tissue mass, which is the consequence, rather than the cause, of enzyme changes. Besides the theoretical significance of these observations the following practical points can be summarized:

1. Changes in phosphatase activities are most strikingly demonstrated in whole tissue cultures inoculated with poliomyelitis virus.
2. There is causal relationship among infection, enzyme changes, and transformation of cell physiology.
3. The biochemical approach provides a quantitative measure of the extent of cell damage, before visible CPE is detectible.
4. Unapparent and active infections with poliomyelitis virus could be differentiated from normal controls by this method.
5. By various manipulations (freezing, long incubation) the difference between normal and infected TC can be enhanced. Suitable technical methods were proposed for various types of investigations.

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