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# Evidence for two waves of induction of DNA enzymes in stimulated human lymphocytes

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## ABSTRACT

The stimulation of human lymphocytes with phytohaemoagglutinin induces the appearance or increase of several enzymes of DNA metabolism [Pedrini et al., Biochem. Biophys. Res. Comm., 47:1221(1972)]. With long times of stimulation, two phenomena are observed; an increase in the levels of DNA polymerase, of a DNase acting on single-stranded DNA, and of an endonuclease, occurring between the third and fourth day, in parallel with a wave of DNA synthesis; a second wave of increase of the same enzymes and of DNA ligase, occurring between the fifth and eight day when the DNA replication rate, as measured by thymidine-pulses, has decreased to values close to the background.

## INTRODUCTION

When human leukocytes are treated with a mitogenic agent like phytohaemoagglutinin (PHA), the transformation of the small lymphocytes into blast cells and the subsequent mitotic activity are paralleled by a drastic increase in the rate of DNA synthesis, as determined by thymidine pulses[1]; DNA polymerase levels rise in parallel with DNA synthesis rate[2, 3, 4, 5]; also DNA ligase increases by a factor of 100 or more but its rise is delayed with respect to DNA synthesis rate and to the DNA polymerase level[4]. The reasons for this delay are obscure and worth further inquiry. We have extended our study to other enzymes of DNA metabolism and to longer times of PHA stimulation, in order to better collate the levels of the different enzymes with the physiological variations occurring in the lymphocytes. These studies have led to the demonstration of a wave of increase of some enzymes of DNA metabolism, that occurs later than the peak in DNA synthesis rate, and, at least in some cases, at a time when DNA replication rate has

returned to levels close to the background.

## MATERIALS AND METHODS

Glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase were purchased from Böhringer Corp., Munich; pancreatic DNase (800 U/mg) from Miles Corp., Slough; micrococcal DNase (NFCP code) from Worthington Biochemical Corp., Freehold.

Unlabeled deoxynucleotide triphosphates were products of Böhringer Corp.; [$^3$H]-dTTP (15 Ci/mmol) and [$^3$H] methyl-thymidine (15.5 Ci/mmol) were purchased from the Radiochemical Center, Amersham; ATP from California Corp. for Biochemical Research, Los Angeles; dithiothreitol from Miles Corp.; highly polymerized salmon sperm DNA (grade A) from California Corp. for Biochemical Research; cellulose - [$^3$H]poly(dT).poly(dA) with an interrupted poly(dT) strand, for the ligase assay (25 cpm/pmol) and the similar, uninterrupted reagent for the "nickase" assay (154 cpm/pmol) were gifts of Drs. F. Campagnari and L. Clerici, Biology Division, Euratom, Ispra. [$\gamma$-$^{32}$P]-ATP ($10^5$ cpm/pmol) was prepared according to Glynn and Chappel[6]. [$^3$H]-DNA from Bacillus subtilis (23 cpm/pmol) was prepared by the procedure of Mazza et al.[7]; GF/C filters were purchased from Whatman Corp., Maidstone-Kent.

Native, 3'OH terminated DNA for DNA polymerase assays was prepared as follows: salmon sperm DNA, at a concentration of 300 $\mu$g/ml, was incubated in a 1,340 ml reaction mixture, containing 500 $\mu$g/ml bovine serum albumin, 5mM $MgCl_2$, 50 mM Tris-HCl buffer, pH 7.6, 5 ng/ml pancreatic DNase. The mixture was incubated at 37 °C for 70 min, sufficient to cause 14% of the $A_{260nm}$ to become acid soluble; the reaction was stopped by making the mixture 25 mM in EDTA and heating at 72 °C for 20 min. The DNA was extracted by addition of an equal volume of a 20:1 chloroform-isoamylol solution; the aqueous phase was concentrated by dialysis vs. a 40% solution of polyethylene glycol (Carbowax 6000, Union Carbide) in 20 mM NaCl, 1 mM EDTA, down to a volume of 300 ml; it was finally dialyzed vs. 10 mM NaCl, 0.1 mM EDTA;

the yield was of 230 mg.

5'OH terminated DNA, for kinase assays, was prepared as follows: native DNA, at a concentration of 300 $\mu$g/ml was incubated in a 20 ml mixture containing 50 mM glycine-NaOH buffer, pH 9.2, 15 mM $CaCl_2$, 40 units of micrococcal nuclease (as defined by Worthington Biochemical Corp.) for 80 min at 37 °C, enough to cause the acid solubilization of 30% of the $A_{260nm}$; the mixture was then made 50 mM in EDTA, and dialysed *vs.* 0.02 M NaCl.

Blood samples were obtained from young healthy male donors. To 100-300 ml of peripheral blood collected in Plasma-Vac containers (Lab. Don Baxter, Trieste), haeparin (sodium salt, Fluka Corp., Buchs, Switzerland) was added to a final concentration of 10 mg/ml. White cells were separated by spontaneous sedimentation for one hour at room temperature; the lymphocytes trapped in the sediment were recovered by twofold dilution with 1.5% Dextran 500 (Pharmacia Corp., Uppsala) followed by spontaneous sedimentation. For the PHA stimulation experiments, the total white cells were suspended at the concentration of $10^6$/ml in Eagle's medium supplemented with 16% of serum (autologous plasma plus calf serum). PHA standard solution (Difco) was added to the appropriate cultures at the final concentration of 1% (V/V). For DNA synthesis and morphological studies, separate 5 to 10 ml cultures were prepared for each point. For the enzymic assays, separate 30 to 40 ml cultures were prepared for each point, after the culture times the cells were washed with saline, centrifuged and stored at -20 °C.

Determination of DNA synthesis rate was, in the experiments reported in figures 1, 2A and 2B as described by Pedrini *et al.*[4]; in the experiments of figures 3 and 4 instead, 0.5 ml of culture, after 3 hours of incubation with [$^3$H]-thymidine were directly collected on HAWP 025 Millipore filters, washed with cold balanced saline solution, 5% TCA, 95% ethanol. After drying at room temperature, the radioactivity remaining on the filters was measured. Appropriate controls showed that the pattern of variation and the absolute values of DNA synthesis rate were the same with

the two types of technique.

For the analysis of cell morphology the cell preparations were fixed with 3:1 methanol-acetic acid solution and stained with orcein.

The cell extracts for the enzyme assays were obtained by hypotonic lysis in two ways: in the experiments of figures 1, 2A and 2B, as reported by Pedrini et al.[4], i.e., to 0.2 g of cells (wet weight) we added 0.6 ml of 50 mM sodium bicarbonate buffer pH 8.7, 5 mM EDTA, 5 mM DTT; the cells were homogenized by pipetting them for 5 min through a narrow

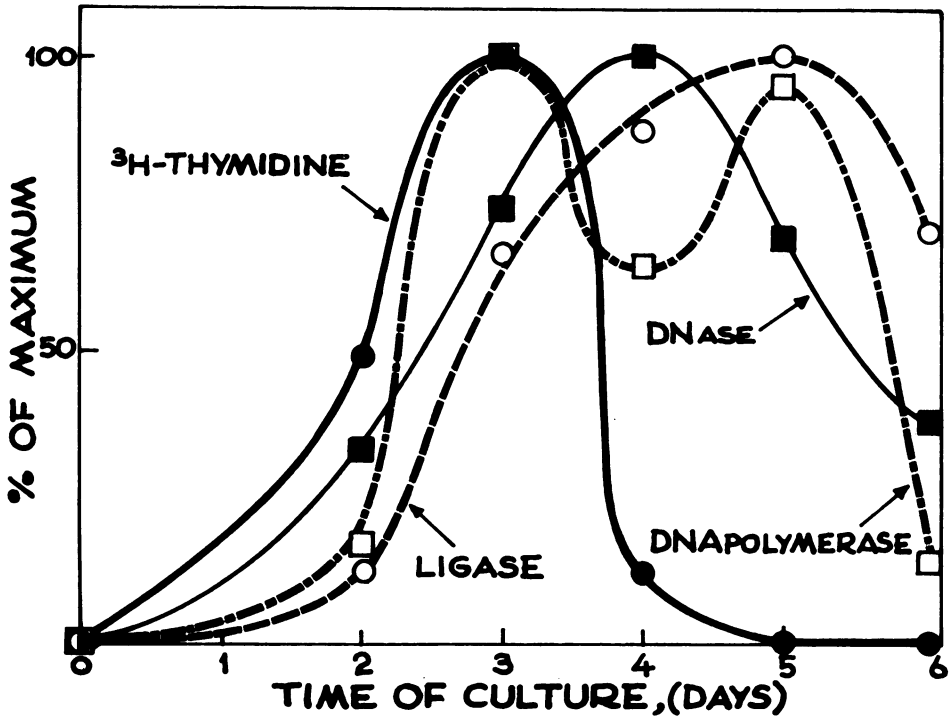



Fig. 1 - Variations in the specific activity of some DNA enzymes during PHA stimulation. Cultures of human leukocytes were treated with PHA for the indicated times; the cells were collected and assayed for the specific activity of the indicated enzymes, as described in the text. ●—● Incorporation of $^3$H -thymidine; ■—□ DNA polymerase; ■—■ DNase acting on single-stranded DNA; ●—○ DNA ligase. The data are expressed as percent of the maximum value. These values, as well as the zero time values are reported in Table I.

bore (0.5 mm diameter) 0.5 ml pipette. In the experiments of figures 3 and 4, a gentle homogenization was performed for 10 min in a tight - fitting Potter homogenizer with a teflon pestle; in this case, 1 M ammonium sulfate was then added to the suspension up to 0.3 M, and the suspension was left in ice for 50 min, with further 1 min homogenizations at 10 min intervals. In both cases, after homogenization, the suspension was spun at 17.000 x g for 10 min, and the supernatant was dialyzed overnight vs. 100 volumes of 50 mM potassium phosphate buffer, pH 7.5, 2.5 mM EDTA, 1 mM DTT.

DNA polymerase was assayed according to Gold and Helleiner[8]; in the experiments of figures 1, 2A and 2B, denatured calf thymus DNA was used as template; for the experiments of figures 3 and 4, activated salmon sperm DNA was used (see above for the activation procedure). In the experiment of figure 3 DNA polymerase was assayed also with denatured calf thymus DNA: the activity was 50% of that observed with activated salmon sperm DNA, but the shape of the curve was identical. The 0.125 ml reaction mixture contained 450 ug/ml of DNA, 50 uM each of the four deoxynucleotide triphosphates ([$^3$H] dTTP 160 cpm/pmol), 12 mM $MgCl_2$, 15 mM potassium phosphate buffer pH 7.2, 0.5 mM DTT and between 0.006 and 0.13 unit of enzyme (1 unit is the amount of enzyme incorporating 1 nmol of labeled nucleotide in 30 min); after 30 min at 37 °C, the incubation was stopped by addition of 1 ml of 2 M HCl and the acid insoluble radioactivity was collected on GF/C filters. Polynucleotide kinase was assayed as described by Richardson[9]; one unit is the amount of enzyme transferring 1 nmol of [$^{32}$P] to DNA, in 30 min. The ligase assay was as reported by Spadari et al.[10]; one unit is the amount of enzyme binding 1 nmol of [$^3$H]-poly(dT) (expressed as nucleotides) to cellulose in 30 min. DNase acting on single-stranded DNA was assayed as reported by Pedrini et al.[4]; one unit is the amount of enzyme transforming 1 nmol of DNA (expressed as nucleotides) into an acid soluble form in 30 min. "Nickase" activity, i.e. the formation of single strand endonucleolytic breaks on double-stranded DNA, was assayed on a substrate composed of a [$^3$H]

labeled poly(dT) covalently attached on the 5' end to cellulose and annealed to unlabeled poly(dA); an endonucleolytic break in the [$^3$H]-poly(dT) helix releases, in alkali, a labeled poly(dT) chain which may be easily removed from the cellulose by centrifugation and may be still large enough to be acid precipitable. One unit is the amount of enzyme detaching 1 nmol of acid-precipitable [$^3$H] -poly(dT), (expressed as nucleotides) from the cellulose in 30 min. The preparation of the substrate, the conditions of the assay and the properties of the main enzyme responsible for this activity will be published elsewhere.

RESULTS

Figures 1 to 4 report results obtained in different stimulation experiments with blood samples from 4 different subjects; figures 1 and 4 report the data of the stimulation of the lymphocytes from the same subject obtained at different times. Table I reports the absolute values of DNA synthesis rate and of the specific activities of the different enzymes, at the zero time and at their respective maxima for all the experiments. The factors of increase are quite variable, but a common general pattern can be discerned.

DNA synthesis rate - This was measured by thymidine pulses:from a zero time value ranging between 300 and 1,000 cpm/$10^6$ cells, it raises by a factor of at least forty fold (figure 3) and at most 200 fold (figures 2A and 4) that value;the time at which the maximum is reached varies between the 3rd day (figures 1, 2A and 2B) and the 5th day (figures 3 and 4). These two basic patterns have already been observed[4]. It is worth noticing that cells belonging to the same subject (figures 1 and 4) show the two different patterns at different times indicating that the type of response is not a property typical of a certain individual. What are the physiological factors determining the timing of the response is not clear. Unstimulated lymphocytes, as already shown, do not undergo this variation, nor those of DNA enzyme levels (see figure 4). In the experiments showing an "early" response, the rate of DNA synthesis goes down rapidly to the background level, which is reached at the fifth day (figures 1 and 2).

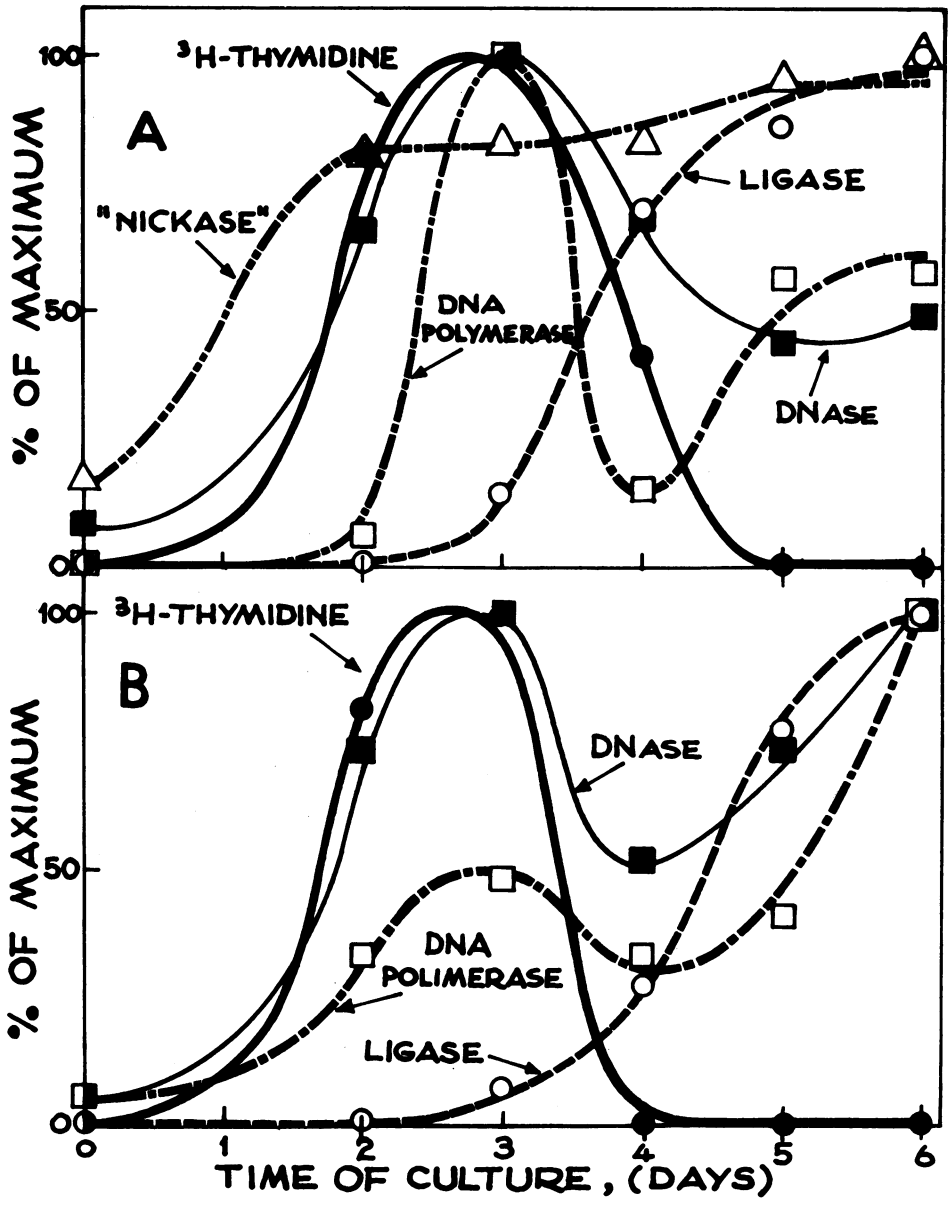
Table I

Values of the different parameters at zero time and at their maximum obtained in 5 different experiments

| Number of figure reporting the experiment | | [$^3$H]thymidine incorporation | DNA polymerase | Single stranded DNA DNase | DNA ligase | "Nickase" | DNA kinase |
|---|---|---|---|---|---|---|---|
| | | cpm/$10^6$ cells | U/mg | | | | |
| 1 | Zero time | 550 | < 0.005 | 0.14 | < 0.1 | - | - |
| | Maximum | 90,000 | 0.42 | 6.90 | 24.4 | - | - |
| 2A | Zero time | 370 | < 0.005 | 0.15 | < 0.1 | 3.2 | - |
| | Maximum | 120,000 | 0.15 | 2.0 | 1.5 | 19.1 | - |
| 2B | Zero time | 340 | 0.007 | 0.15 | < 0.02 | - | - |
| | Maximum | 11,000 | 0.11 | 2.1 | 0.78 | - | - |
| 3 | Zero time | 1,300 | 0.05 | 0.64 | < 0.5 | 1.7 | 2.8 |
| | Maximum | 46,000 | 3.6 | 4.7 | 20.4 | 10.5 | 5.0 |
| 4 | Zero time | 690 | < 0.05 | 8.7 | < 0.3 | - | - |
| | Maximum | 150,000 | 3.8 | 19.0 | 2.3 | - | - |

When the response is "late" the decrease is also relatively delayed and the background levels are not reached even after 10 days if at all (figures 3 and 4). The peak of DNA synthesis rate corresponds in all the experiments to the mitoses peak. Figure 5 shows a typical pattern of the morphological changes that occur in leukocyte cultures stimulated with PHA. This morphological analysis was performed on the same cell samples utilized for the enzymic assays reported in Fig. 4. Large blast cells appear during the first 24 hours of culture and their fraction increases up to 100%. In some experiments, at the latest times of culture, small lymphocytes and pyknotic cells are also present.

DNA polymerase - As already described, DNA polymerase levels raise in all cases, from values below the threshold of detectability, up to 100 fold higher levels, reaching a maximum at the same time as the DNA synthesis rate, whether this happens at the third or at the fifth day. The parallelism with the rate of DNA replication is quite good, in agreement with our previous observations, and those of other authors[2,3,4,5]. By prolonging the incubation times, DNA synthesis rate decreases univocally; the level of DNA polymerase instead, after an initial decrease, undergoes a second increase, which reaches a maximum that can be even higher than the previous one, and is two or more days delayed with respect to the first peak. This is true for all experiments, except the one reported in figure 3, where only one peak is observed. In all other cases, the second polymerase peak occurs at a time when DNA synthesis rate is returned to a value as low as the experimental background level in most cases (figures 1, 2A and 2B). In the experiment of Fig. 4, the thymidine incorporation rate has not yet reached the background level at the time of the second polymerase peak, but is definitely in the decreasing portion of the curve. The type of template used (denatured DNA for the experiments in figures 1, 2A and 2B, native activated DNA for the experiments reported in figures 3 and 4) does not affect the shape of the curve: in figure 3, the assays were performed also with denatured DNA; the specific activity obtained was reduced to 50%, but the pattern of the curve was identical to the one




Figs. 2A and 2B - Same as Fig. 1, with different donors. Also the "nickase" activity ( ) is reported in Fig. 2A. Other symbols as Fig. 1. For the absolute values at zero time and at maximum, see Table I.

reported in the figure.

Ligase - The delay in the rise of ligase, already reported, is confirmed in all the experiments, though more or less pronounced; when the peak in DNA synthesis rate is "early" (figures 1, 2A and 2B) the displacement of the ligase peak is quite pronounced. It is noteworthy to observe that the ligase curve corresponds rather well to the second wave of polymerase in all the three cases. The maximum is in fact attained for ligase at times when the DNA synthesis rate is returned to the background level; this is also the case for the second wave of DNA polymerase as shown above. When the peak in DNA synthesis rate is "late" (figures 3 and 4), the shift is less pronounced but the delay of ligase is still clearly observed: in figure 3, ligase raises from the background level at least one day after DNA synthesis rate and DNA polymerase, reaches the maximum value one day later than DNA polymerase and remains at the maximum level for one day after polymerase and DNA synthesis rate have begun to decrease. In figure 4, the pattern is more complex: the ligase curve shows two peaks, the first one corresponding to the DNA synthesis peak, the second and highest one, delayed by two days, occurring when DNA synthesis is decreasing. The DNA polymerase also follows a pattern similar to ligase, with a "shoulder" (probably corresponding to the second wave more clearly observed in figures 1, 2A and 2B) coincident with the second peak of ligase. In spite of the variability of patterns, ligase confirms in all cases its tendency to a delayed rise, and it shows a maximum at a late time, when DNA synthesis rate is down to a minimum or clearly in the decreasing phase.

Single stranded DNA DNase - The DNase assay is rather specific for single stranded DNA, since with native DNA the activity is reduced to less than 15%. The main activity so far described in mammalian cells specific for single stranded DNA was characterized by Lindahl in rabbit liver[11] and defined as DNase III, i.e. an exonuclease attacking DNA from 3'OH end and producing 5' mononucleotides; we have not characterized the activity we assay and therefore we cannot say that this corre-

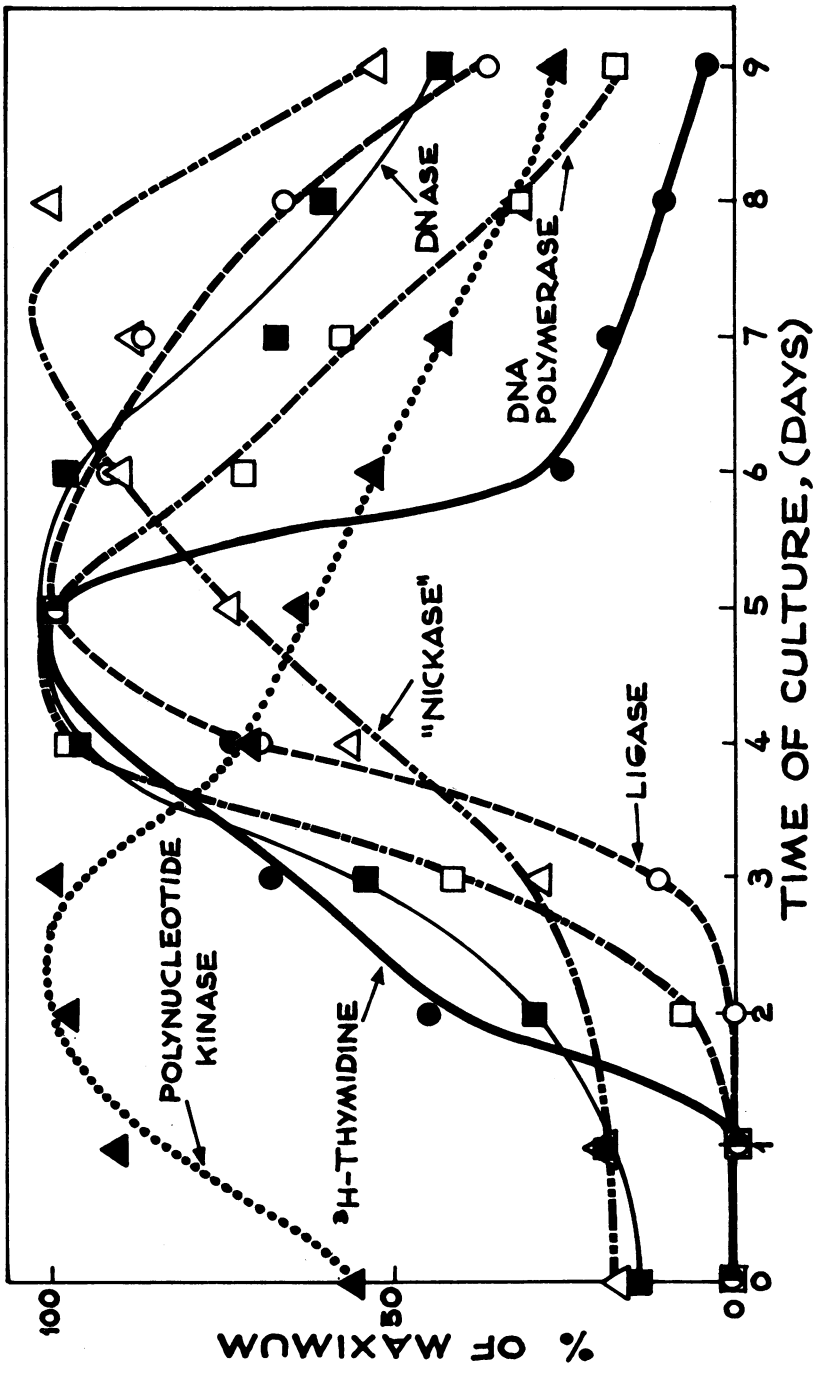



Fig. 3 - Same as Figs. 1 and 2, with different donor. Also the polynucleotide kinase activity (••▲•••▲••) is reported. Other symbols, as in the previous figures. For the absolute values at zero time and at maximum see Table I.

sponds to DNase III. It is quite likely in fact that our assay measures a variety of enzymes undergoing possibly different physiological variations during the PHA stimulation; the patterns observed are thus even more difficult to interpret than the other ones. The zero time level is relatively high and the overall increase is less pronounced than for the other enzymes. Nonetheless, the general pattern of variation of this enzymic activity is rather parallel to that of DNA polymerase; thus it is always at a peak (the only one for figures 1 and 3, a second one for the other cases) later than DNA synthesis rate. In the experiment of figure 4, the DNase level at zero time is particularly high; on the other hand, in the unstimulated control, it decreases down to less than 3 per cent of the original level, whereas it increases more than twofold that level in the stimulated cells. The most likely explanation is that the relatively high zero time level is due to the cells that lyse in culture in the following days; in fact, the rate of decrease of DNase in the controls is similar to the rate of lysis of granulocytes in the same experiment, as shown in figure 5. The real increase in DNase in the PHA responding cells is therefore much higher than one would guess from the simple observation of the curve of the stimulated lymphocytes.

Endonuclease -The "nickase" assay suffers from the same kind of uncertanties as the previous one, though to a lesser extent; it represents certainly a different enzyme(s) than the one just described, in view of its specificity towards native DNA; it is certainly not an exonuclease. We have assayed this activity only in two experiments (figures 2A and 3); the level is appreciable also at the zero time, but the increase is certainly impressive; also, the enzyme remains at high level and reaches a maximum at a time when DNA synthesis rate is down to the background level or decreasing.

Polynucleotide kinase - The variations in the level of this activity (that is rather specific for DNA, since DNase I treatment of the substrate reduces it to less than 10%), are the least important and most atypical. From an appreciable level at zero time (figure 3) it increases by a fac-

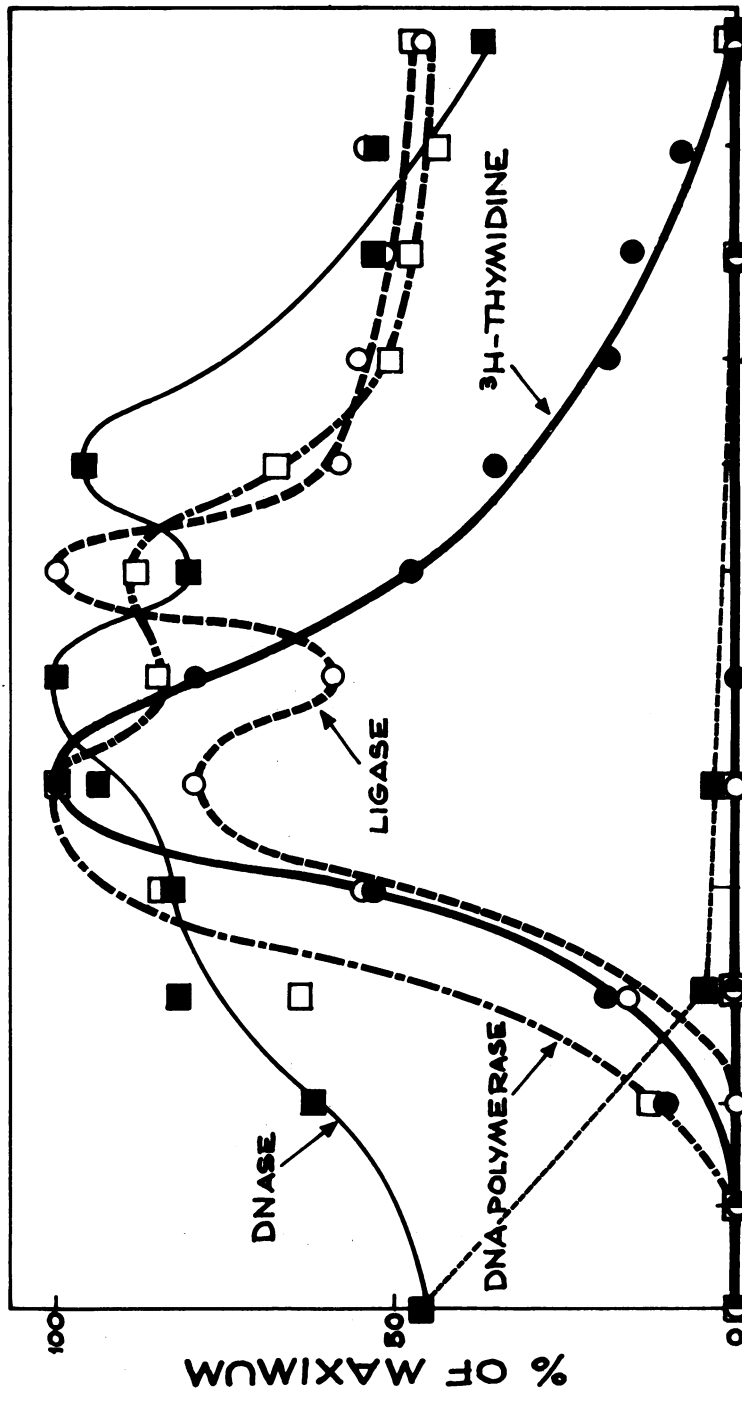



Fig. 4 - Same as Figs. 1 to 3, with the same donor as Fig. 1. For the absolute values at zero time and at maximum of the stimulated cells, see Table I. In the unstimulated controls, the values for $^{3}H$ thymidine decreased after the zero time below the 150 cpm/$10^{6}$ cells threshold; for DNA polymerase, the values were always below the 0.05 U/mg threshold; the values of DNase (--■---■--) after the zero time decreased below the 0.5 U/mg threshold; for ligase, they were all below the 0.3 U/mg threshold.

tor less than two at the third day (in an experiment in which DNA synthesis rate is "late" and has its peak at the 5th day) and then decreases continuously for all the subsequent time. The most interesting consideration concerning this enzyme is that it shows that the tendency to a drastic increase in specific activity during PHA stimulation is not a general property of all the enzymes acting on DNA; this indicates a contrario, that the increase of the other studied enzymes must reflect some sort of specificity and migh have a certain physiological significance.

DISCUSSION

The original aim of this work was to establish whether the changes in enzyme levels during the variations in DNA synthesis rate could give indications of the function of the enzymes under study in the DNA replication process. The results raise novel problems, but a general pattern is observed which allows some tentative conclusion.

DNA polymerase increases with a first wave which raises and begins to decrease with a good parallelism to DNA synthesis rate. Chang et al.[12] have reported that, of the two main polymerases isolated from human cells, the one with a larger molecular weight increases during the early days of stimulation; it seems then likely that this latter enzyme is directly involved in replicative DNA synthesis.

The meaning of the second wave of DNA polymerase is more obscure; it is a qualitatively reproducible phenomenon, in spite of the great variability of patterns, and it occurs when DNA synthesis rate is not showing any increase. That the late wave must have a definite physiological significance is demonstrated by its parallelism with ligase activity. There is no evidence so far whether this polymerase represents the same large M.W. enzyme raising in the first wave, or it is another molecular species.

The rise of ligase, as already shown in our previous work, is constantly delayed with respect to DNA synthesis rate, and corresponds to the second wave of DNA polymerase, when this is evident. A function of ligase in DNA replication is still very likely from all the data obtained

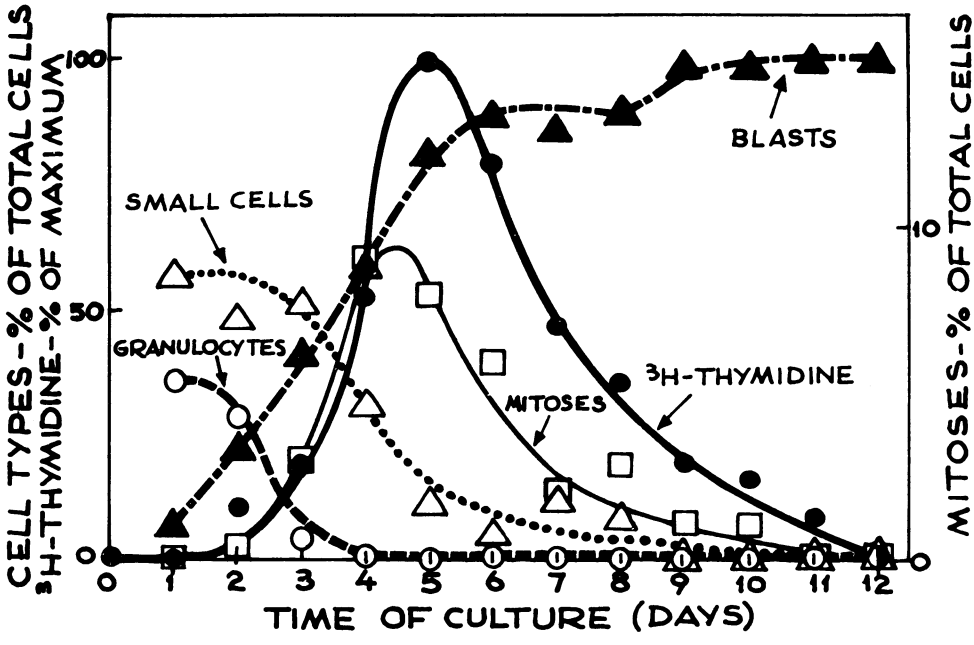



Fig. 5 - Morphological study of a PHA stimulated culture. Same stimulation experiment as Fig. 4. For the different types of cells, the data are expressed as percent of the total: -○- - -○- polymorphonuclear leukocytes; ••△••••△•• monocytes, small and intermediate size lymphocytes, pyknotic cells; -▲- - -▲- -large blast cells; -□——□- mitoses, reported on an enlarged ordinate. For comparison, also the rate of $^3$H - thymidine incorporation ( -●——● ) is reported, expressed as percent of the maximum value.

with microorganisms and from the fact that its rise is correlated also in these experiments to a DNA replication wave. There is no evidence for a multiplicity of ligases in human cells, since the two forms of the enzyme that can be demonstrated in such cells are most likely the product of a conversion of a larger dimeric form into a monomer[13]. So, if ligase is requested for some other function concerning DNA metabolism at later times of lymphocyte culture together with a particular DNA polymerase activity, it is not surprising to observe that ligase continues to raise, since no other isozyme is available to the cell for this hypotheti -

cal function.

The same argument can apply to the DNases: nucleolytic enzymes can have a function during DNA replication, e.g., they could introduce "nicks" that could work as swivels in between the different tandem - organized replication units of eukariotic chromosomes, or they could serve to remove possible tangles left by the priming of the Okazaki pieces. In fact, in bacteria, the maximum of activity of several DNases is reached at the time of maximum DNA replication rate[14]. The later peaks could reflect an involvement of the same or similar enzymes in the hypothetical late process.

The variations of the enzymes described above are probably specific: first of all, we observe changes in specific activity, which means that many other proteins are not increasing at the same rate or at all; furthermore, at least one enzyme of DNA metabolism, polynucleotide kinase, does not follow these variations and we must infer that it does not play its main role neither in the replication process, nor in the later unexplained one.

Finally, what is the nature of the late process, involving enzymes specific for handling DNA molecules in a non-destructive fashion? We can advance two simple hypotheses.

1) The late wave of enzymes is the preparation of a second wave of DNA replication that either

a) aborts for some reason in culture or

b) is not observable by thymidine pulses, because the thymidine metabolism could be changing at late incubation times (inactivation of thymidine kinase? activation of thymidine degrading enzymes? ) so that only <u>de novo</u> pathways of dTTP synthesis would be active at this time.

2) A process involving rearrangements of chromosome structure, either through breakage and reunion of analogous sequences or through degradation and repair of certain regions, is taking place at the late times of culture, without any net DNA replication.

If the second process is analogous to the first one (hypothesis 1 )

we would expect that the same type of enzyme be involved: so, the second wave of DNA polymerase should be due to the same molecular species as the first one. If the second wave (hypothesis 2) represents a form of repair - type process involving breakage and reunion of DNA molecules we would expect that the cells at this stage should show a higher amount of repair - type synthesis than usual. It would be tempting to correlate such a process to the specific function in immunoglobulin production of the blast cells at this stage.

We intend to pursue our work in these two directions:determine whether the same types of enzyme are involved in the two processes revealed by this study; measure the extent and ability to perform repair - type synthesis by lymphocytes at different stimulation stages.

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