Acid-Soluble Deoxynucleotides and DNA Synthesis in Growing
Yeast after X-Irradiation, II

Synthesis of Deoxynucleoside Tri- and Monophosphates in Synchronized and Asynchronously Growing Cells

Hans Eckstein, Sybille Ahnefeld, and Karin Albietz-Loges

Institut für Physiologische Chemie der Universität, Hamburg

The behaviour of acid-soluble DNA precursors in synchronized and asynchronously growing yeast after X-irradiation is investigated by labeling techniques with $^{32}P_i$ and by enzymic estimation. In prelabeled synchronized growing cells, radioactivity associated with deoxynucleoside triphosphates increases to maximum values shortly before each DNA replication, followed by a drastic decrease during S-phase. Radioactivity associated with monophosphates fluctuates, too, but with an opposite rhythm. These fluctuations apparently reflect quantitative changes of the DNA precursor pool during a cell cycle, as judged from the following findings: 1. Acid-soluble phosphorus is augmented stepwise. 2. "Specific" radioactivity from acid-soluble phosphorus compounds decreases steadily, indicating a continuous dilution of the labeled phosphorus pool with "cold" phosphorus. 3. Radioactivity associated with ribonucleotides fluctuates, too, but with a divergent rhythm.

In X-irradiated synchronice growing yeast, the fluctuations of the deoxynucleotide-associated ^{32}P are disturbed only little. Maximum values appear nearly at the same time as in the control, they decrease to minor values even if DNA augmentation is delayed. This decrease is less drastic, however, than that during DNA replication in unirradiated yeast, yielding a slightly increased average label per generation time. At the same sime a rapid augmentation of monophosphate label is observed.

A pronounced increase of deoxynucleotide-^{32}P is seen with X-irradiated asynchronously growing yeast, pointing to distinct radiosensitivities of the DNA/DNA precursor system in different cell stages. Neither ^{32}P-fluctuations nor ^{32}P-accumulation during DNA delay can be explained by corresponding observations with acid-soluble phosphorus or with ribonucleotide pools. Studies on ^{32}P-incorporation also exclude radiation effects on cellular phosphorus uptake. Enzymic estimations of the deoxynucleoside triphosphate pools from asynchronously growing yeast rather exhibit a considerable increase of these substances during the radiation-induced delay of DNA augmentation. This accumulation of DNA precursors probably is caused by undisturbed synthesis, but reduced incorporation into DNA. The possible role of DNA repair in this system is discussed.

Introduction

Irradiation of synchronized yeast with 50 kr of X-rays destroys the proliferation ability of the growing cells and induces some "uncoupling" between DNA polymerase activity in cell extracts and DNA increase in the growing cells[1], the mechanism of which is not yet understood. Using the diphenylamine reaction it was shown, that the radiation-induced delay of DNA augmentation is caused probably not by deficiency of the yeast cells with DNA precursors, since augmentation of acid-soluble purine deoxyribose derivatives is continued in a synchronized rhythm, leading to an excessive accumulation of these compounds in the X-irradiated growing cells[2]. On the other hand, X-irradiation of bacteria apparently results in an accumulation of deoxynucleosides and deoxynucleoside monophosphates[3-6]. The well-known fundamental investigations on regulation of deoxynucleotide biosynthesis[7-13] postulate that, if DNA replication is delayed, synthesis of excess deoxynucleoside triphosphates should be prevented by feedback, or might occur only, if total cell mass increases — as is the case with X-irradiated yeast. Indeed, synchronized mouse embryo cells X-irradiated in G_1 show augmentation of dATP and dTTP retarded, and increase of ribonucleotide reductase activity in cell extracts im-

paired [14]. Thus, further investigations seemed necessary to elucidate this problem. In the present paper, the behaviour of single deoxynucleoside tri- and monophosphates in synchronized and asynchronously growing yeast after X-irradiation is studied, by means of radioactive labeling, and by enzymic estimation of the deoxynucleotide pools. Parts of the results were reported in a preliminary form [15, 16].

Experimental

Synchronized yeast was prepared from growing baker's yeast (*Saccharomyces cerevisiae*, "Germania-Hefe", Deutsche Hefewerke, Hamburg) by temperature shifts, as described earlier [17]. Asynchronous cultures were grown from a cell clone originally derived from baker's yeast [18]. Growth conditions were as previously [17], except that growth medium was only 0.01 M in phosphate. Irradiation was performed with a RT 200 X-ray machine (Röntgen-Mueller, Hamburg) at 1750 r/min (20 mA, 200 kV, filter 2 mm Al) as described earlier [1].

Radioactive labeling

Three series of experiments were performed:

Uniformly labeled exponentially growing cells were obtained by growing an appropriate cell number overnight (15 to 16 hours, corresponding to 9 to 10 generation times) in 1 l of growth medium containing 2.0 mCi $Na_2H^{32}PO_4$ (100 mCi/mmol, The Radioactive Centre, Amersham, England). At about 20×10^6 cells per ml the yeast was harvested by centrifugation (10 min $15\,000 \times g$, 2 °C), washed with 250 ml of icecold water, and resuspended with water to 1×10^8 cells/ml. 100 ml of the suspension were taken for X-irradiation.

In a second series of experiments, cells grown overnight without ^{32}P were harvested and washed as above, X-irradiated, and incubated with $Na_2H^{32}PO_4$ (1 mCi/l growth medium).

The third series of experiments concerning synchronized growing yeast proved difficult because no simple and rapid method is available for large-scale synchronization of exponentially growing cultures. Radioactive labeling therefore could be performed only by feeding baker's yeast with ^{32}P during the synchronizing treatment, with 2.5 mCi $Na_2H^{32}PO_4/$ 500 ml medium (20×10^6 cells/ml, 0.01 M phosphate). The synchronized labeled cells were washed twice with 350 ml of icecold water, and were finally resuspended in 100 ml water. 50 ml of this suspension were taken for X-irradiation. Identical results were obtained whether the labeled cells were washed with water, or with 10^{-3} M phosphate buffer pH 6.5.

Radioactivity was measured in a Packard TriCarb liquid scintillation spectrometer with an efficiency of 80%. Considering the radioactive decay, the data were calculated as cpm/200 ml culture (10^9 cells at "zero time" of each experiment). "Specific" radioactivity was calculated as cpm/nmol phosphate, and cpm/nmol nucleotide, resp.

Cell extraction

Yeast from samples containing $0.5 \times 10^9 - 2.0 \times 10^9$ cells was harvested by centrifugation (10 min $10\,000 \times g$, 2 °C), and washed with 10 ml of icecold water. Acid-soluble cell constituents were extracted with 3.0 ml 5% TCA 30 min at 25 °C. The acid-insoluble material was washed twice with 2.0 ml of icecold 5% TCA, twice with 5.0 ml of ethanol, and taken for DNA estimation. The TCA supernatants were pooled yielding the "extract".

Separation of acid-soluble deoxynucleotides

The extract was applied to a Sephadex G-25 column (12×550 mm), and the nucleotides were separated from TCA and other contaminants by elution with water at 2 °C, at a flow rate of 3 drops per min. Separation was controlled by recording 254 nm absorbance and pH of the effluent. The UV absorbing fractions exhibiting pH values above 2.5 were pooled, and evaporated to dryness in a rotary vacuum evaporator at $30 - 33$ °C. The residue was redissolved with 0.5 ml of icecold water yielding the "concentrate".

In several experiments TCA was removed from extracts with ether, and the aqueous phase was then evaporated immediately. Results obtained in this way did not differ significantly from those gained after gel filtration.

Acid-soluble deoxynucleotides were separated from the concentrates by two-dimensional thin-layer chromatography according to Randerath [19–21]: Commercial thin-layer plates (0.1 mm polyethylene-imine impregnated cellulose, pre-coated plastic sheets 20×20 cm, Polygram CEL 300 PEI, Macherey u. Nagel, Düren, Germany) were washed with water and dried under a warm air draft. 10 to 50 μl concentrate and 5 μl of a carrier nucleotide solution containing 5 nmol each of the four ribo- and deoxyribonucleoside mono- and triphosphates were applied to one plate. The carriers were omitted, when nucleotide phosphorus had to be determined. Triphosphates were isolated by running in the first dimension with 1 M LiCl in 2 M HCOOH at pH 2.0, and in the second dimension with 0.37 M H_3BO_3 in 1.715 M LiCl. Monophosphates were separated in the first dimension with 1 M LiCl, and with 0.215 M

H_3BO_3 in 0.67 M LiCl in the second one. The flow distance was 15 cm in both directions. After the chromatograms were developed in the first dimension they were dried, and washed with methanol.

The nucleotides were identified under a UV lamp (260 nm) by their R_F values. For further analysis including UV absorption, phosphorus content and ^{32}P-radioactivity, the nucleotide spots were moistened with 5 or 10 μl water, scraped off with a sharp spatula, and handled in the appropriate way. When standard nucleotides were supplied to extracts, at least 95% of the additional phosphorus were recovered together with the original compound, even after two-dimensional thin-layer chromatography. Likewise, radioactivity from ^3H- or ^{14}C-labeled nucleotides was found to more than 90% at the respective nucleotide spot.

Phosphorus determination

Phosphorus was determined from extracts, and from nucleotide spots on the chromatograms, resp., according to [22, 23], modified as follows: 0.02 ml of the acid cell extract, or one scraped-off nucleotide spot, resp., were heated at 280 °C with 4 drops of concentrated H_2SO_4 for 5 min. The samples were cooled, 2 drops of 60% $HClO_4$ were added, and the material was combusted 15 min at 280 °C. The samples were diluted with 1.2 ml water, 0.4 ml of a solution of 2.5% ammoniummolybdate in 0.1 M sodium acetate, and 0.4 ml of a 10% ascorbic acid solution were added. After 3 hours at 37 °C, OD was measured at 820 nm with 2 cm cells. The data were calculated as nmoles phosphate from a KH_2PO_4 standard. Nucleotide phosphorus was calculated as nmoles nucleotide from a corresponding nucleotide standard. Phosphorus determinations from standard KH_2PO_4 and from equivalent amounts of nucleotide standards yielded comparable results.

Estimation of DNA

DNA at first was estimated by the diphenylamine reaction essentially as described earlier [1, 24]; OD was read at 600 nm. In the later experiments, DNA was estimated according to Burton [25], as described elsewhere [26]. Results from both methods did not differ significantly.

Enzymatic estimation of deoxynucleoside triphosphates

Differing from the procedure described above, "concentrates" were prepared by resolving the residues from ether-extracted, vacuum-evaporated cell extracts with 0.6 ml 0.1 M tris-HCl-buffer containing 0.01 M $MgCl_2$ at pH 7.6. Deoxynucleoside triphosphates were estimated from these "concentrates" by the DNA polymerase reaction according to [27]. The standard assay for dATP contained, in a final volume of 0.30 ml, and at a final pH of 7.4 – 7.5: 5 nmol dTTP supplemented with 500 nCi $[^3H]$dTTP ($[^3H$-methyl]-thymidine-5'-triphosphate, ammonium salt, Amersham, England), 2×10^{-3} OD units poly-d(A-T) (more than 0.3 nmol P, Na-salt, Boehringer, Mannheim, Germany), 500 nmol mercaptoethanol, 2.5 μmol $MgCl_2$, 28 μmol tris(hydroxymethyl)aminomethan (as a buffer solution, pH 7.6), and 5 to 50 μl "concentrate". Control assays were performed with "concentrates" gained from extracts, which were supplemented with 5 nmol dATP either immediately at cell extraction, or after evaporation to dryness, resp. They served for estimation of deoxynucleotide loss during the preparation steps. The reaction was started by addition of 2.5 units of enzyme (DNA polymerase I from *E. coli* MRE 600, EC 2.7.7.7, purified to step VII according to Jovin *et al.* [28], Boehringer, Mannheim), and by incubating the sample at 37 °C. After 0, 5, 10, 20, 30, and 45 min of incubation, 50 μl each of the assay were placed on a filter paper (No. 589, Schleicher u. Schuell, Germany, 2×3 cm), which immediately was immersed into icecold 10% TCA. The filter papers were washed, twice with icecold 5% TCA, twice with ethanol, and finally with ether, dried under a warm air draft, and counted in a Packard liquid scintillation spectrometer. cpm were calculated as nmoles dATP from standard assays containing 0 – 200 pmol dATP instead of "concentrate".

dTTP was estimated by an analogous procedure, with $[^3H]$dATP (500 nCi, 5.0 nmol, $[8-^3H]$deoxyadenosine-5'-triphosphate, ammonium salt, Amersham, England) as a marker. Estimations of dGTP and dCTP were performed in a similar manner, with poly-d(I-C) (Na-salt, Boehringer, Mannheim, Germany) as a primer, and with $[^3H]$dCTP ($[5-^3H]$deoxycytidine-5'-triphosphate, ammonium salt, Amersham, England) and $[^3H]$ dGTP ($[8-^3H]$deoxyguanosine-5'-triphosphate, ammonium salt, Amersham, England) resp., as a marker. Estimation of dCTP required a tenfold of enzyme concentration per assay.

Incorporation of ^3H-label into acid-insoluble material increased linearly with increasing deoxynucleotide content, in the range up to 2.0 nmol/assay. With limiting amounts of enzyme, incorporation proceeded linearly for at least 60 min. With excess of enzyme, however, product degradation by contaminating exonuclease activity became evident with increasing incubation time. All deoxynucleo-

side triphosphate calculations therefore are based on maximum values found for each assay.

Results

When acid-soluble deoxynucleotides from ^{32}P-labeled synchronized growing yeast are separated by thin-layer chromatography, radioactivity associated with triphosphates shows rhythmic fluctuations (Fig. 1): The label decreases considerably

Fig. 1. Fluctuating behaviour of ^{32}P-label associated with deoxynucleoside tri- and monophosphates, and stepwise DNA augmentation in synchronized growing yeast. Acid-soluble deoxynucleotides from prelabeled cells were separated chromatographically, and radioactivity was counted. DNA was estimated by the diphenylamine reaction according to [1, 24]. All data were calculated for 200 ml culture, corresponding to 1×10^9 cells at "zero time" of incubation. For further details see "Experimental". Closed symbols: Triphosphates. Dotted lines: Monophosphates. Open symbols: DNA.

during each DNA replication. It begins to increase, again, at the end of the S-phase, and rises to maximum values shortly before the next DNA replication. As the labeled phosphorus pool is diluted

during the cell growth with non-radioactive phosphorus, maximum values become lower with each new cell generation.

Radioactivity associated with deoxynucleoside monophosphates shows a fluctuating behaviour, too (Fig. 1). Maximum values mostly appear, however, when radioactivity with triphosphates is rendered low, *i. e.* during the S-phase. The average amount per cell generation of dAMP-label is nearly equal to that of dATP. The other deoxynucleoside monophosphates differ, however, from their corresponding triphosphates by factors of about 0.5 (dGMP and dTMP) and 0.3 (dCMP), resp. Since triphosphate degradation during the preparation steps can be excluded largely (*cf.* "Experimental"), these findings indicate an unexpectedly high deoxynucleoside monophosphate pool in the growing yeast cells.

In contrast to the fluctuating behaviour of the deoxynucleotide label, acid-soluble total phosphorus is augmented stepwise in the synchronized growing yeast (Fig. 2). Consequently, the "specific" radio-
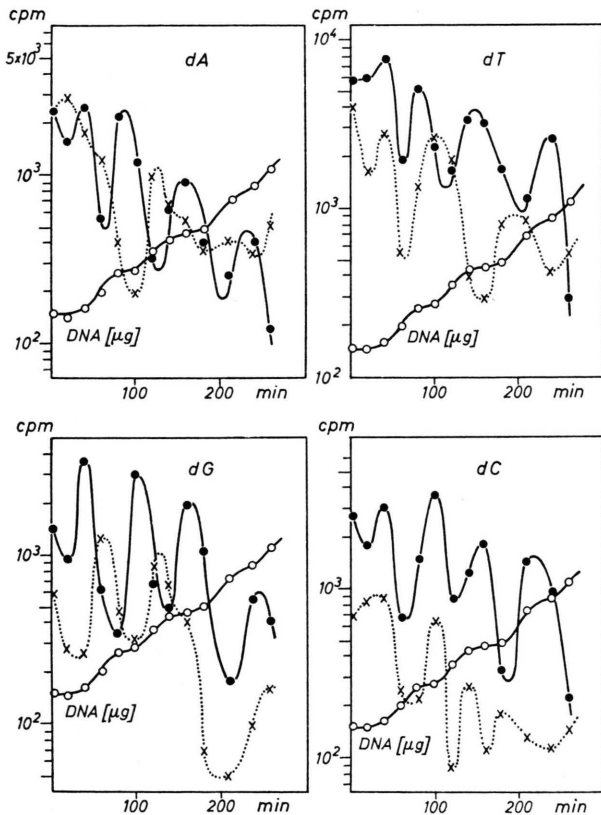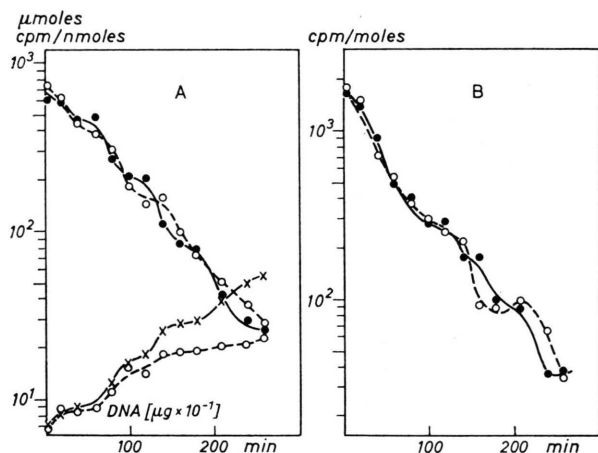

Fig. 2. Acid-soluble phosphorus from prelabeled synchronized growing yeast after X-irradiation. A. Augmentation of acid-soluble total phosphorus, and decrease of its "specific" radioactivity. B. "Specific" radioactivity from ^{32}P-labeled ATP. All data were calculated for 200 ml culture. For details see "Experimental". Closed symbols: Unirradiated control. Open symbols and broken lines: X-irradiated (35 kr).

activity of soluble phosphorus from prelabeled cells decreases stepwise, the slope of the curve reflects exactly that of phosphorus augmentation. "Specific" radioactivity of important acid-soluble phosphorus containing cell constituents, *e. g.* ATP, decreases stepwise, too, but with a different rhythm.

Irradiation of ^{32}P-labeled synchronized yeast with 35 kr of X-rays just destroys the proliferation ability of the cells. The fluctuating behaviour of ^{32}P-activity associated with deoxynucleotides is but affected only little. As shown in Fig. 3, the triphos-

Tab. I. Mean radioactivity associated with the four deoxynucleoside triphosphates from prelabeled synchronized growing yeast. The figures represent the average label between 0 min and 140 (260) min of incubation time, expressed as cpm/200 ml culture.

	dTTP	dATP	dCTP	dGTP
Control	(3285.0)	(9932.7)	(1505.3)	(15097.4)
	4222.8	13564.6	1867.1	18345.9
Irradiated	(3701.1)	(10771.6)	(1609.0)	(11908.1)
	4826.6	15420.2	1818.4	15586.7
Irradiated % of control	(112.6)	(108.4)	(106.8)	(78.8)
	114.2	113.6	97.3	84.8

radioactivity only between 20 and 100 min of cell growth, and during late growth stages, whereas with dGTP a remarkable decrease of the average radioactivity is observed.
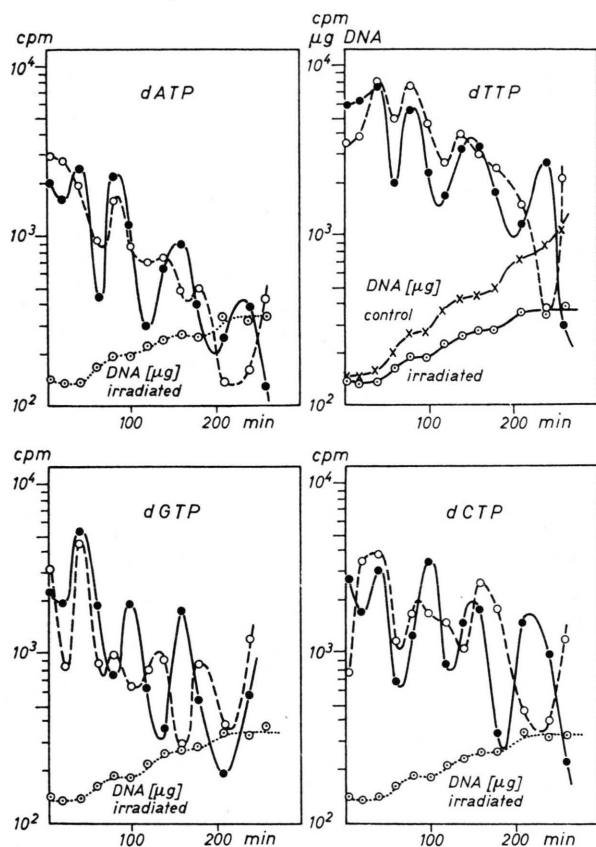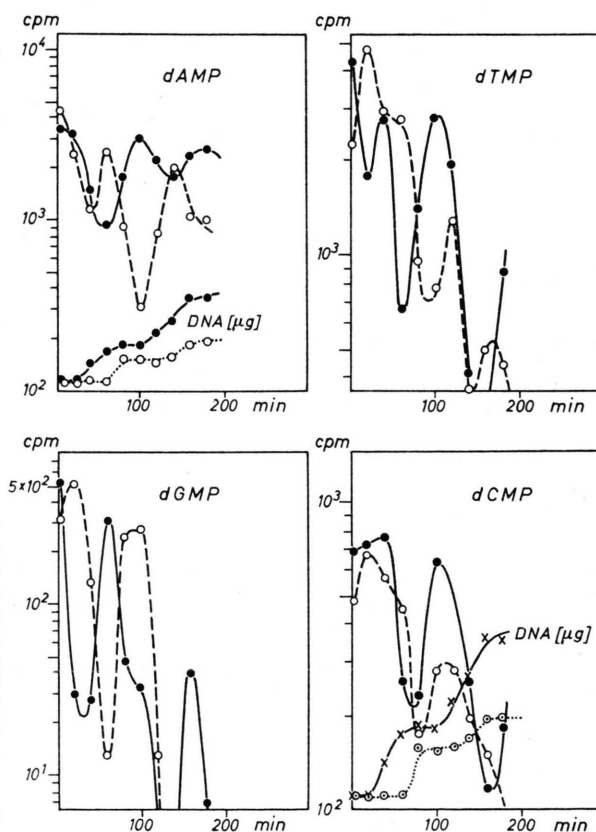

Fig. 3. ^{32}P-label associated with deoxynucleoside triphosphates, and DNA augmentation in prelabeled synchronized growing yeast after X-irradiation. ^{32}P-labeled synchronized yeast was irradiated with 35 kr of X-rays. Acid-soluble deoxynucleotides were extracted from the growing cells at the indicated times, separated chromatographically, and counted for radioactivity. DNA was estimated by the diphenylamine reaction according to [1, 24]. All data were calculated for 200 ml culture (1 × 10^{9} cells at "zero time" of incubation). For details see "Experimental". Closed symbols: Unirradiated control. Open symbols, broken and dotted lines: X-irradiated.

phate label is at maximum nearly at the same times as in the control. It decreases to minor values, even if the DNA content in the irradiated yeast does not rise. This diminution is less drastic, however, than that during DNA replication in the unirradiated control, yielding a slightly elevated average label of the DNA precursors, at least of dATP and dTTP (Table I). dCTP exhibits an increased average

Fig. 4. ^{32}P-label associated with deoxynucleoside monophosphates, and DNA augmentation in prelabeled synchronized growing yeast after X-irradiation. Cell extracts from the same experiment as in Fig. 3 were analyzed for radioactivity associated with deoxynucleoside monophosphates, as described in "Experimental". All data were calculated for 200 ml culture (1 × 10^{9} cells at "zero time" of incubation). Closed symbols: Unirradiated control. Open symbols, broken and dotted lines: X-irradiated.
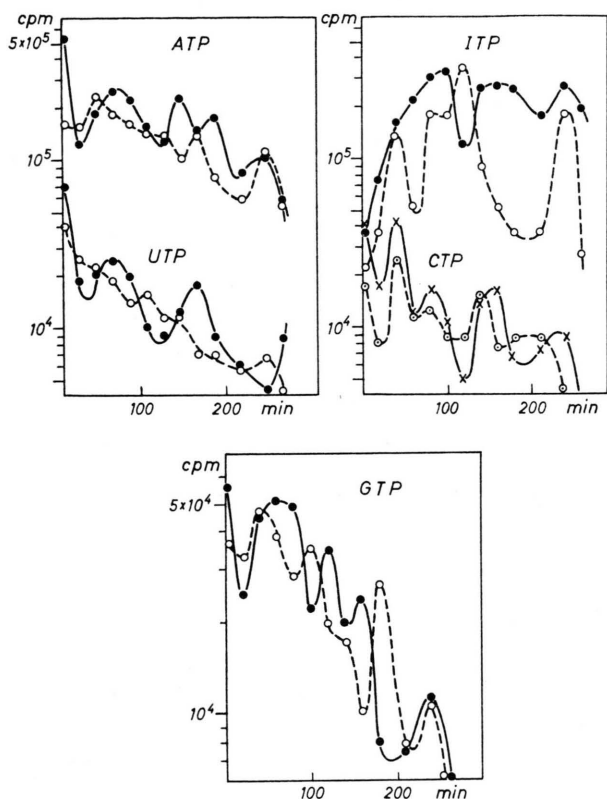
Fig. 5. ³²P-label associated with ribonucleoside triphosphates from prelabeled synchronized growing yeast after X-irradiation. The same cell extracts as in Figs 3 and 4 were analyzed for radioactivity associated with ribonucleotides, as described in "Experimental". All data were calculated for 200 ml culture (1×10^9 cells at "zero time" of incubation). Closed symbols: Unirradiated control. Open symbols: X-irradiated.

The fluctuating behaviour of radioactivity associated with deoxynucleoside monophosphates from X-irradiated cells differs from that of triphosphates in the following points (Fig. 4): Maximum values in general are reached, when the unirradiated control is rendered low. At least radioactivities associated with dTMP and with dGMP overgrow the control considerably just during the first time of cell growth, as long as DNA replication is delayed. A similar increase with dAMP and dCMP can be perceived at the end of the first DNA lag. When DNA augmentation is delayed a second time (80 – 120 min of incubation time), the monophosphate label increases again, though the values now remain beneath the control throughout.

Ribonucleoside triphosphates from prelabeled synchronized growing yeast also show fluctuating ³²P-radioactivities (Fig. 5). The time points of maximum and minimum values, however, do not coincide with those from the corresponding deoxynucleotides, neither in control cells nor after X-irradiation, at least with ATP, UTP and GTP. Deoxynucleotide fluctuations hence cannot be explained simply by artefacts arising from possible experimental insufficiencies. Furthermore, the values from X-irradiated cells do not increase above those from the unirradiated control, ITP excepted. The label rather is dimnished, especially when cell growth is continued over several hours, as evident mostly with ATP.

Fig. 6. ³²P-label associated with deoxynucleoside triphosphates, DNA augmentation, and decrease of "specific" radioactivity from acid-soluble phosphorus and ATP, in asynchronously growing yeast after irradiation with 35 kr of X-rays. Asynchronously growing cells were prelabeled with ³²P, X-irradiated, and analyzed as described in "Experimental". All data were calculated for 200 ml culture (1×10^9 cells at "zero time" of incubation). Closed symbols: Unirradiated control. Open symbols, broken and dotted lines: X-irradiated.
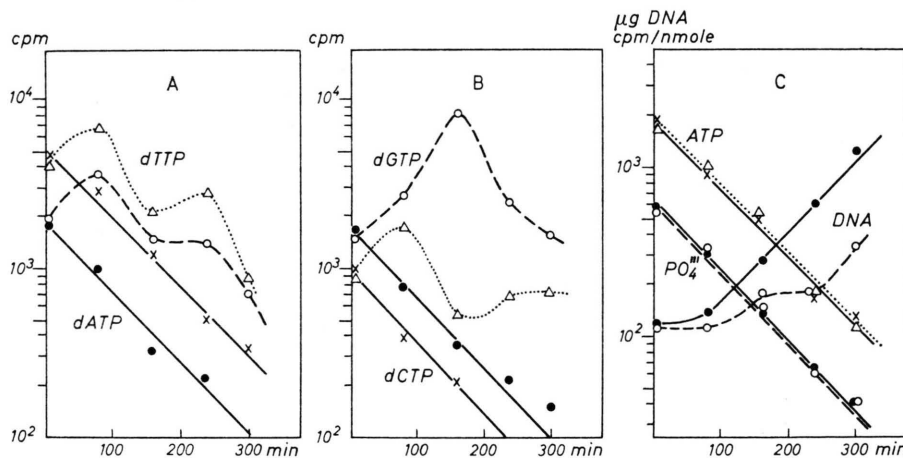
Fig. 7. ^{32}P-incorporation into acid-soluble phosphorus and ribonucleoside triphosphates from asynchronously growing yeast after X-irradiation. Asynchronously growing cells were irradiated with 50 kr of X-rays, and incubated with ^{32}P-phosphate. Cell extracts were prepared from samples taken at the indicated times, and analyzed for the various parameters, as described in "Experimental". All data were calculated for 200 ml culture (1×10^9 cells at "zero time" of incubation). Closed symbols: Unirradiated control. Open symbols, broken and dotted lines: X-irradiated.
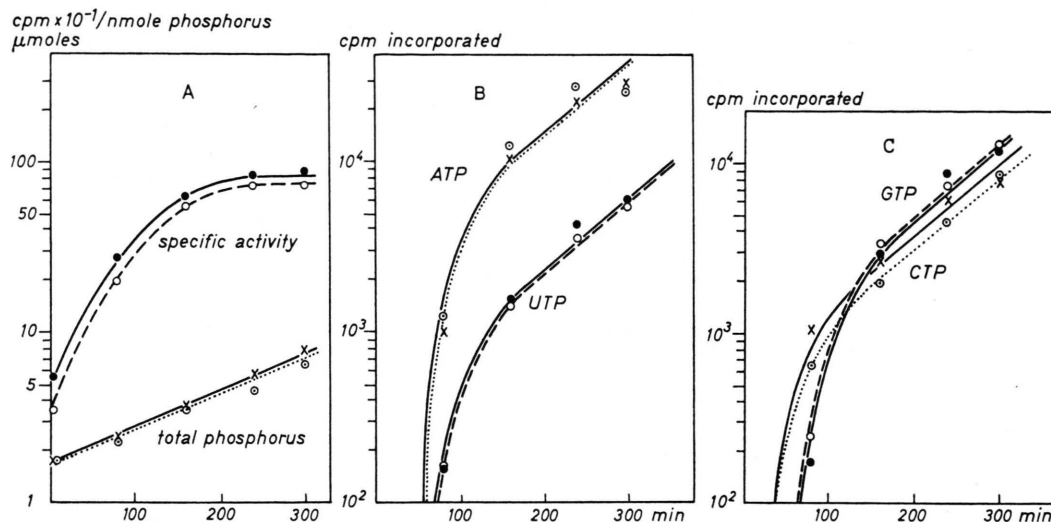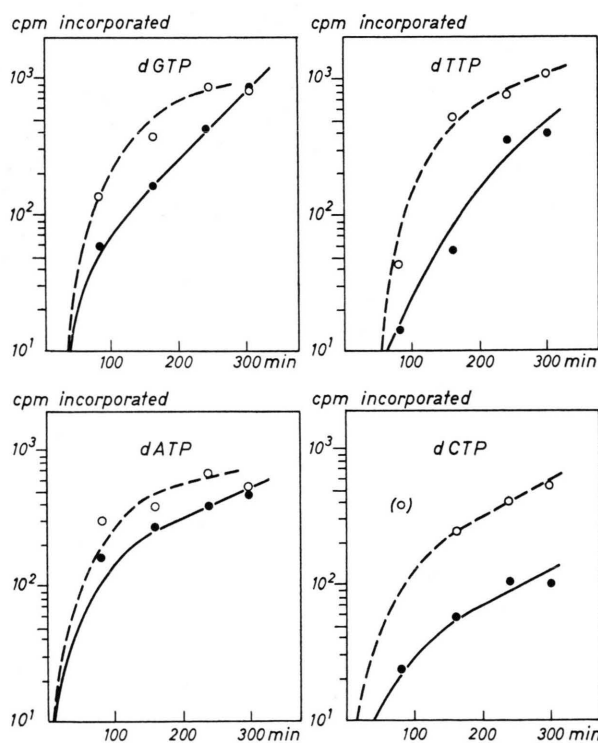

Fig. 8. ^{32}P-incorporation into acid-soluble deoxynucleoside triphosphates from asynchronously growing yeast after X-irradiation. Extracts from yeast irradiated with 50 kr of X-rays and grown asynchronously with ^{32}P-phosphate were prepared, and analyzed for radioactivity associated with DNA precursors, as described in Fig. 7 and in "Experimental". All data were calculated for 200 ml culture (1×10^9 cells at "zero time" of incubation). Closed symbols: Unirradiated control. Open symbols: X-irradiated.

Acid-soluble phosphorus from X-irradiated synchronized growing yeast increases stepwise and — for the first two hours of incubation — with the same rate as in the unirradiated control (Fig. 2). Later on, phosphorus augmentation becomes retarded, but for at least 240 min it is not abolished. "Specific" radioactivity of acid-soluble phosphorus and of ATP, respectively, decreases in prelabeled X-irradiated cells with the same rate as in the control.

In asynchronously growing prelabeled cells, ^{32}P-radioactivity associated with deoxynucleoside triphosphates decreases steadily, and by the same rate as "specific" radioactivity of acid-soluble total phosphorus (Fig. 6). Irradiation of the cells with 35 kr of X-rays results in a rapid increase of deoxynucleotide-bound radioactivity, as long as DNA augmentation is delayed. At the same time when DNA augmentation is resumed, ^{32}P-label of all four DNA precursors decreases sharply. This decrease is delayed, if DNA augmentation once more is retarded. Increase of acid-soluble phosphorus, and decrease of its "specific" radioactivity, however, remain undisturbed.

Incorporation of ^{32}P$_i$ into the acid-soluble fraction from asynchronously growing yeast cells shows no radiation-induced change (Fig. 7 A). Radioactive labeling of ribonucleotides likewise is undisturbed after X-irradiation of the cells (Fig. 7, B and C). ^{32}P-label associated with DNA precursors, however, increases more rapidly in irradiated yeast

than in the unirradiated control, thus overgrowing the control just in the first time of cell growth (Fig. 8). When DNA augmentation is resumed to a normal degree, the rate by which DNA precursors are labeled is reduced to that from unirradiated yeast. Nevertheless, the level of [^{32}P]deoxynucleotide remains increased for at least 5 hours of cell growth.

Enzymic deoxynucleotide estimations from asynchronously growing yeast cultures exhibit small but unobjectionably demonstrable pools of all four DNA precursors, ranging between 0.7 and 3.0 nmol/10^9 cells (Fig. 9). dTTP represents the largest pool, being about two times the dATP values, whereas

dGTP amounts to about 75% of dATP. The smallest pool is represented by dCTP, making about 50% of dATP. During asynchronous cell growth, all four deoxynucleoside triphosphates are augmented, the ratio between the various compounds being maintained.

Surprisingly, the increase of dTTP, dCTP and dGTP does not follow exactly that of cell number and DNA content, but after a burst in the first time of cell growth it is delayed for about one generation time. As shown in Fig. 10, this slope of the curve results from standard experimental conditions, which are necessary to use in the irradiation experiments: When cell growth is not interrupted by washing the pregrown cells with icecold water and keeping them at 4 °C overnight, deoxynucleoside triphosphates are augmented continuously and without "bursts" and delays.

After X-irradiation of the cells deoxynucleotide augmentation appears accelerated, resulting in a remarkably elevated level of DNA precursors at the end of the first generation time (90 min). At the same time, when DNA augmentation is started, the rate of further deoxynucleotide increase drops to minor values. The DNA precursor pools maintain their elevated levels, however, even if cell growth and DNA augmentation are continued.
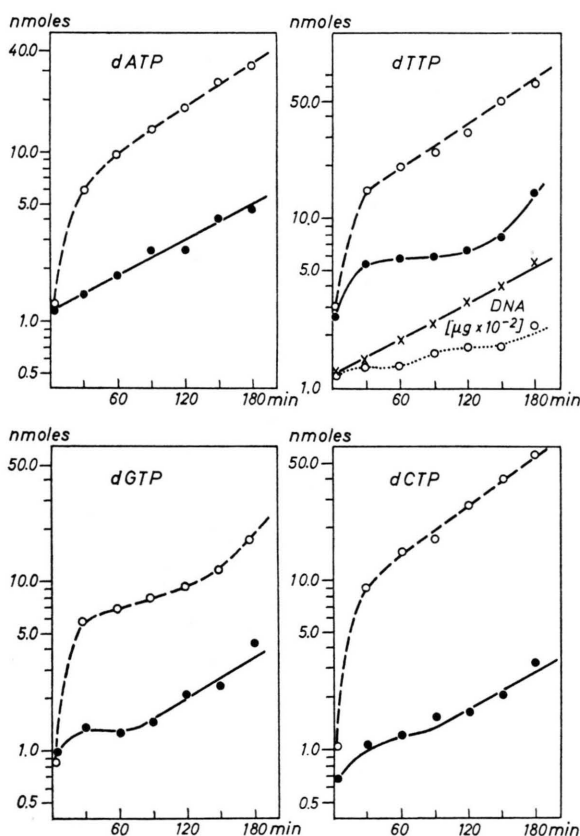

Fig. 9. Increase of deoxynucleoside triphosphates, and DNA augmentation, in asynchronously growing yeast after irradiation with 70 kr of X-rays. X-irradiated asynchronously growing yeast cells were extracted, and concentrates were prepared and immediately brought to pH 7.5. Deoxynucleotides were estimated enzymatically. DNA was estimated according to Burton [25, 26]. All data were calculated for 200 ml culture (1×10^9 cells at "zero time" of incubation). Standard deviation 5.0−6.5%, standard error 1.0−1.5%. For further details see "Experimental". Closed symbols: Unirradiated control. Open symbols, broken and dotted lines: X-irradiated.
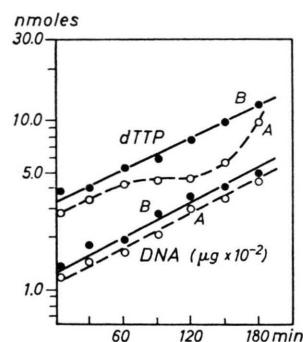

Fig. 10. Augmentation of deoxynucleoside triphosphates in asynchronously growing yeast under various growth conditions. A: Open symbols and broken line: Standard experimental conditions (*cf.* "Experimental"). Cells were pregrown, harvested by centrifugation, washed twice with icecold water, and kept cold overnight as an aqueous suspension (1×10^9 cells/ml). Growth was started again by suspending the cells in fresh cold growth medium and incubation at 31 °C with rigid aeration and stirring. B: Closed symbols: Cells from the same pre-culture as in A were grown continuously overnight. The experiment was started simultaneously with A, by transferring an aliquot from the growing yeast into fresh growth medium, which was preheated at 31 °C, and incubation as above. Deoxynucleoside triphosphates were estimated enzymatically, as described in "Experimental",

Discussion

Studies on acid-soluble deoxynucleotides from growing cells render difficult by extremely low intracellular concentrations of these substances. Recently an enzymic method for estimation of deoxynucleoside triphosphates was developed [27], but when we started our investigations, the only approach to these key substances of cell proliferation was labeling with radioactive precursors in vivo and chromatographic separation of the extracted material [11, 21]. This circumstantial method yet has the essential advantage, that deoxynucleoside mono-, di-, and triphosphates and the corresponding ribonucleotides can be studied simultaneously. Extensive preliminary experiments using commercially available [3]H- and ^{14}C-labeled deoxynucleotides [18] gave certainty, that an application of this procedure to the acid soluble DNA precursor pool from growing yeast proved practicable.

An essential prerequisite for these investigations are uniformly labeled cells, in order to exclude interferences from various intracellular precursor pools. When asynchronously growing yeast was labeled with ^{32}P, steady state conditions were obtained after $2-3$ cell generations. With synchronized yeast, however, prelabeling of the cells was possible for technical reasons only during the synchronizing treatment. Possibly resulting distinctly labeled phosphorus pools, *e. g.* ribonucleotides, nucleic acids and polymetaphosphate (*cf.* 53), and severe over-all changes of phosphorus metabolism in the X-irradiated synchronized growing yeast, should be detected by discriminating investigation of the acid-soluble phosphorus. But neither acid-soluble total phosphorus, nor "specific" radioactivities of its main components, show any irregularities in the prelabeled synchronized growing yeast. Different labeling of those phosphorus pools, which contribute essentially to phosphorus turnover, therefore appear unlikely.

When acid-soluble deoxynucleotides from ^{32}P-labeled synchronized growing yeast are separated chromatographically, the radioactive label of mono- and triphosphates shows fluctuations, which correlate to the rhythmic DNA replication. Since acid-soluble total phosphorus is augmented stepwise, and "specific" radioactivities of total phosphorus and of most important acid-soluble phosphorus transfering substances, *e. g.* ATP, decrease stepwise,

the fluctuating behaviour of the deoxynucleotide-label cannot be explained by a similar behaviour of the phosphorus pool. Similar to deoxynucleotides, the label associated with ribonucleotides fluctuates, too. The timing of these fluctuations differs considerably, however, from that of deoxynucleotides, thus excluding any simple and direct connection between ^{32}P-increases in both species of cell constituents. With ATP the fluctuating behaviour of ^{32}P closely resembles rhythmic alterations of the ATP amount in synchronized growing yeast described earlier [17]. Similarly, if unspecific effects on deoxynucleotide-^{32}P can be excluded, increasing and decreasing ^{32}P-label of the four DNA precursors should reflect fluctuating deoxynucleotide levels in the synchronized growing cells, possibly arising from periodical shifts between synthesis and incorporation into DNA. Unfortunately, no experimental data on this are available for the present. But rhythmic fluctuations of DNA polymerase activity observed with synchronized growing yeast [1], and periodically increasing deoxynucleotide synthetizing enzyme activities described from other cell systems [29-34], strengthen to this view. Accordingly, cyclic fluctuations of the four DNA precursors recently were described with highly synchronous HeLa cell cultures [35].

When DNA augmentation of the growing yeast is delayed by X-irradiation, the fluctuating behaviour of ^{32}P-label associated with deoxynucleoside triphosphates is maintained with an undisturbed rhythm. An influence of X-irradiation can be ascertained only in so far, as the rate of the rhythmic decrease is reduced as long as DNA synthesis is delayed. Consequently, the average ^{32}P-label associated with deoxynucleotides is raised. This but relatively slight radiation effect appears strongly pronounced, when asynchronous yeast is X-irradiated. This difference unquestionably results primarily from different lengths of single cell stages, which determine the composition of an asynchronous population and thereby average pool size. Different radiosensitivity of the deoxynucleotide synthetizing enzyme system during a cell cycle, as shown with animal cells [14], might play an additional role herein. In any case, since with synchronized as well as with asynchronously growing cells neither augmentation of acid-soluble phosphorus, nor decrease of its "specific" radioactivity in prelabeled cells, nor ^{32}P-uptake are affected after X-irradiation, our findings

cannot be explained by general radiation effects on phosphorus turnover of the growing yeast cells (*cf.* 37). Radiation-induced general changes of nucleotide metabolism likewise cannot serve for an explanation of the enlarged ^{32}P-label with DNA precursors, because [^{32}P]ribonucleotide becomes depressed after X-irradiation, and its fluctuations appear retarded. These observations particularly emphasize an independency between ^{32}P-fluctuations of ribo- and deoxynucleotides.

Taken together, the increased ^{32}-label associated with deoxynucleotides from X-irradiated yeast very likely reflects an enlarged DNA precursor pool, presumably caused by continued de novo synthesis and restricted incorporation into DNA. Results obtained with asynchronously growing cells and by enzymic deoxynucleotide estimations confirm this interpretation. At zero time of cell growth, nearly the same amounts of deoxynucleoside triphosphates are found enzymatically and by calculation from "specific" radioactivity of acid-soluble phosphorus. The degree, by which all four deoxynucleoside triphosphates from X-irradiated yeast overgrow the control, is from the same order of magnitude as that observed by labeling techniques.

In the unirradiated control, deoxynucleoside triphosphates (DNA precursors) range between 0.7 and 3 nmol/10^9 cells. When related to DNA, these general data agree well with those reported from animal cell systems [14, 31, 35, 54, 55]. However, differing from animal cells, not dGTP, but dCTP represents the smallest pool in yeast. The other triphosphates follow in the order: dCTP < dGTP <

dATP < dTTP. Whether dCTP acts as a limiting factor of DNA synthesis remains to investigate.

Summarizing, the radiation-induced delay of DNA augmentation cannot be caused by deficient supply of the growing cells with DNA precursors. On the other hand, the rhythmic increase of the DNA precursor pool seems not to function as a trigger for DNA augmentation, though a regulatory influence of the precursor pool size on DNA polymerase activity *in vivo* cannot be excluded from our data.

Unexpectedly, a rhythmic, slight decrease of DNA precursors is seen in synchronized yeast after X-irradiation, even if DNA augmentation is delayed. Since at the same time the deoxynucleoside monophosphate label rises to maximum values, degradation of DNA precursors could be suggested to play a role in control of triphosphate pool size after X-irradiation. On the other hand, the coincidence of rhythmic decrease of triphosphate-^{32}P with increase of DNA polymerase activity in X-irradiated yeast (*cf.* 1) suggests an incorporation of parts of DNA precursors into DNA by repair replication processes. Repair replication after X-irradiation is reported repeatedly [38-51]. Observations on DNA repair in X-irradiated growing yeast recently were published in a preliminary form [15, 16, 52]; details will be described elsewhere [26].

[1] H. Eckstein, V. Paduch, and H. Hilz, Europ. J. Biochem. **3**, 224 [1967].

[2] H. Eckstein, Z. Naturforsch. **26 b**, 1266 [1971].

[3] C. W. Bishop and J. N. Davidson, Brit. J. Radiol. **30**, 367 [1957].

[4] M. G. Ord and L. A. Stocken, Biochim. biophysica Acta [Amsterdam] **29**, 201 [1958].

[5] J. J. Jaffè, L. G. Lajtha, J. Lascelles, M. G. Ord, and L. A. Stocken, Intern. J. Radiat. Biol. **1**, 241 [1959].

[6] D. Billen, Biochim. biophysica Acta [Amsterdam] **55**, 960 [1962].

[7] E. Racker, Nature [London] **167**, 408 [1951].

[8] E. Racker, J. biol. Chemistry **196**, 347 [1952].

[9] St. C. Hartman and J. M. Buchanan, Annu. Rev. Biochem. **28**, 365 [1959].

[10] E. C. Moore and R. B. Hurlbert, J. biol. Chemistry **241**, 4802 [1966].

[11] J. Neuhard, Biochim. biophysica Acta [Amsterdam] **129**, 104 [1966].

[12] P. Reichard, The Biosynthesis of Deoxyribose, Ciba Lectures in Biochemistry, John Wiley and Sons, Inc., New York 1967.

[13] R. L. Blakeley and E. Vitols, Annu. Rev. Biochem. **37**, 201 [1968].

[14] J. Slaby, L. Skoog, and L. Thelander, Europ. J. Biochem. **21**, 279 [1971].

[15] H. Eckstein, U. Koll, and S. Ahnefeld, Hoppe-Seyler's Z. physiol. Chem. **353**, 702 [1972].

[16] H. Eckstein, U. Koll, and S. Ahnefeld, Abstr. Commun. Meet. Fed. Europ. Biochem. Soc. **8**, 1017 [1972].

[17] H. Hilz and H. Eckstein, Biochem. Z. **340**, 351 [1964].

[18] H. Eckstein, unpublished results.

[19] E. Randerath and K. Randerath, J. Chromatogr. **16**, 126 [1964].

[20] J. Neuhard, E. Randerath, and K. Randerath, Analyt. Biochem. [New York] **13**, 211 [1965].

[21] K. Randerath, Dünnschichtchromatographie, Verlag Chemie GmbH, Weinheim 1965.

[22] O. H. Lowry, N. R. Roberts, K. Y. Leiner, M. L. Wu, and A. L. Farr, J. biol. Chemistry **207**, 1 [1954].

[23] P. S. Chen, T. Y. Toribara, and H. Warner, Analyt. Chem. **28**, 1756 [1956].

[24] W. C. Schneider, Meth. Enzymol. (ed. S. P. Colowick and N. O. Kaplan), **Vol. 3**, p. 680, Academic Press, New York 1957.

[25] K. Burton, Meth. Enzymol. (ed. L. Grossman and K. Moldave), **Vol. XII B**, p. 163, Academic Press, New York 1968.

[26] H. Eckstein, Z. Naturforsch. **29 c**, 48 [1974].

[27] U. Lindberg and L. Skoog, Analyt. Biochem. [New York] **34**, 152 [1970].

[28] T. M. Jovin, P. T. Englund, and Leroy L. Bertsch, J. biol. Chemistry **244**, 2996 [1969].

[29] T. P. Brent, J. A. V. Butler, and A. R. Crathorne, Nature [London] **207**, 176 [1965].

[30] T. P. Brent, Cell Tissue Kinet. **4**, 297 [1971].

[31] B. A. Nordenskjöld, L. Skoog, N. C. Brown, and P. Reichard, J. biol. Chemistry **245**, 5360 [1970].

[32] St. Rei-chi Shen and R. R. Schmidt, Arch. Biochem. Biophysics **115**, 13 [1966].

[33] B. Clausnizer, K. H. Dönges, U. Remy, and W. Sachsenmaier, Hoppe-Seyler's Z. physiol. Chem. **349**, 1242 [1968].

[34] W. Linser, G. Finkenstedt, H. Madreiter, H. Wolf, and W. Sachsenmaier, Abstr. Commun. Meet. Fed. Europ. Biochem. Soc. **8**, 1035 [1972].

[35] G. Bray and Th. P. Brent, Biochim. biophysica Acta [Amsterdam] **269**, 184 [1972].

[36] N. L. R. Bucher and N. J. Oakman, Biochim. biophysica Acta [Amsterdam] **186**, 13 [1969].

[37] E. Spoerl, H. W. Nields, and J. J. Plisich, J. cellular comparat. Physiol. **56**, 55 [1960].

[38] H. Harrington, Biochim. biophysica Acta [Amsterdam] **41**, 461 [1961].

[39] H. S. Kaplan, Proc. nat. Acad. Sci. USA **55**, 1442 [1966].

[40] R. A. McGrath and R. W. Williams, Nature [London] **212**, 534 [1966].

[41] W. K. Sinclair, Proc. nat. Acad. Sci. USA **58**, 115 [1967].

[42] S. Nakai and S. Matsumoto, Mutation Res. **4**, 129 [1967].

[43] R. G. Evans and A. Norman, Nature [London] **217**, 455 [1968].

[44] J. F. Scaife and H. Brohée, Canad. J. Biochem. **47**, 237 [1969].

[45] S. Nakai and E. Yamaguchi, Jap. J. Genetics **44**, 355 [1969].

[46] P. Alexander, C. J. Dean, A. R. Lehman, M. G. Ormerod, P. Feldschreiber, and R. W. Serianni, Radiat. Protect. and Sensitization (ed. H. L. Moroson and M. Quintiliani), p. 15—34, Barnes and Noble, New York 1970.

[47] S. Kondo, H. Ichikawa, K. Iwo and T. Kato, Genetics **66**, 187 [1970].

[48] Ch. D. Town, K. C. Smith, and H. S. Kaplan, Science [Washington] **172**, 851 [1971].

[49] E. Cabela, E. Wawra, and H. Altmann, Biophysik **8**, 88 [1972].

[50] H. Matsudaira and I. Furuno, Biochim. biophysica Acta [Amsterdam] **272**, 202 [1972].

[51] Ch. D. Town, K. C. Smith, and H. S. Kaplan, Radiat. Res. **52**, 99 [1972].

[52] U. Yamaguchi-Koll, Dissertation, Freie Universität, Berlin 1972.

[53] U. Till, W. Köhler, J. Ruschke, A. Köhler, and W. Lösche, Eur. J. Biochem. **35**, 167 [1973].

[54] K. L. Skoog, B. A. Nordenskjöld, and K. G. Bjursell, Eur. J. Biochem. **33**, 428 [1973].

[55] E. Liss, S. Brammer, and H. Schmidt, Hoppe-Seyler's Z. physiol. Chem. **354**, 682 [1973].