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STUDIES OF
EXCISION REPAIR OF DNA
IN CULTURED MAMMALIAN CELLS

by

Anthony Cho-Lai Chan

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Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
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ABSTRACT

This study examines the inhibition of DNA synthesis by 5'-fluorodeoxyuridine (FdUrd) and the process of DNA excision repair in differentiated and subcultured mammalian cells.

FdUrd was used to suppress DNA synthesis in the study of repair. Since it depletes the cellular thymidine pool, it should stop all DNA synthesis. Yet, after 16h of incubation with 10^{-6}MFdUrd, the rate of [^{32}P]-orthophosphate uptake into DNA isolated from L-cells amounted to 15% of that of an untreated culture, although cell division had stopped several hours earlier. All 4 deoxynucleotides were present in this DNA but its nucleotide composition, as measured by enzymatic digestion and chromatography, reflected a decreased thymidine precursor pool in the FdUrd treated cells. Sedimentation analysis in alkaline sucrose gradients revealed that the DNA formed in the presence of FdUrd had a sedimentation coefficient of 10S corresponding to a single-stranded molecular weight of 5×10^5 daltons. This DNA could be "chased" into a high molecular weight DNA if the FdUrd block was bypassed with added BrdUrd or thymidine. Other analyses failed to detect RNA covalently linked to the DNA fragments at a level of more than 5% RNA or about 90 ribonucleotides. Since the inhibition of DNA synthesis

by FdUrd is not complete, it is not surprising that excision repair of DNA can occur in the presence of the drug.

Repair synthesis induced by 4-nitroquinoline-1-oxide (4NQO) was measured by [^{3}H]-thymidine incorporation into unreplicated DNA. The level of repair synthesis in L_6 myoblasts was reduced after the cells had fused into myotubes. The terminal addition of radioactive nucleotides into DNA strands and the dilution of [^{3}H]-thymidine by intracellular nucleotide pools were shown not to be responsible for the observed decrease. Both the initial rate and the overall incorporation of [^{3}H]-thymidine were found to be 50% lower in the myotubes. A similar reduction was observed when primary rat fibroblasts were subcultured. On the other hand, the low level of repair synthesis in normal human lymphocytes was elevated when the cells were stimulated by phytohemagglutinin.

4NQO treatment induced modifications in the DNA which were observed as single-strand breaks during alkaline sucrose sedimentation. After the myoblasts were allowed a post-treatment incubation, most of the single-strand breaks were no longer apparent. In contrast, a post-treatment incubation of myotubes did not change the extent of single-strand breakage seen. Both myoblasts and myotubes were equally effective in repairing single-strand breaks induced by X-radiation. It would appear

that when myoblasts fuse, a repair enzyme activity is lost, probably an endonuclease that recognizes one of the 4NQO modifications of DNA. The result observed is a partial loss of repair synthetic ability and a complete loss of ability to remove the modification that appears as a single-strand break in alkali.

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Chapter 1

GENERAL INTRODUCTION

The carcinogenicity and mutagenicity of radiation and chemicals have been linked to their reactions with cellular DNA. When cells were exposed to these agents, a variety of DNA modifications could be identified. Some examples of these modifications are:

Agents	Modification
X-rays	single-strand breaks and altered base (e.g. 5,6-dihydroxydihydrothymine).
Ultraviolet light	pyrimidine dimers and other undefined lesions.
Alkylating agents (e.g. methymethane sulfonate)	deletion of bases, 6-methoxyguanine and other derivatives.
4-nitroquinoline-1-oxide	purine adducts not fully identified.

Many if not all of the DNA modifications listed above are potential genetic hazards; it is therefore important that the DNA be restored to its original form. This is accomplished by a series of reactions termed DNA repair, and it should not be surprising that an impairment of the repair reaction could result in harmful events including carcinogenesis. At least two human diseases, Xeroderma Pigmentosum and Ataxia telangiectasia, have been causally linked to a deficiency of repair enzymes (Cleaver, 1969; Paterson et al., 1976). The present study is aimed towards a better understanding of the repair system in mammalian cells, and the remainder of this chapter will describe the rationale behind the investigation.

In biological systems, DNA modifications induced by radiation and chemical agents are dealt with in several ways. These include photoreactivation, excision repair, and post-replication repair. In photoreactivation, pyrimidine dimers caused by ultra-violet irradiation (Regan et al., 1968; Cleaver & Trosko, 1970) are reverted in a single enzymatic step (Sutherland et al., 1972). Alternatively, excision repair involves a multi-step 'cut and patch' process by which the modified sequence is corrected (Boyce & Howard-Flanders, 1964; Setlow & Carrier, 1964). Post-replication repair, on the other hand, accounts for the existence of unremoved modifications. Apparently, the modified sequence is not copied during normal replication (Howard-Flanders et al., 1968; Iyer & Rupp, 1971; Smith & Meun, 1970; Bridges & Sedgwick, 1974), and the gap left in the daughter strand is filled in by the homologous stretch of nucleotides derived from the other parental strand initially opposite the modification (Rupp & Howard-Flanders, 1968). The 'changing over' is similar to genetic recombination (Rupp et al., 1971; Ley, 1974), except that the sequence removed from the parental strand has to be resynthesized. The modification is not removed but is eventually diluted out of the population (Ganesan, 1974). Thus, it appears that these mechanisms

are complementary processes that are employed in maintaining the integrity of genetic information.

At least for procaryotes, the existence of the above-mentioned processes is supported by a large body of evidence (Howard-Flanders, 1968; Grossman, 1974). For mammalian cells, the situation is less clear. Photoreactivation was thought not to occur in placental mammals (Cook, 1970; 1972) until recently when the photoreactivation of dimers was detected in human leukocytes (Sutherland, 1974; Sutherland et al., 1975, 1976). Furthermore, UV-irradiation of mammalian cells causes a delay in DNA replication (Chiu & Rauth, 1972). Newly synthesized DNA in mammalian cells after irradiation has a lower molecular weight than that synthesized by the control, indicating that gaps are formed in the daughter strand (Cleaver & Thomas, 1969; Meyn & Humphrey, 1971). It appears that these gaps are eventually filled by de novo synthesis, (Lehmann, 1972; Buhl et al., 1972; Buhl & Regan, 1973) but there is evidence that recombinational exchange is also involved (Meneghini & Hanawalt, 1976). Thus, the mechanism of post-replication repair in mammalian cells is not clear. Perhaps the best studied repair mechanism in mammalian cells is excision repair. A brief summary of the present state of knowledge in this respect is given in the following discussion.

Using an autoradiographic technique, Rasmussen and

Painter (1964) observed that usually only 20-30% of the nuclei in a culture of human Hela cells were heavily labelled by radioactive thymidine. After irradiation, however, a lesser amount of labelling was evident in the other non-S-phase nuclei. This radiation-induced "unscheduled synthesis" (Djordjevic & Tolmach, 1967) was interpreted as a consequence of excision repair. Several years later, Cleaver (1968) found that cells from humans with the rare genetic disorder Xeroderma pigmentosum (XP) did not display this phenomenon. A defective excision repair system was thus implicated in these cancer cells which were hypersensitive to U.V. light (Cleaver, 1969). This link prompted considerable interest in excision repair and this emphasis has since remained in the study of mammalian DNA repair. The experiments described in this volume represent one such study.

The process of excision repair is initiated by the introduction of a nick in the modified strand. This priming step opens up the long chain of esterified deoxynucleotides and exposes the sequence which is to be replaced in subsequent steps. Nicks in DNA are made through the action of endonucleases (Strauss & Robbins, 1968; Paterson et al., 1973). These enzymes recognize local distortions induced in the helical structure by pyrimidine dimers or other bulky DNA adducts (Paterson & Setlow, 1972; Regan & Setlow, 1974); their action then

effects a nick in the strand 5' to the distortions (Carrier & Setlow, 1970; Kushner et al., 1971). Strand breaks may also occur non-enzymatically. For the kind of damage typified by that caused by X-rays and the monofunctional alkylating agent methyl methane-sulfonate (MMS), the loss of a single base results in a break in the sugar-phosphate backbone (Lawley & Brooks, 1963; Regan & Setlow, 1973), and the intervention by an endonuclease is not necessary. Thus, XP cells which are capable of repairing X-ray damage (Kleijer et al., 1970) but not UV damage (Bootsma et al., 1970) are considered to be endonuclease deficient (Cleaver, 1974). Nonetheless, the significance of break formation lies in that it is the limiting step for the excision repair process.

Once the nick is made, a 5' → 3' exonuclease proceeds to remove the modification and neighbouring nucleotides (Kaplan et al., 1971; Kushner et al., 1973). The extent of cleavage ranges from one or a few nucleotides in the case of X-ray or MMS induced damage (Painter & Young, 1972; Fox & Fox, 1973; Regan & Setlow, 1973), to as much as 40-100 in the case of UV modifications (Cooper & Hanawalt, 1972; Regan et al., 1971). Cleavage is immediately followed by resynthesis of the missing sequence. In E. coli, excision and synthesis may be simultaneously carried out by DNA polymerase I which also contains a 5' → 3' exonuclease (Kelly et al., 1969). Finally, the

renewed sequence is welded back into the still severed strand by polynucleotide ligase, the same enzyme that catalyses the joining of Okazaki's fragments in normal replication (Segev et al., 1973; Hariharan & Cerutti, 1974).

Based on the above working model, many methods have been described for studying excision repair. In the present study, 4-nitroquinoline-1-oxide (4NQO) was used to induce repair. Tanooka et al. (1975) reported that the active form of 4NQO in microorganisms is a serylated derivative of 4-hydroxylaminoquinoline-1-oxide which can react with DNA, RNA or protein, but the exact reaction products are unknown. Notwithstanding, 4NQO is an interesting compound because of its mutagenicity and carcinogenicity and that it mimics ultraviolet light in its heightened lethality for XP cells, relative to normal cells (Stich & San, 1971). It is also comparatively easy to handle.

The replacement of nucleotides during excision repair was measured by using a density label and the technique of isopycnic centrifugation (Pettijohn & Hanawalt, 1964; Roberts et al., 1968). The incorporation of radioactive 5'-bromouracil into replicating DNA increases the buoyant density of the molecule which is then separable from unreplicated DNA by centrifugation in a CsCl solution. The small amount of bromouracil

incorporation during repair, however, does not increase significantly the buoyant density of DNA. Consequently, the amount of label in the parental DNA gives an estimate of the extent of repair. Another method used to study repair was alkaline sucrose gradient sedimentation (McGrath & William, 1966; Lett et al., 1967). This method involves first the incubation of labelled cells in an alkaline detergent solution that is layered on top of a preformed sucrose gradient. After 6-12 hours of lysis, the gradient is centrifuged at low speed (15,000 rpm) for a few hours (Palcic & Skarsgard, 1972). Under these conditions, single-stranded DNA released from normal cells has a maximum sedimentation coefficient of 160-165S (Lett et al., 1970); sedimentation at a lower rate would indicate that strand breakage has occurred. Thus, the induction and rejoining of strand breaks can be followed with this procedure which, when used in conjunction with the density labelling method, provides an overview of the excision repair process.

As calculated by Cleaver, "unscheduled synthesis" amounts to at most a few percent of the total DNA synthesis in an unsynchronized population of cells (Cleaver, 1974). It is therefore desirable to suppress normal replication if repair is to be quantitated in terms of nucleotide incorporation. Unlike procaryotes, mutants that are impaired in their DNA synthetic ability

(e.g. polA$^-$ of E. coli) are not available in mammalian strains. Thus, metabolic inhibitors are usually used to prevent DNA replication. In this respect, hydroxyurea and 5'-fluorodeoxyuridine (FdUrd) are used most often (Regan et al., 1971; Brandt et al., 1972; Scudiero & Strauss, 1974). Cleaver (1969) claimed that these agents are specific inhibitors of replication, having no effect on repair synthesis. A closer examination of their modes of action, however, leaves one in doubt. Hydroxyurea inhibits the enzyme ribonucleotide reductase which converts ribonucleoside diphosphates into deoxyribonucleoside diphosphates (Young et al., 1967). Fluorodeoxyuridine, being a thymidine analogue, inhibits the enzyme thymidylate synthetase (Santi et al., 1974) which is responsible for the conversion of deoxyuridylate to thymidylate, and ultimately the production of dTTP. Both agents essentially inhibit the formation of deoxynucleotide triphosphates, the immediate precursors of DNA replication. Yet, there is no apparent reason why repair synthesis is not influenced. Repair also requires deoxynucleotides. Earlier studies showed that almost complete inhibition of thymidylate synthetase and over 90% inhibition of DNA synthesis could be effected by less than 10^{-7}M FdUrd (Hartman & Heidelberger, 1961; Mukherjee & Heidelberger, 1962). More recent studies, however, showed that FdUrd did not inhibit DNA synthesis completely (Amaldi et al., 1972) and although the level of thymidylate synthetase dropped initially when cells were

treated with FdUrd, it returned to the control value within twelve hours (Conard & Ruddle, 1972). In the present study, when the incorporation of [^{32}P]-phosphate into DNA was measured in the presence of 10^{-6}M FdUrd, as much as 15% incorporation, compared to an untreated control, was evident. This observation led to a reexamination of the action of FdUrd. It was subsequently observed that the 15% incorporation was due to de novo DNA synthesis that was incomplete resulting in the accumulation of intermediary fragments. It has been suggested that when the cellular nucleotide pool is depleted, or severely reduced, the rate of replicon initiation supercedes that of chain elongation (Olivera et al., 1973), and the intermediary fragments are somehow left unjoined. This notion is also supported by similar results reported for hydroxyurea (Coyle & Strauss, 1970; Ben-Hur & Ben-Ishai, 1971). Details of the experiments with FdUrd are described in Chapter 2 of this volume.

The study with FdUrd shows that although the cellular dTTP pool is reduced, the amount of nucleotide left is still sufficient for a small amount of DNA synthesis, but, for the study of repair synthesis, it was desired to stop normal replication. Here, the use of differentiated cell types emerges as a possible alternative. One example is the human peripheral blood lymphocytes. Normal lymphocytes are usually in a resting

state and do not replicate their DNA (Darzynkiewicz, 1969). The lack of semiconservative synthesis in these cells is a property which could be used to advantage. A similar phenomenon is observed when myogenic cells fuse to form myotubes (Yaffe, 1968). That the latter are successfully carried in tissue culture (Richler & Yaffe, 1970) further prompted their use in our study.

In addition, the relationship between differentiation and excision repair is itself an interesting subject of study. It is possible that enzyme changes that usually accompany differentiation (Shainberg, 1971) may have an influence on excision repair. From the scheme of excision repair earlier described, the involvement of enzymes is obvious. But, the use of cycloheximide has failed to show a dependency of the process on protein synthesis (Gautschi et al., 1973). Of course, the enzymes that catalyse repair may have been the long-lived kind which are turned over slowly (Powell, 1962). On the other hand, rather 'permanent' genetic repression is presumably responsible for the altered enzyme profiles in differentiated cells. Therefore, measurement of repair in differentiating cells might provide information about the enzyme requirement of excision repair as well as the influence of differentiation on repair. Toward this end, excision repair was studied in Yaffe's L_6 myoblasts (Yaffe, 1968) before and after cellular fusion.

In the context above, the action of mitogens on lymphocytes is interesting. Under the influence of mitogen, small lymphocytes undergo blastic transformation that is characterized by a flood of enzyme production and DNA synthesis (Douglas, 1972; Pauli & Strauss, 1973). It is technically almost a reversal of the phenomena exhibited during the fusion of myoblasts. Thus, it is possible that DNA repair in lymphocytes is influenced by the addition of mitogens. In this study, repair synthesis was measured in human lymphocytes before and after they were treated with phytohemagglutin (PHA). This serves as an interesting comparison to the rat myogenic system.

Up to this point, excision repair has been discussed as the dominant repair process in mammalian cells. This may be true for human cells but there is room for doubt in the case of rodent cells. In studies which measure the removal of pyrimidine dimers, mouse and hamster cells in culture, with few exceptions, always perform poorly (Painter & Cleaver, 1969; Trosko & Chu, 1973). This means either that the damage is excised in forms other than the one assayed for, or that another form of DNA repair (e.g. post-replication repair) is more important for these cells. Interestingly , primary mouse cells are competent in removing thymine dimers, but the ability is lost when the cells are subcultured (Ben-Ishai & Peleg, 1975). In contrast, human cells do not display this

phenomenon. Freshly explanted human fibroblasts and the established lines Hela and WI38 all have the same ability to excise thymine dimers and perform DNA repair (Cleaver, 1974). It was asked to which of these categories rat cells belong, and how general among species is this phenomenon. Since rat cells (L_6) were used in this study, knowledge of their potential for repair, and in which way rat cells are influenced by subculturing would be helpful.

The experiments reported in Chapter 3 depict the study on DNA repair in rat L_6 myoblasts, human lymphocytes and primary rat fibroblasts. The immediate goal is to determine how the process of excision repair is influenced by differentiation and subculturing.

Chapter 2

DNA SYNTHESIS IN L-CELLS IN THE PRESENCE OF 5'-FLUORODEOXYURIDINE

2.1 INTRODUCTION

The mechanism by which FdUrd inhibits the synthesis of DNA is by a prior inhibition of the enzyme thymidylate synthetase with a consequent depletion of the cellular pool of dTTP. After mammalian cells have been exposed to FdUrd for a few hours, inhibition of DNA synthesis appears to be complete because cell division has ceased. The addition of exogenous thymidine to the blocked cultures results in a prompt resumption of DNA synthesis and cell division follows after a time dictated by the cell cycle characteristics. A very careful analysis of these events in L-cells led Till et al. (1963) to conclude that "the probable distribution of cells in the cycle after 16h of unbalanced growth in the presence of FdUrd was as follows: cells that were in S-phase at the time of addition of FdUrd were randomly distributed through S, and addition of FdUrd simply prevented further progress of the cells through S as soon as the intracellular pools of thymidine had been depleted...the cells not in S at the time FdUrd was added proceeded around the cycle until they reach the beginning of S, whereupon they were prevented from entering S". The renewed interest in this question stemmed from a desire to be able to deplete completely the dTTP pool in cultured mammalian cells as part of a study of

repair synthesis of DNA. But, when the inhibition by FdUrd of DNA synthesis in L-cells was measured, it was found that even after a 16h exposure to 10^{-6}M FdUrd, the rate of DNA synthesis was about 15% of the control value although cell division has ceased several hours before. However, much of the DNA that was made in the presence of FdUrd had a single stranded molecular weight of about 5×10^5 daltons (10S). Since these small DNA molecules could be chased into much larger DNA molecules, they probably represented one of the intermediate forms that are joined to make the final high molecular weight product.

2.2 MATERIALS AND METHODS

Cells and Labelling

Strain L mouse cells, were maintained in thymidine-free 1066 medium containing 7% horse serum, streptomycin and penicillin (Till et al., 1963). Suspension cultures in spinner flasks were incubated at 37°C and measurements with a Coulter counter (Coulter Electronics, Hialeah, Florida, U.S.A.) indicated that the generation time was about 18 to 20 hours. Cultures in the logarithmic phase of growth were used and cell density was adjusted to approximately 2×10^5 cells/ml at the beginning of every experiment.

For pulse labelling, cultures containing 6×10^7

cells were centrifuged and resuspended in 20 ml of medium. Radioactive materials were added, and the concentrated cultures were further incubated for the desired length of time before the cells were collected.

Extraction of nucleic acids

Cell pellets obtained by centrifugation were dispersed in 16.2 ml of 5% sodium p-aminosalicylate and shaken with an additional 1.8 ml of 10% sodium dodecylsulfate. Nucleic acids were then extracted by two changes of a mixture of phenol, cresol, and 8-hydroxy-quinoline. The resulting aqueous layer was mixed with 1.5 volumes of cold ethoxyethanol. The precipitate was dissolved in SSC x 10^{-1} (15 mM NaCl, 1.5 mM sodium citrate), treated with ribonuclease, and reextracted (Walker & Ewart, 1973a).

For extraction of the DNA-RNA mixture to be later separated by Cs_2SO_4 centrifugation, the aqueous layer resulting after the first two extractions with phenol reagent was precipitated by two volumes of ice-cold absolute alcohol. The precipitate was spooled on a glass rod and dissolved in SSC x 10^{-1}.

Base composition analysis

Digestion of DNA samples with deoxyribonuclease and phosphodiesterase, and chromatography were carried out as previously reported (Walker et al., 1973), except that

incubations with enzymes were maintained for twice the length of time.

Equilibrium centrifugation

Centrifugation in CsCl and collection of fractions were done as described previously (Walker et al., 1973) then each fraction was neutralized with 2 ml of 2 x 10^{-4} N HCl. After adding 50 µg of salmon sperm DNA as carrier, 2 ml of 10% trichloroacetic acid was used to precipitate the DNA which was then caught on glass fibre filters (Gelman Instrument Co., Ann Arbor, Michigan) and assayed by liquid scintillation counting.

For Cs_2SO_4 centrifugation, a mixture of 2.2 ml saturated Cs_2SO_4 solution ($\rho = 2.06$ g/cc), 0.5 ml dimethyl sulfoxide, 0.5 ml buffer (1.0M Tris-HCl, 0.1M EDTA, pH 7.6), and 1.3 ml heat-denatured, labelled nucleic acid sample were placed in a cellulose nitrate tube, covered with mineral oil, and centrifuged in a Ti50 rotor at 36,000 rpm and 15°C for 45h. Carrier DNA was added to the fractions collected, and the acid insoluble radioactivity trapped on membrane filters was measured by liquid scintillation counting.

Sucrose gradient centrifugation

4.2 ml sucrose gradients (5-20%) in a solution containing 0.3M NaOH, 0.01% sodium dodecylsulfate, and

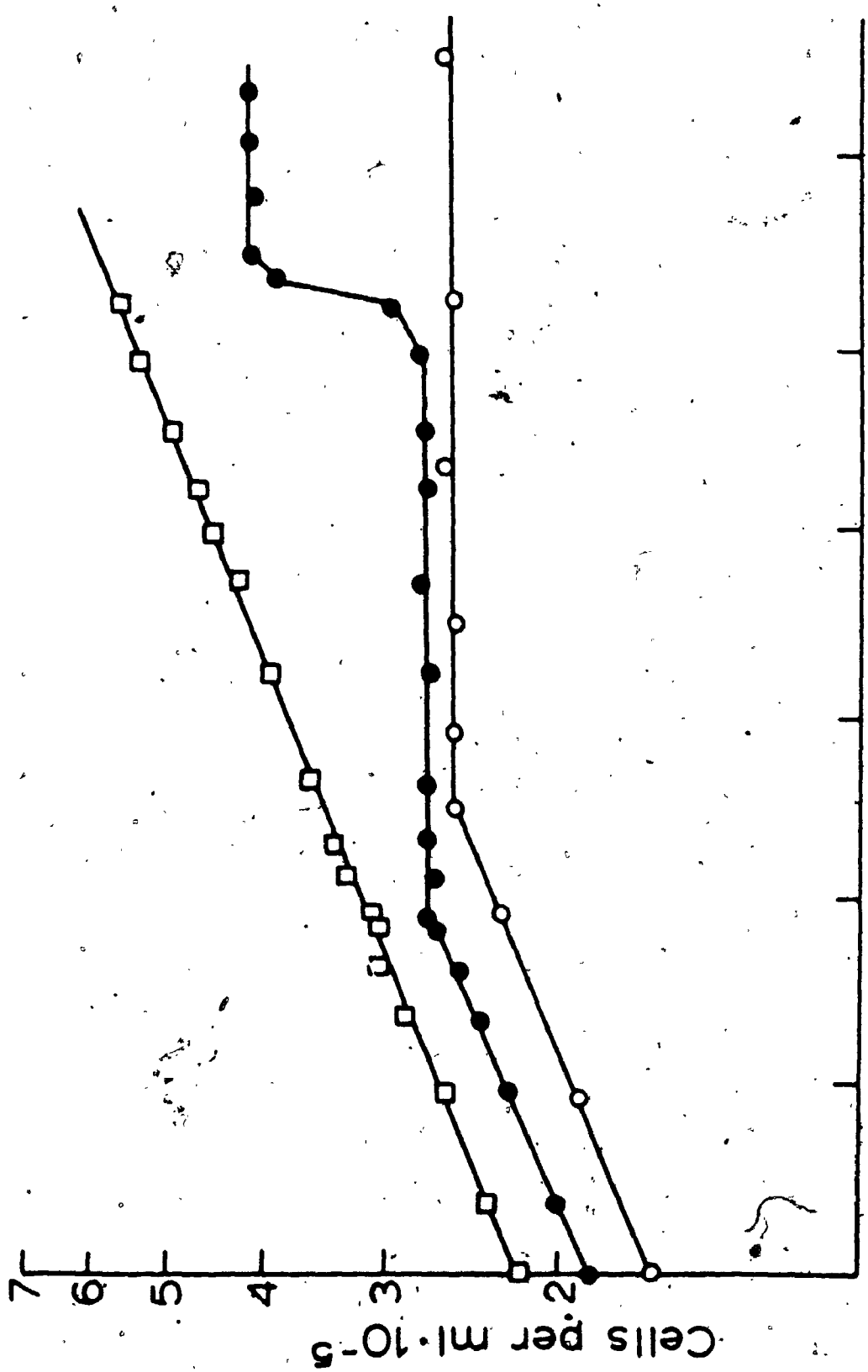
0.001M EDTA were made by an ISCO 570 gradient former (Instrumentation Specialties Co., Lincoln, Nebraska), and 0.3 ml of a lysing solution containing 0.5M NaOH, 0.2% SDS and 0.01M EDTA was layered carefully on top of each gradient. Cells cultured and treated on 60 mm plastic petri dishes were collected by trypsinization and 20 µl of the resulting cell suspension was delivered into the lysing solution. The gradients were allowed to stand at room temperature for 6-7h before centrifugation was carried out at 30,000 rpm and 20°C for 2h in a SW 50.1 rotor. Fractions (0.2 ml) were collected from the top using an ISCO 640 fraction collector, and the acid insoluble radioactivity in each fraction was assayed as before.

2.3 RESULTS

Fig. 1 shows the effects of FdUrd on the growth of L-cells. Before growth ceased there was an approximately 40% increase in cell density which was due presumably to the preexisting population of G_2 cells plus some cells in S-phase which had sufficient amounts of DNA precursors available to allow completion of S-phase. The figure shows that inhibition of cell division persists until at least the 35th hour after adding FdUrd or until the addition of thymidine which bypasses the metabolic blockade. The incorporation of [^{32}P]-orthophosphate into DNA was

Fig. 1. Effect of FdUrd on growth of L-cells.

Cell densities were determined periodically with a Coulter counter. □—□—□, Control, no FdUrd. ●—●—●, 10^{-6}M FdUrd at Oh, thymidine (10µg/ml) at 19h. o—o—o, 10^{-7}M FdUrd at Oh, no thymidine.


Cells per ml·10⁻⁵
7
6
5
4
3
2
5
10
15
20
25
30
Hours

measured from the 16th to the 17th hour following addition of FdUrd (10^{-6}M) to a culture (Table 1). Compared to control cells the FdUrd treated cells incorporated about 15% as much [^{32}P] into DNA. To ensure that the [^{32}P] was indeed incorporated into DNA and not present as contaminating RNA, orthophosphate or other phosphorus containing compounds, samples of the DNA were treated with NaOH or were dialysed against an orthophosphate solution. These treatments did not change the specific activity of the DNA. When incorporation measurements were made at 5, 15 and 20h after adding FdUrd (10^{-6}M) it was found that the inhibition of DNA synthesis was virtually the same at all times. The effectiveness of different concentrations of FdUrd were compared. It was found that the specific activity of the DNA synthesized after a 16 hour exposure to FdUrd at concentrations of 10^{-7}M, 10^{-6}M and 10^{-5}M were 19%, 13% and 10% respectively. In subsequent experiments the concentration of FdUrd used was 10^{-7}M because it was effective in blocking cell division and was not excessively toxic (Till et al., 1963).

The [^{32}P]-labelled nucleotide profile of the DNA synthesized in the presence of 10^{-7}M FdUrd was determined by adding [^{32}P]-orthosphosphate to a culture 8 hours after adding the FdUrd. DNA was isolated 12 hours later, and degraded enzymatically to nucleotides. Chromatographic analysis revealed that all four nucleotides were present

Treatment of DNA Solution	Specific Activity ([^{32}P] CPM/µg DNA)	
	Control	10^{-6}M FdUrd
Trichloroacetic acid precipitation, membrane filtration	373	58.0
Overnight treatment with 0.1N NaOH, precipitated as above	370	56.9
Dialysed overnight against 50 volume of 0.05M Na_2HPO_4, precipitated as above	371	57.1

TABLE I. Incorporation [^{32}P]-Orthophosphate into DNA of L-Cells with or without FdUrd Treatment.

Cells were incubated for 16h in medium containing 10^{-6}M FdUrd, [^{32}P]-orthophosphate (25 µCi/ml) was added and incubation was continued for 1h. DNA was isolated as described in the text and was dissolved in SSC x 10^{-1}. Its concentration was determined by measuring absorbance at 260 nm.

but the proportions, based on [^{32}P] content were not those expected for L-cell DNA. The proportions found were dAMP=21%, dTMP=41%, dGMP=16% and dCMP=22%. L-cell DNA has a molar nucleotide composition in which dAMP=dTMP=28% and dGMP=dCMP=22% (Walker et al., 1973). The nucleotide profile of the DNA synthesized under these conditions in the presence of FdUrd is not a valid measure of the composition of the DNA and its peculiarity is simply a reflection of the size of the unlabelled precursor pools at the time of adding [^{32}P]-orthophosphate. Thus, the small pool size of unlabelled thymine nucleotides would result in the formation of [^{32}P]-labelled thymidylate with a high specific activity. The specific activity of the other nucleotides would be correspondingly smaller.

It seemed possible that the source of the thymidylate for DNA synthesis in the presence of FdUrd was from a degradation and recycling of preexisting DNA. This possibility was excluded by the results of the following experiment. Cellular DNA was labelled by growing the cells in medium containing [^{3}H]-thymidine; the cells were transferred to medium containing FdUrd and after 16h, bromodeoxyuridine (BrdUrd) was added and incubation was continued for 3h. BrdUrd released the FdUrd-inhibition and because the DNA strands so formed had a greater density than the parental strands, the two kinds of DNA were readily separated by density gradient centrifugation.

If parental DNA contributed labelled thymidine to a precursor pool the DNA synthesized after BrdUrd was added would have acquired some label. Fig. 2 shows that this did not occur; the newly synthesized, BrdUrd-containing DNA at the bottom of the gradient, contains no label. The next question asked was, was the DNA synthesized in the presence of FdUrd complete, or was it some kind of replicative intermediate? The experimental results shown in Fig. 3 demonstrate the latter alternative to be correct. After a culture was treated with FdUrd for 16h, the cells were labelled for 3h with [^{32}P]-orthophosphate. The cells were washed thoroughly and resuspended in fresh medium. One half of the culture received BrdUrd, the other half received thymidine and both were incubated for 3h. Control cultures were treated similarly but did not receive the FdUrd. DNA was isolated from the cells in all 4 cultures and it was analysed by isopycnic centrifugation. Fig. 3A shows that after BrdUrd was added to the FdUrd treated cells, about 75% of the [^{32}P] was found at a density corresponding to a DNA virtually fully substituted with BrdUrd. This indicates that at least 75% of the DNA formed in the presence of FdUrd was in the form of a fragment that could become part of a much denser and hence larger BrdUrd-containing fragment. In the corresponding control (Fig. 3B), only about 30% of the [^{32}P] was found in the dense fractions. Therefore, in the presence of FdUrd, DNA fragments accumulated. In

Fig. 2. Stability of preformed DNA during incubation with FdUrd. A culture labelled for 20h with [^{3}H]-thymidine (3µCi/ml was centrifuged, washed in saline, resuspended in medium and then divided into two parts and to one of these, FdUrd was added to a final concentration of 10^{-7}M. The incubation was continued for 16h when BrdUrd (5µg/ml) was added and the incubation continued for 3h. DNA was then extracted, purified and centrifuged in alkaline CsCl at 37,000 rpm and 25°C for 36h. Successive fractions were collected from the bottom of the tube and these were each neutralized by 2 ml of 2.4×10^{-4}N HCl. Optical densities were read at 260 nm then the DNA was precipitated with 5% trichloroacetic acid and caught on membrane filters for liquid scintillation counting. If the [^{3}H] thymidine resulted from the break-down of labelled DNA was not diluted by the intracellular thymidine pool before it was reused, the recycling of less than 1% of labelled DNA would have been detected.

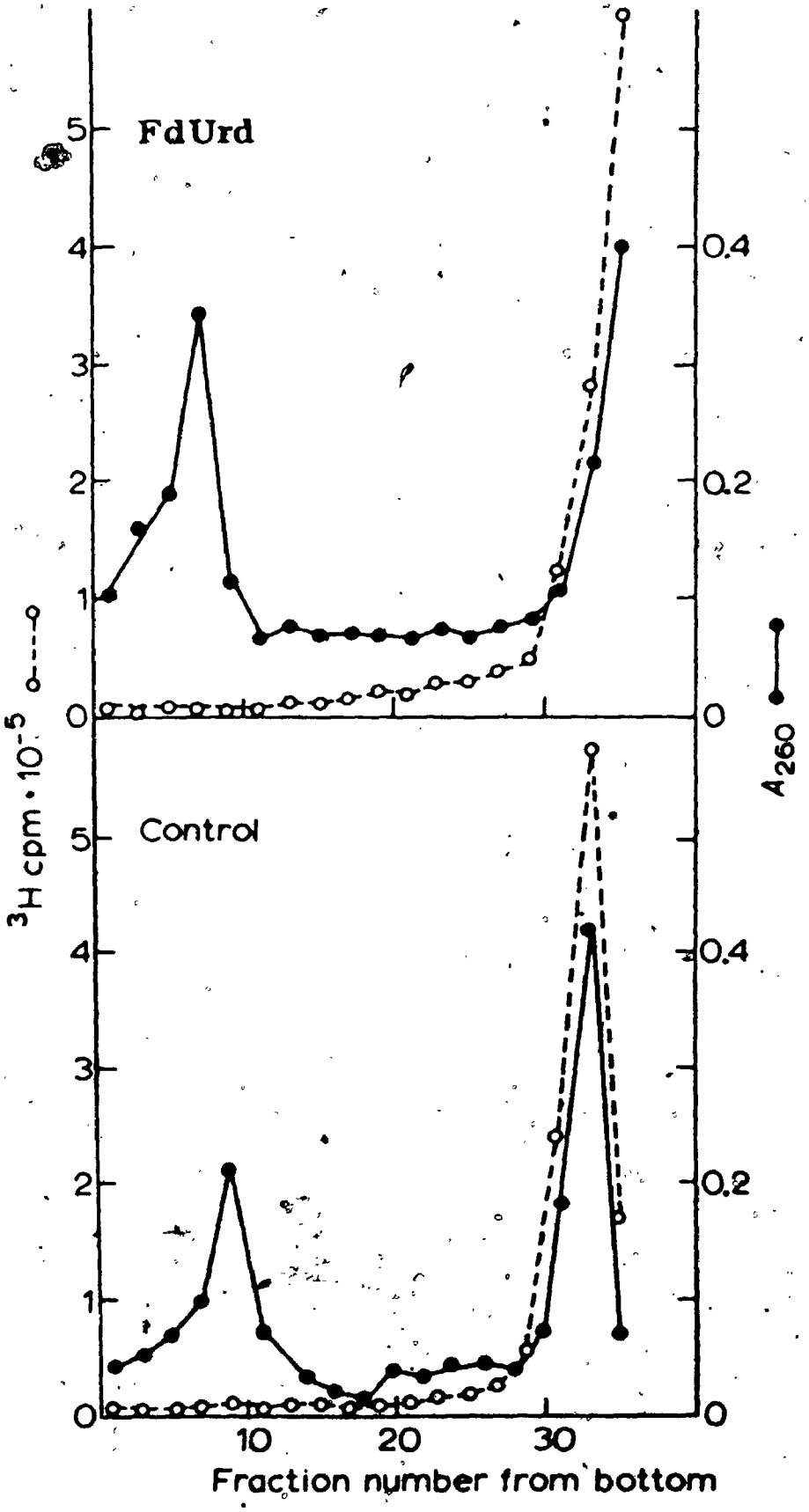
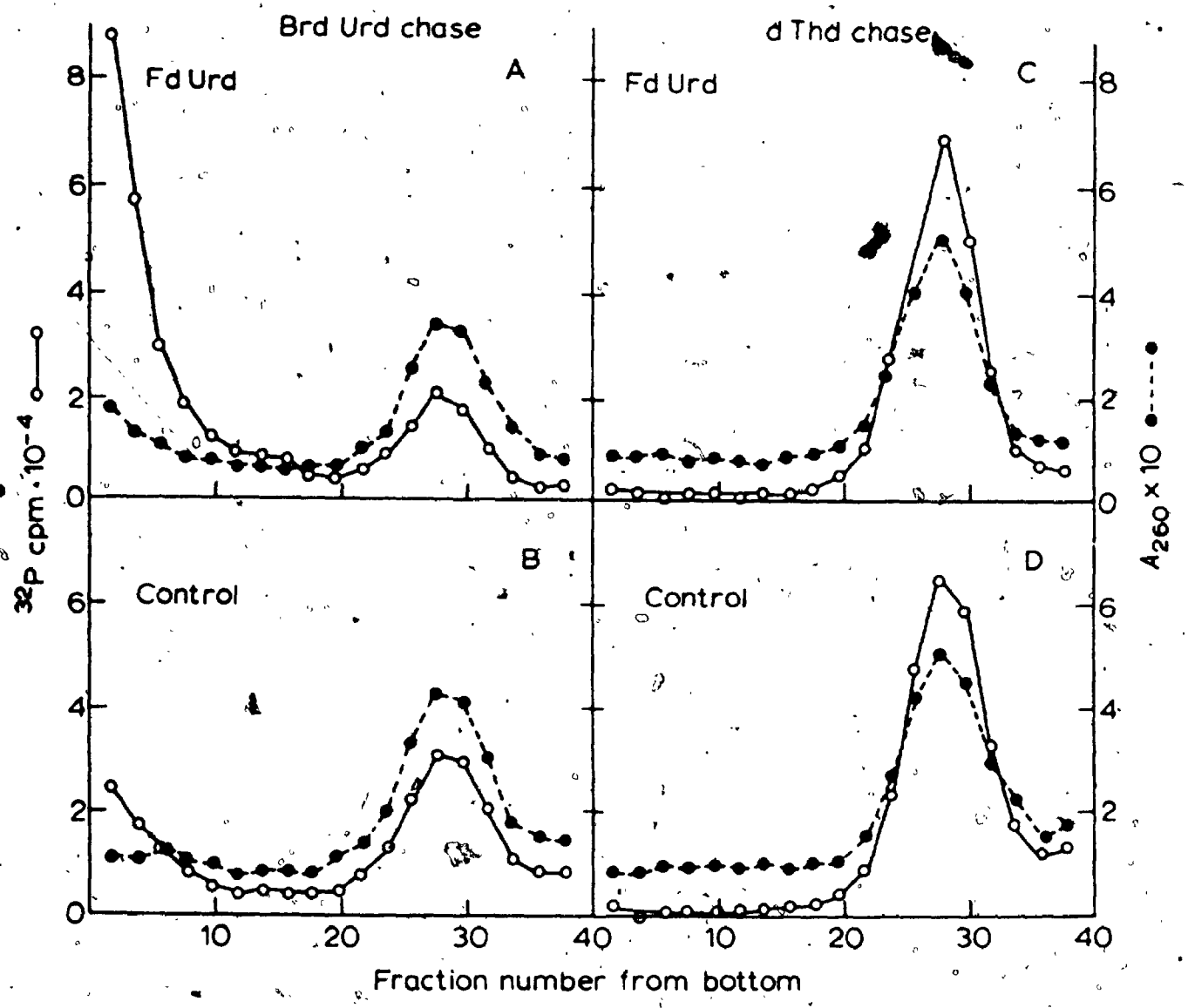

FdUrd
Control
^{3}H cpm · 10^{-5}
A_{260}
0
1
2
3
4
5
0.2
0.4
10
20
30
Fraction number from bottom

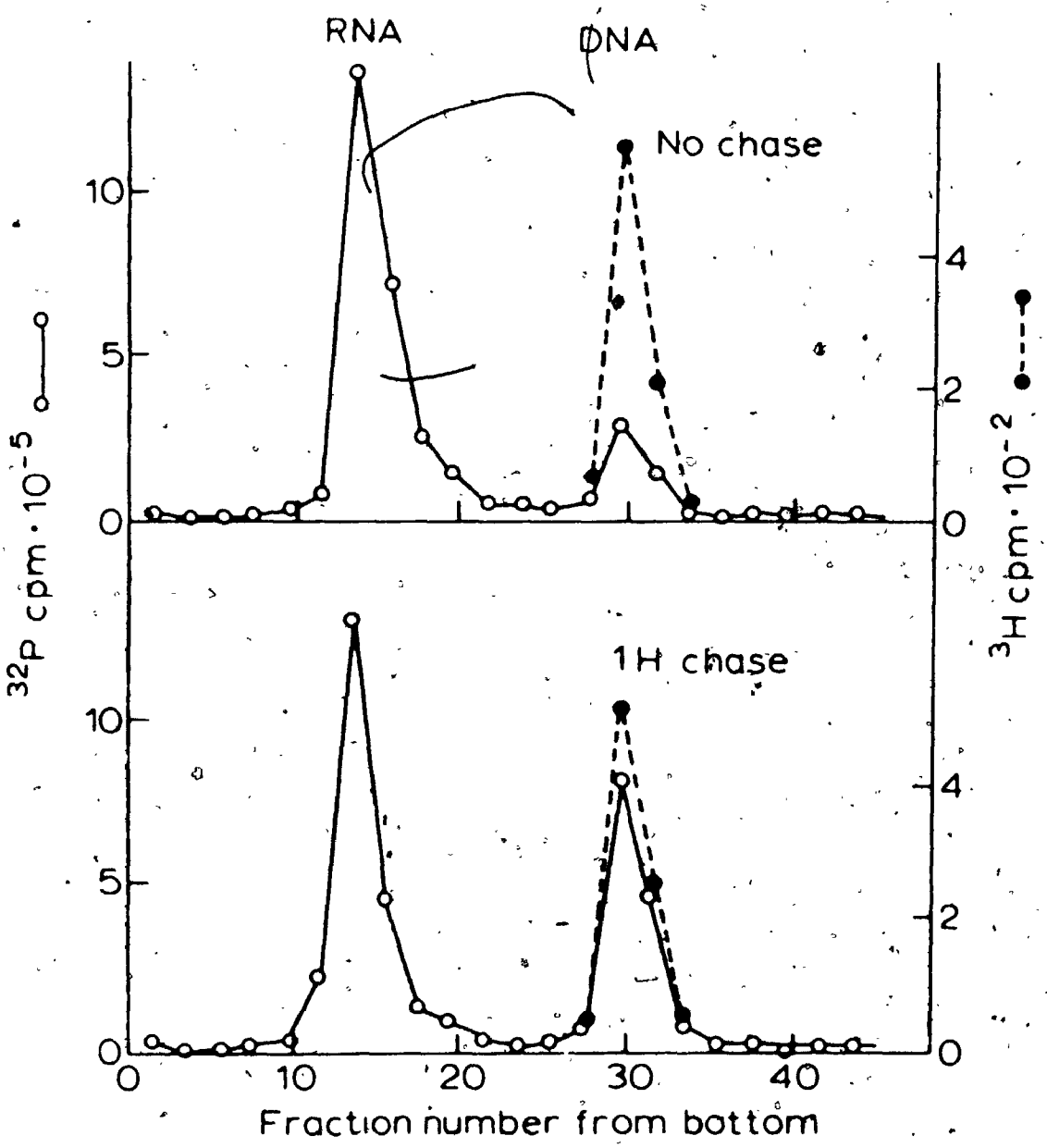
Fig. 3. Conversion of small DNA molecules formed in the presence of FdUrd into large DNA molecules. Cells were incubated for 16h in medium containing 10^{-7}M FdUrd then [^{32}P]-orthophosphate (10μCi/ml) was added and incubation was continued for 3h. The cells were washed and resuspended in medium containing 10^{-6}M FdUrd and either BrdUrd (5μg/ml) or thymidine (10μg/ml). After a 3h incubation DNA was isolated and analysed by density gradient centrifugation in alkaline cesium chloride solution. Control cultures were not treated with FdUrd before the 3h incubation with [^{32}P]-orthophosphate. Successive fractions were collected from the bottom of the tube. The DNA in each was precipitated with 5% trichloroacetic acid and caught on membrane filters for liquid scintillation counting.


Brd Urd chase
d Thd chase
Fd Urd
A
Fd Urd
C
Control
B
Control
D
^{32}P cpm · 10^{-4}
A_{260} x 10
Fraction number from bottom
0
2
4
6
8
10
20
30
40

the experiment with thymidine there was, naturally, no change in the density when the incomplete fragments of DNA became large. However, the thymidine profiles should be compared with the corresponding BrdUrd profiles (3A with 3C and 3B with 3D) to confirm the conclusion that DNA fragments were converted into larger pieces. That is, DNA appeared in the dense fractions at the expense of DNA in the fractions of normal density.

A further demonstration of the small size of the DNA that accumulates in the presence of FdUrd was provided during initial attempts to isolate this DNA. In the experiment illustrated by Fig. 4, L-cells were prelabelled with [^{3}H]-thymidine before an incubation in FdUrd for 16h and then the cells were pulse-labelled for 2h with [^{32}P]-orthophosphate. From one portion total nucleic acids were extracted with phenol and precipitated with ethanol. Another portion of the cells was washed thoroughly to remove non-incorporated [^{32}P] and then was incubated for 1h in medium containing thymidine. Total nucleic acids were then isolated. The nucleic acids from both portions of cells were heat-denatured and centrifuged in neutral cesium sulfate solution. The figure shows that much more [^{32}P]-DNA, which was synthesized in the presence of FdUrd, was isolated after the FdUrd block had been bypassed by adding thymidine. The [^{3}H]-prelabelled DNA provides an internal control to show that similar

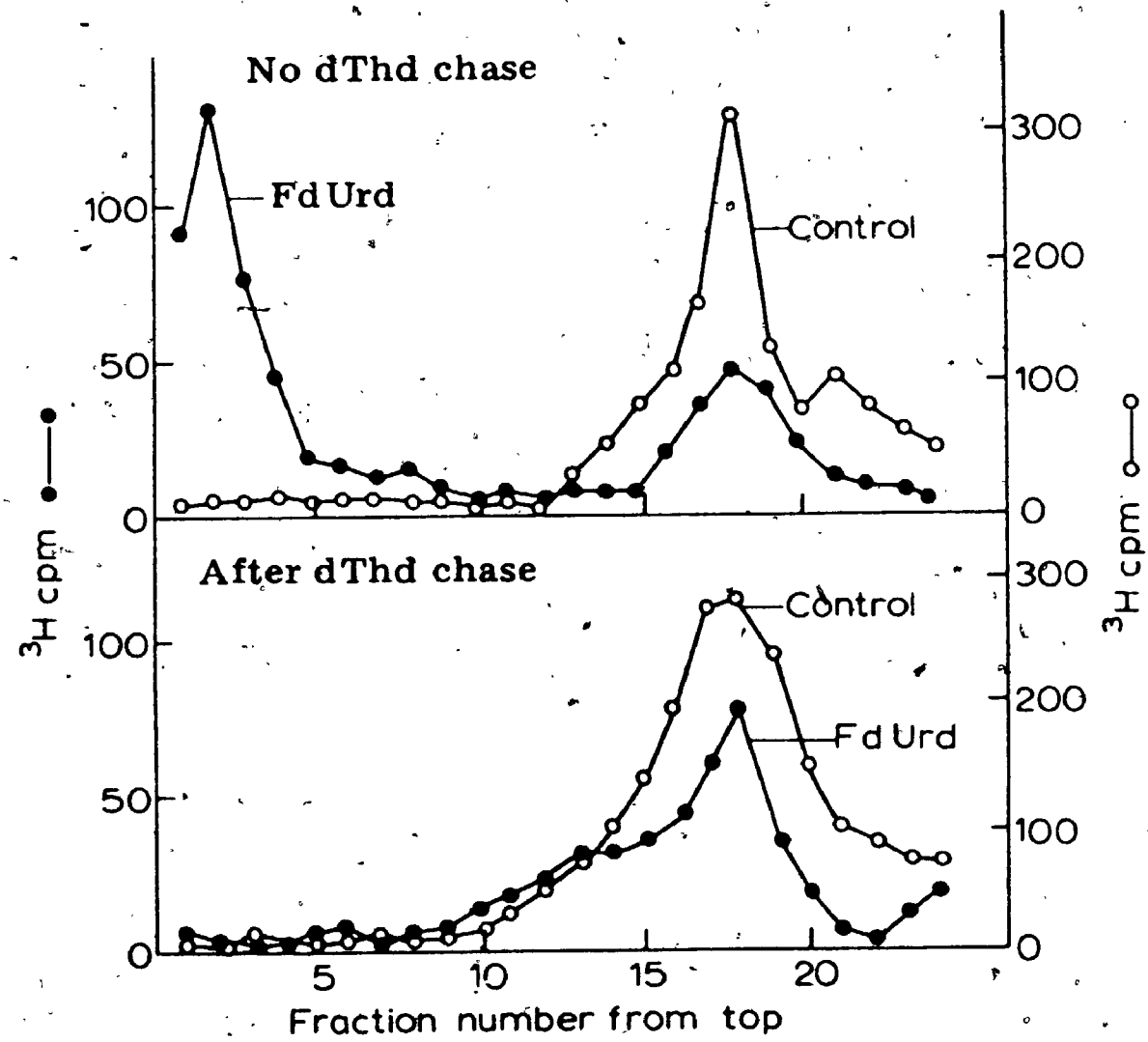
Fig. 4. Effect of a thymidine chase on the extraction of nucleic acids formed in the presence of FdUrd. Cells prelabelled with [^{3}H]-thymidine (1μCi/ml for 24h) were incubated with 10^{-7}M FdUrd for 16h, and then [^{32}P]-orthophosphate (10μCi/ml) was added and the incubation continued for 2h. After washing the cells thoroughly a portion of these was incubated in thymidine (10μg/ml) containing medium for 1h. From both groups of cells nucleic acids were then extracted, denatured and centrifuged to equilibrium in neutral cesium sulfate as described in the methods section. Successive fractions were collected from the bottom of the tube. The DNA in each was precipitated with 5% trichloroacetic acid and caught on membrane filters for liquid scintillation counting.


RNA
DNA
No chase
1H chase
10
5
0
4
2
0
^{32}P cpm · 10^{-5}
^{3}H cpm · 10^{-2}
0
10
20
30
40
Fraction number from bottom

amounts of pre-formed DNA were isolated in either case. Presumably, small pieces of DNA synthesized in the presence of FdUrd were not isolated by the phenol procedure. However, after thymidine was added, the small pieces of DNA were "chased" into larger pieces which were isolated readily. This notion was again supported by the results shown in Fig. 5. In this experiment, cells were incubated with FdUrd for 16h and then labelled for 2h with 5[^{3}H]-deoxycytidine. The cells were lysed on top of an alkaline sucrose gradient (5-20%) and centrifuged for 2h at 30,000 rpm. A substantial amount of acid precipitable label from FdUrd treated cells was recovered near the top of the gradient but this same material was diminished after a 1h chase with thymidine. A corresponding increase in radioactivity was then found near the position where high molecular weight DNA sedimented.

In order to characterize the small DNA fragments formed in the presence of FdUrd, cells were cultured on plastic petri dishes, treated with FdUrd for 16h and labelled with [^{3}H]-deoxycytidine for 2h. After trypsinization, the resulting cell suspension was lysed. The lysate was centrifuged at high speed to sediment the high molecular weight DNA (Fox et al., 1973), and the supernatant was poured off for further analyses. A portion of the supernatant was layered on a 5-20% alkaline sucrose gradient and centrifuged. After 3h and 5h of

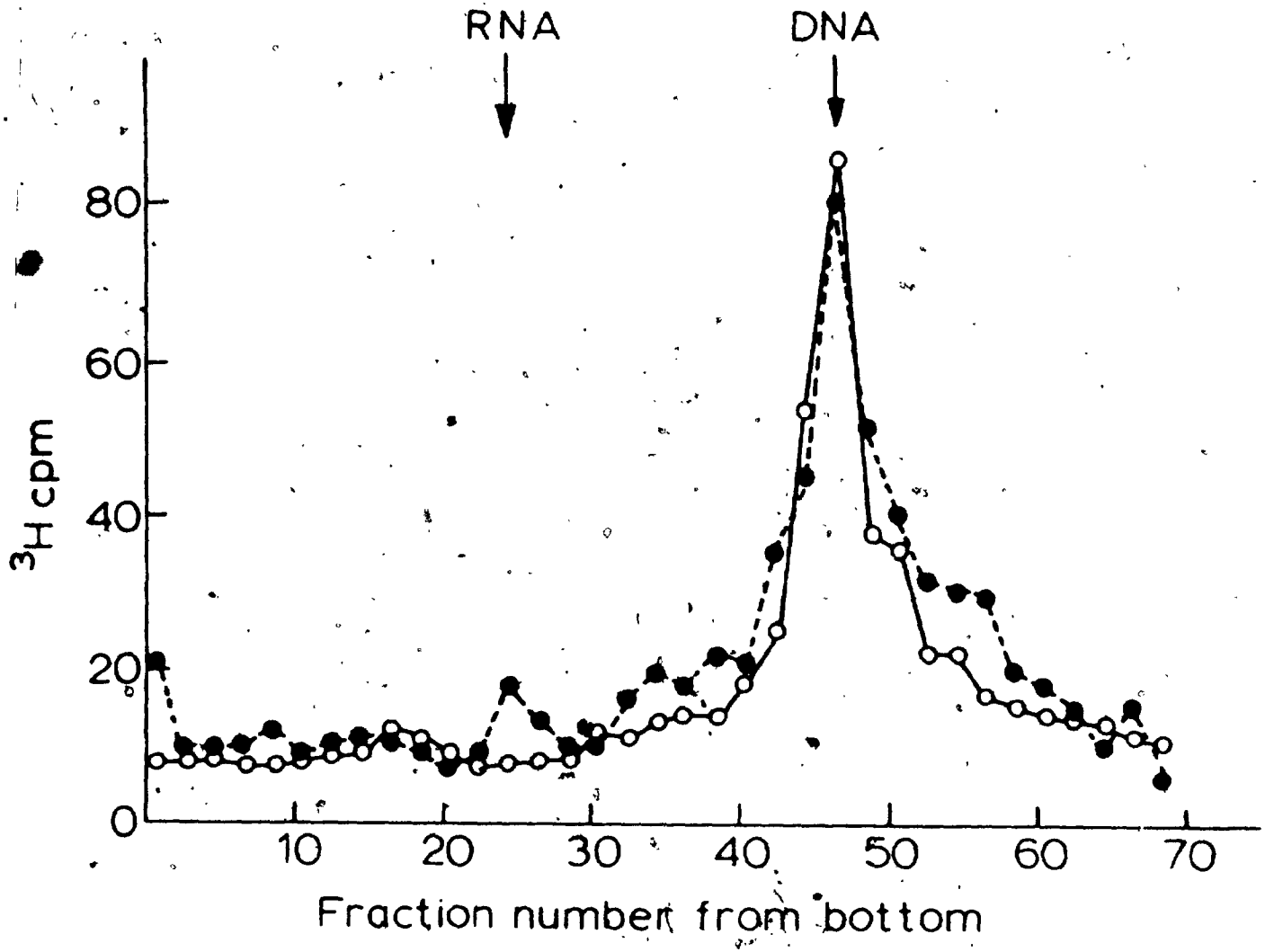
Fig. 5. Sedimentation pattern of DNA formed in the presence of FdUrd. Upper panel; cells (1×10^6) cultured on plastic petri dishes were incubated with or without FdUrd (10^{-7}M) for 16h before [^{3}H]-deoxycytidine (21.2 Ci/mmol) was added to a final concentration of 20 µCi/ml. After 2h of labelling, the monolayers were washed and trypsinized. Cells were lysed on top of 5-20% alkaline sucrose gradients for 6h before centrifugation and precipitation were carried out as described in the methods section. (o) DNA from control cells. (●) DNA from FdUrd treated cells. Lower panel; the protocol was the same as that above but after 2h labelling period the cells were washed and incubated for 1h in medium containing 5µg/ml of thymidine. Symbols as in upper panel.


No dThd chase
Fd Urd
Control
After dThd chase
Control
Fd Urd
^{3}H cpm
^{3}H cpm
0
50
100
0
100
200
300
5
10
15
20
Fraction number from top

centrifugation at 45,000 rpm, the radioactivity was found at 7.42 cm and 9.03 cm respectively from the center of rotation. The meniscus was 6.33 cm from the centre. Utilizing the sedimentation equation $\log x = \omega^2 s \cdot t + c$, a plot of log of distance sedimented against time yielded a sedimentation coefficient of 10S. This value, admittedly just a rough estimate, probably corresponds to a molecular weight of approximately 5×10^5 daltons.

The discovery of RNA-linked DNA fragments as synthetic intermediates (Magnusson et al., 1970; Sugino et al., 1972; Pigiet et al., 1973; Fox et al., 1973; Sadoff & Cheevers, 1973) prompted an examination of whether the DNA fragments isolated above also were attached to a stretch of ribonucleotides. The cell lysate obtained earlier was centrifuged to equilibrium in neutral cesium sulfate after it was heated for 5 min. at 100°C with or without the addition of NaOH to a concentration of 0.3M. Should these DNA fragments be linked to RNA, they would be expected to equilibrate at a slightly higher density than normal DNA in a neutral Cs_2SO_4 gradient. Treatment with alkali would remove the RNA and the fragments would have a buoyant density like that of normal DNA. As will be seen in Fig. 6, the RNA peak and the DNA peak are separated by 22 fractions and a shift of 1 fraction in the position of the DNA peak to the left would correspond to a DNA-RNA molecule containing 5% RNA. The size of the

Fig. 6. Isopycnic centrifugation of DNA formed in cells in the presence of FdUrd. Cells on 2 petri dishes (1×10^6/plate) treated and labelled as in Fig. 6 were pooled after washing and trypsinization. They were diluted into 4 volumes of a solution containing 0.5% sodium dodecylsufate, 0.5mM EDTA, 1mM Tris-HCl (pH 7.5). This lysate was then centrifuged at 45,000 rpm for 2h in the SW50.1 rotor of a Spinco centrifuge to sediment the high molecular weight DNA.[5] A portion of the supernatant was heated for 5 min. at 100°C (●) and centrifuged to equilibrium in neutral Cs_2SO_4 solution (Methods). A second portion was heated similarly in 0.3M NaOH, neutralized with HCl and centrifuged (o). Successive fractions were collected from the bottom of the tube. The DNA in each was precipitated with 5% trichloroacetic acid and caught on membrane filters for liquid scintillation counting.


RNA
DNA
80
60
40
20
0
^{3}H cpm
10
20
30
40
50
60
70
Fraction number from bottom

DNA being examined is about 5×10^5 daltons or 1800 nucleotides in length. Since no shift in the position of the DNA peak was observed, it is concluded that if a stretch of ribonucleotides is present there must be less than about 90 of them (5% of 1800).

2.4 DISCUSSION

It has been shown that DNA synthesis persists in L-cells exposed to a concentration of FdUrd that completely blocks cell division. Much of the DNA formed under this condition is small, with a single stranded molecular weight of about 5×10^5 daltons (10S). The rate of its synthesis relative to the rate in control cells was found to be about 15%. The estimated size of this DNA and the fact that it could be "chased" into a high molecular weight DNA by releasing the block of DNA synthesis, leads one to believe that it is a DNA unit that is formed in the normal course of synthesis and is ligated to similar units to form eventually the giant DNA molecules found in chromosomes (Kidwell & Mueller, 1969; Schandl & Taylor, 1969; Nuzzo et al., 1970; Cheevers et al., 1972; Fox et al., 1973). Goldstein and Rutman (1973) have described the assembly process in Ehrlich ascites cells as follows: dNTP's $\overset{a}{\rightarrow}$ x(9S) $\overset{b}{\rightarrow}$ y(30S) $\overset{b}{\rightarrow}$ 44S, where a is DNA polymerase, b DNA ligase, x = 20 and y = 3. The 44S unit is the size attained by parental DNA under the conditions employed by

Goldstein and Rutman. The DNA actually becomes much larger, at least 165S, but the size found in the centrifuge depends on the technique (Palcic & Skarsgard, 1972).

The accumulation of 10S DNA units in the presence of FdUrd is puzzling. Since the units were formed at all, there was obviously a pool of all 4 deoxynucleoside triphosphates. Why then did not the units become joined together? Obviously a portion of them did as evidenced by the results shown in Figs. 3 and 4 which showed that some of the DNA formed in the presence of FdUrd had a high molecular weight before the block was released. There appears therefore, to have been an inhibition of, or a delay in the joining process during the prolonged exposure to FdUrd. The mere presence of FdUrd cannot be the cause however, because when thymidine or BrdUrd is added to the inhibited culture without removing FdUrd, DNA of a large size is rapidly made. Also, during the initial hours of exposure to FUdR, DNA is made normally. A similar phenomenon was noted by Coyle and Strauss (1970) when HEp-2 cells were treated with hydroxyurea. A small molecular weight DNA accumulated in the presence of hydroxyurea but when the hydroxyurea was removed, the small DNA fragments were "chased" into large pieces. Hydroxyurea exerts its effect by decreasing the deoxynucleoside triphosphate pool by preventing the reductive conversion of ribonucleoside diphosphates to

deoxyribonucleoside diphosphates (Young et al., 1967). Thus in the case of both hydroxyurea and FdUrd, associated with a decrease in the precursor pool there is an unexpected inhibition of a late step concerned with formation of the final giant sized DNA molecule. In fact, it was shown recently by autoradiography that both hydroxyurea and FdUrd could inhibit DNA chain elongation (Hand & Tamm, 1973). One explanation for this effect is that in the presence of limiting precursor pool the rate of DNA chain initiation is greater than the rate of chain elongation. An analogous situation has been described by Olivera et al. (1973) for the in vitro synthesis of DNA with limiting amounts of deoxynucleoside triphosphates.

It has been suggested by Baumunk and Friedman (1971) that FdUrd does not in fact decrease the level of dTTP in HeLa cells, however their findings were questioned by Tattersall and Harrop (1973) who criticized their method of analyzing for dTTP. In this study it is certain that FdUrd decreases the dTTP pool size, first, because of the results of the radioactive nucleotide analysis performed on DNA synthesized in the presence of FdUrd. The excessive radioactivity of the thymidylic acid fraction of DNA reflected a decreased pool size of dTTP in the presence of FdUrd. Second, in a previous study using L-cells, it was found that the incorporation of BrdUrd into DNA was greatly enhanced in the presence of FdUrd

(Walker & Ewart, 1973a). This would not have been the case if FdUrd had not decreased the dTTP pool size.

It was asked whether the small DNA molecules synthesized in the presence of FdUrd would be joined covalently to RNA as was first reported by Sugino et al. (1972) for E. coli and later by others for mammalian cells (Fox et al., 1975; Pigier et al., 1973) and polyoma virus (Sadoff & Cheevers; 1973). What was looked for was material that in a neutral cesium sulfate gradient would have a slightly greater density than single stranded DNA and which would revert to the density of single stranded DNA after treatment with alkali (Sugino et al., 1972; Fox et al., 1973). No such material was seen. However, it is likely that in mammalian cells the RNA-DNA species has a size of 4S (Fox et al., 1973) and that when this unit has been converted to the 10S unit, the RNA has been removed.

This study has clarified a feature of repair synthesis of DNA that has long been a puzzle. Cleaver (1969) reported that FdUrd inhibited semi-conservative DNA synthesis but not repair synthesis. Since both processes presumably utilize dTTP as one of the precursors the finding of Cleaver did not make sense. The explanation now is, that in the presence of FdUrd the pool of dTTP is diminished but is sufficient for a normal rate of repair synthesis but not for semi-conservative synthesis. A similar explanation could apply to the effect of hydroxyurea

which also inhibits semi-conservative but not repair synthesis of DNA (Rasmussen et al., 1970). The use of these agents in the study of repair is illustrated by the experiments described in the following chapter.

Chapter 3

EFFECTS OF DIFFERENTIATION AND SUBCULTURING ON DNA EXCISION REPAIR IN MAMMALIAN CELLS

3.1 INTRODUCTION

During the differentiation of muscle cells, a number of enzyme activities rise and others fall (Shainberg, 1971). In the latter category is an enzyme or enzymes associated with the repair of DNA. Thus, Hahn et al. (1971) reported that methyl methanesulfonate-stimulated unscheduled DNA synthesis was readily apparent in freshly cultured rat embryo myoblasts, but this activity declined considerably as the myoblasts fused. Stockdale (1971) compared ultraviolet light stimulated unscheduled DNA synthesis in cloned chick embryo myoblasts and the multinucleated myotubes derived from them. The former were 2-4 times more active. In a subsequent paper, Stockdale and O'Neill (1972) showed that the unscheduled DNA synthesis was due to repair synthesis. The isolation by Yaffe (1968) of L_6 cells, an established line of myoblasts which has retained the ability to fuse and form myotubes, has provided a promising system for studying the differentiation of muscle cells. These cells display the same characteristic changes in biochemistry as freshly explanted embryonic muscle cells in culture (Shainberg, 1971), but are more amenable to experimental manipulation. It was decided therefore to examine the ability of cultured L_6 muscle cells, in the undifferentiated myoblast form and the differentiated myotube form, to repair

DNA.

In the same vein, repair synthesis was measured in human lymphocytes. Lymphocytes are able to remove chemical modifications from their DNA (Lieberman et al., 1971; Lieberman & Dipple, 1972) and to perform unscheduled synthesis (Spiegler & Norman, 1969; Clarkson & Evans, 1971; Slor, 1973). More interesting is that the level of repair incorporation in these cells is elevated after treatment with mitogens such as phytohemagglutinin (Darzynkiewicz, 1971; Jacobs et al., 1972) or concanavalin A (Scudiero et al., 1976). Since the lymphocyte is a very specialized cell type and the action of mitogen in these cells is essentially a re-initiation of DNA synthesis and cell growth, the increase in repair reciprocates the decrease seen during the differentiation of the myoblasts. Thus, by measuring repair synthesis in lymphocytes before and after they were stimulated by PHA, it was intended to illustrate further the effect of differentiation on DNA repair.

The influence of cellular changes on DNA repair was studied in a third system, that of subcultured fibroblasts. Ben-Ishai and Peleg (1974) have reported that although primary mouse embryo cells are competent in removing UV -induced thymine dimers from their DNA, the ability was lost after the cells were subcultured several times. It is also known that the established line

of mouse L-cells and certain hamster lines are unable to excise thymine dimers (Trosko et al., 1965; Klimek, 1966). This phenomenon of loss of repair after subculturing or after formation of an established line does not seem to apply to human cells (Painter et al., 1973; Clarkson & Painter, 1974). It was considered therefore that a study with primary rat fibroblasts would answer whether or not the effect of subculturing on repair could be observed in another species. The study would possibly also provide helpful information for interpreting the results obtained with the L_6 rat muscle cells.

Repair synthesis was measured by the incorporation of [^{3}H]-thymidine into unreplicated DNA, and the repair of DNA strand breaks was examined by sedimentation in an alkaline sucrose gradient. To induce repair, 4-nitroquinoline-1-oxide (4NQO) was used. This compound is mutagenic and carcinogenic, and its effect on biological systems is similar to that of ultraviolet light (Stich & San, 1973).

3.2 MATERIALS AND METHODS

Cell Cultures

The L_6 line of rat myoblast was carried as a monolayer culture in Dulbecco Modified Eagle's medium supplemented by 10% horse serum and gentamycin (50 µg/ml).

Cells were subcultured at .1 x 10^6 per culture flask ($75cm^2$; Falcon Plastics, Oxnard, Ca., U.S.A.) or at 1 x 10^5 per Petri dish (60 x 10 mm, Falcon), and 2-3-day-old cultures were used as myoblasts. On day 6, when fusion was well in progress, the cultures were treated with 10^{-7}M FdUrd for 24 hours whereby non-fused myoblasts became detached and were removed (Coleman & Coleman, 1968). The remaining myotubes were used on day 7. All experiments were done in Eagle's minimal essential medium supplemented by 10% fetal-calf serum and antibiotics; this second medium did not alter the growth of the cells.

Newborn rats (Wistar strain) were used for the preparation of fibroblasts. The inner abdominal walls of 4 to 6 rats were dissected, minced, and then stirred for 30 minutes in a solution containing 0.5% trypsin in 0.015M sodium citrate and 0.135M KCl (pH = 7.8). After passage through surgical gauze, the preparation was centrifuged. The pellet thus obtained was dispersed in Minimal Essential Medium to a density of 1 x 10^5 cells per ml. The medium was supplemented by penicillin, streptomycin, tylocin, and 10% fetal-calf serum. Portions of this cell suspension (30 ml) were then placed in 400 ml medicine bottles and incubated at 37°C in 5% CO_2, 95% air. Under these conditions, the cells doubled in 20-24 hours. Cells in confluent monolayers thus generated were retrieved by scraping with a rubber policeman. Dilution in halves with

fresh medium then was followed by transfer into clean bottles. After twelve such transfers, the generation time was lengthened to approximately 36 hours.

To isolate lymphocytes, human blood was obtained from healthy adult males. The lower one-third of the plasma layer was collected after the heparinized blood was centrifuged at 1,200 rpm for 10 min., and the plasma was diluted with an equal volume of Balanced Salt Solution (BSS: Favour, 1964). A 5 ml sample of this diluted plasma was then layered on top of 3 ml of Lymphoprep (Nyegaard and Co. As. Oslo.) and centrifuged at 1,700 rpm for 30 min. (Boyum, 1968). The resulting lymphocyte layer at the interface was resuspended and centrifuged twice in BSS before the final pellet was suspended at a density of 1×10^6 cells/ml in medium supplemented as described earlier, and maintained in 25 cm^2 tissue culture flasks (Falcon). For mitogen stimulation, phytohemagglutinin (PHA) was added to a final concentration of 0.4% (v/v), and the culture was incubated at 37°C for 72 hours before each experiment. To prevent clumping, 20% serum was used during the incubation with PHA. Medium supplemented by 10% serum, however, was used in the repair experiments.

Measurement of Repair Synthesis

i. Using [^{3}H]-thymidine

The method used to measure repair synthesis was

similar to that of Roberts et al. (1968). Each flask of cells containing 10 ml of medium was incubated at 37°C with FdUrd (10^{-6}M) and BrdUrd (5 µg/ml) for one half hour, followed by 10^{-5}M 4NQO for one hour. Controls received saline instead of 4NQO solution. The medium was then changed to one containing FdUrd and BrdUrd as before plus hydroxyurea (10^{-2}M) and [^{3}H]-thymidine (5µCi/ml, sp.act. = 21.2 Ci/mmol). The effect of using [^{3}H]-thymidine and BrdUrd simultaneously is equivalent to that of using [^{3}H]-BrdUrd and incorporation of [^{3}H] would essentially indicate substitution by BrdUrd. Hydroxyurea was present to suppress semi-conservative DNA synthesis and the protocol was designed to minimize terminal labelling of incomplete DNA fragments. The incorporation of BrdUrd into replicating DNA increases the buoyant density of the molecule which then, is distinguishable from unreplicated DNA in a CsCl gradient. Since repair synthesis involves the incorporation of only very small amounts of nucleotide precursors, incorporation of BrdUrd during repair is not sufficient to alter significantly the buoyant density of the DNA (Edenberg & Hanawalt, 1972). In other words, radioactivity recovered in DNA of normal density would indicate the repair phenomenon.

The method for isolating DNA was essentially that of Flamm et al. (1969). Two identically treated flask-cultures were pooled for each experiment. After a 3 hour

labelling period, the cells were washed, scraped off, and resuspended in 0.5 ml of a solution containing 80 mM EDTA and 20 mM NaCl. Then, 5 ml of the same solution containing, in addition, 1% triton X-100 (Sigma, St. Louis, Mo., U.S.A.) was added and the samples were kept in ice for 10 min. These broken cell preparations were centrifuged at 1000xg for 10 min., and the nuclei in the pellet were lysed in 0.4 ml of 1 mM Tris-HCl, 0.5 mM EDTA, 0.5% SDS (pH 7.6). This lysate was mixed with 4 ml of CsCl solution (1.44 g CsCl per ml of buffer containing 10mM Tris-Hcl, 1mM EDTA, pH 8, density = 1.77 g/cc), and centrifuged at 10,000 rpm for 30 min. The protein which precipitated from the lysate formed a thin layer at the top of the tube. 4.0 ml of the clear solution (ρ = 1.71 g/cc) was carefully transferred into a cellulose nitrate tube, covered with oil and centrifuged at 45,000 rpm and 20°C for 24 hours. Fractions measuring 0.25 ml were collected and on some of these, refractive indices were measured in order to determine the density. The fractions were then diluted to 1 ml with water and their optical densities were read at 260 nm. A 0.2 ml sample of each diluted fraction was mixed with 10 ml of Aquasol (NEN, Boston, Mass., U.S.A.) for liquid scintillation counting.

ii. Using [^{32}P]-orthophosphate

The measurement of repair synthesis was carried out exactly as described above except that carrier-free

[^{32}P]-phosphate (20μCi/ml of medium) and [^{3}H]-thymidine (10μCi/ml) were used for nucleotide labelling during the post treatment incubation period. After centrifugation in CsCl, the parental DNA fractions (density = 1.70-1.71 g/cc) from myoblasts and myotubes were dialysed against a solution containing 0.0015M sodium citrate, 0.015M NaCl, and then mixed with 2 ml of a buffer containing 0.08M Na_2HPO_4, 0.11N NaOH (pH 12.5) and enough distilled water to give a final volume of 4.5 ml. These preparations were added to 6.5g CsCl and the solutions were centrifuged in a Ti50 rotor at 37,000 r.p.m. and 20°C for 36 hours. Fractions of 0.35 ml were collected and each was neutralized by 0.7 ml of 0.005N HCl. Optical densities were measured at 260 nm. An equal volume of 10% trichloroacetic acid was added to the fractions. The precipitate was caught on fibre glass filters prior to liquid scintillation counting.

Phosphodiesterase digestion

After centrifugation in CsCl, the fraction containing parental DNA (density = 1.70-1.71 g/cc) of the 4NQO treated culture were pooled and dialysed. The sample was mixed with buffer such that the final preparation contained 30mM Tris-HCl, 70mM $MgCl_2$ (pH 8.5). It was then mixed with purified [^{32}P]-DNA and heated for 15 min. at 90°C. The [^{32}P]-DNA was isolated from BHK cells which had been grown for 24 hours in medium containing [^{32}P]-orthophosphate (0.2 μCi carrier free radiophosphate added per ml of medium).

Venom phosphodiesterase (Worthington Biochemical Corp.) was dissolved in the above mentioned buffer and added to the DNA to a final concentration of 20 µg/ml. The reaction mixture was incubated at 37°C. Aliquots measuring 0.5 ml were removed at intervals and mixed with an equal volume of bovine serum albumin (1 mg/ml). After precipitation with trichloroacetic acid, radioactivity in the supernatant was counted in Aquasol.

Incorporation of Thymidine into Total DNA

Separate cultures in petri dishes were treated with 1×10^{-5}M 4NQO or saline for 1 hour before being incubated with [^{3}H]-thymidine (10 µCi/ml) and hydroxyurea (10^{-2}M) for 1,3, and 8 hours. The cells were retrieved by scraping, counted, and then frozen in 2 ml of a buffer containing 30 mM Tris-HCl, 70 mM $MgCl_2$ (pH 8.5). The number of myotube cells was taken to be equal to the number of myoblast cells measured in a replicate culture on the previous day. The cell suspensions were thawed and bovine serum albumin was added to a final concentration of 1 mg/ml. An equal volume of 10% trichloroacetic acid was used for precipitation. After filtration, the filters (25mm, Type A glass fibre, Gelman Instrument Co., Ann Arbor, Michigan, U.S.A.) were counted in Aquasol.

Alkaline Sucrose Gradient Sedimentation Analysis

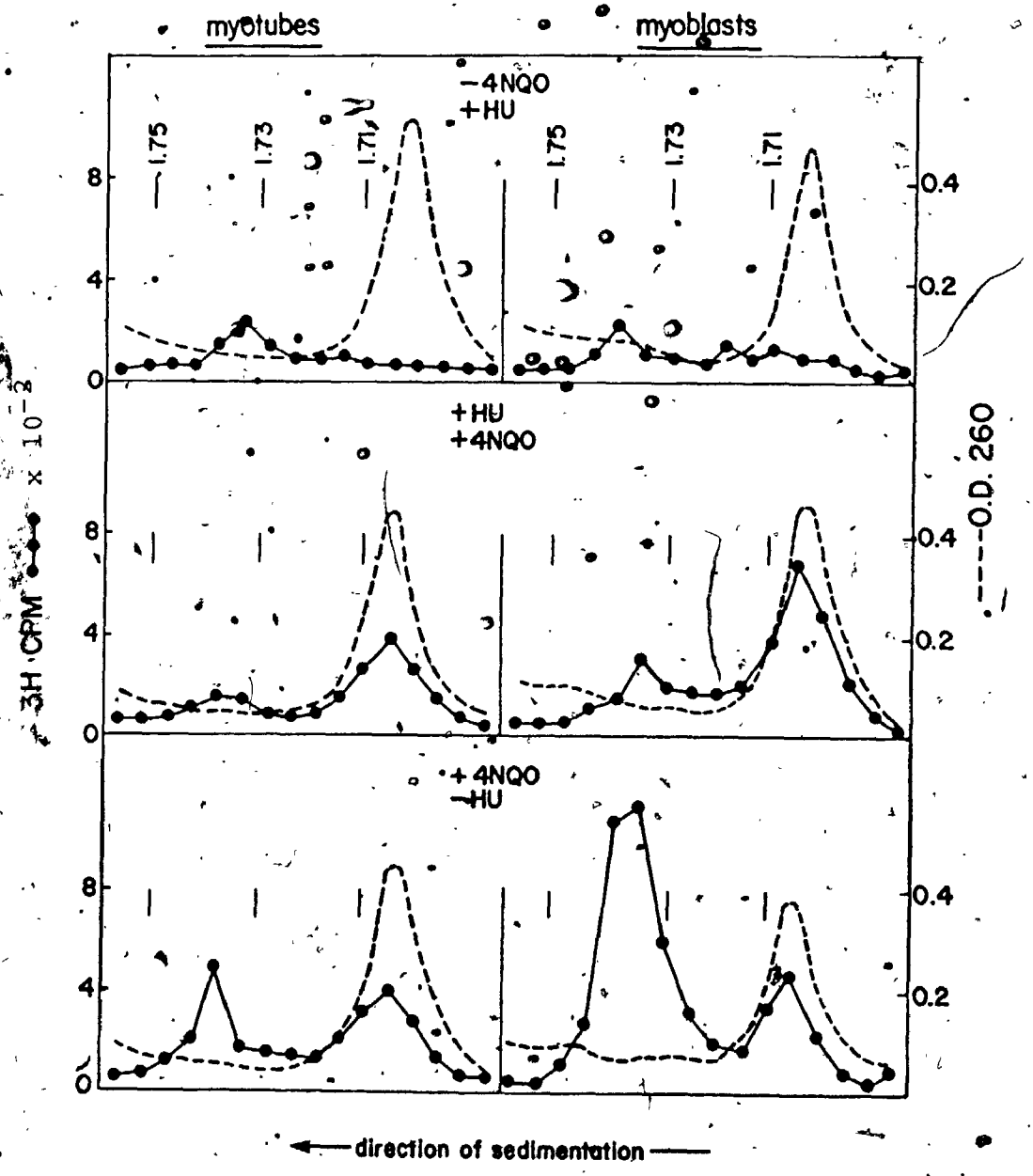
Two-day old cultures in petri dishes were incubated with [^{3}H]-thymidine (0.5 µCi/ml) for 24 hours, washed and replenished with fresh medium. They were used either immediately (myoblasts) or after fusion had occurred (myotubes). Single-strand breaks in DNA were produced by treating the cultures with 1 x 10^{-5}M 4NQO for 1 hour or by exposing them to 6 Krad of gamma radiation. The latter was delivered by a Cobalt^{60} Gamma Cell (Atomic Energy of Canada Ltd.) at a dose rate of 12 Krad/min. The cell samples were analysed then or after a post-treatment period of 3 or 5 hours in fresh medium. Sedimentation analysis was carried out as previously described (Walker & Ewart, 1973b). Briefly, 4.7 ml sucrose gradients (5-20%) in a solution containing 0.3M NaOH, 0.01% sodium dodecylsulfate, and 0.001M EDTA were made by an ISCO 570 gradient former (Instrumentation Specialties Co., Lincoln, Nebraska), and 0.3 ml of a lysing solution containing 0.5M NaOH, 0.2% SDS and 0.01M EDTA was layered on top of each gradient. Approximately 1 x 10^4 cells were delivered onto each gradient. The gradients were allowed to stand at room temperature for 10-12 hours before they were centrifuged at 15,000 rpm and 20°C for 4.5 hours in a SW 50.1 rotor. Fractions (0.2 ml) were collected from the top using an ISCO 640 fraction collector, and neutralized by HCl. Radioactivity in each fraction was then measured by liquid scintillation

counting.

3.3 RESULTS

The ability of the L_6 line of myoblasts and myotubes for repair synthesis of DNA was compared using the method of isopycnic centrifugation to separate the newly replicated DNA from the repaired but not replicated DNA. The profiles in Figure 7 show the banding positions of the DNA in the CsCl gradients. The position of parental DNA is marked by the large peak of $O.D._{260}$ absorbing material occurring at a buoyant density of 1.70-1.71 g/cc. Newly synthesized DNA, containing one bromouracil substituted strand and one ordinary strand has a density of 1.73-1.74 g/cc as indicated by the radioactivity in this region. The radioactivity associated with parental DNA is indicative of repair synthesis. The specific activity of this DNA was measured after the appropriate fractions were pooled and the CsCl removed by dialysis. In the control cultures not treated with 4NQO (top panel), these values in cpm/μg DNA were 2.6 for the myoblasts and 8.8 for the myotubes. In the 4NQO-treated cultures (middle panel), the values were 112 and 66.5 respectively. The myotubes exhibited about one half the repair activity of the myoblasts. The small amount of incorporation in the controls was probably due to incomplete resolution of semi-conservatively synthesized DNA although hydroxyurea

Fig. 7. Isopycnic centrifugation of DNA in neutral CsCl. Gradient profiles of DNA isolated from control cultures (top panel) and from treated cells (middle panel). In two samples (bottom panel), hydroxyurea was omitted during the incorporation period. Numbers and vertical bars in the diagram represent the densities of the corresponding fractions in g/c.c.


myotubes
myoblasts
-4NQO
+HU
1.75
1.73
1.71
+HU
+4NQO
+4NQO
-HU
8
4
0
0.4
0.2
3H CPM x 10⁻³
O.D. 260
direction of sedimentation

was used to inhibit normal DNA synthesis. Similar results were obtained without the use of hydroxyurea (lower panel) but in this case, the fractions containing parental DNA had to be recentrifuged in alkaline CsCl in order to effect a sufficiently clean separation of newly synthesized DNA from parental DNA. The results shown in the lower panel of Fig. 7 also indicate that the myotube culture contained only a few dividing myoblasts because the amount of semiconservative synthesis in the myotube culture was small relative to that in the myoblast culture.

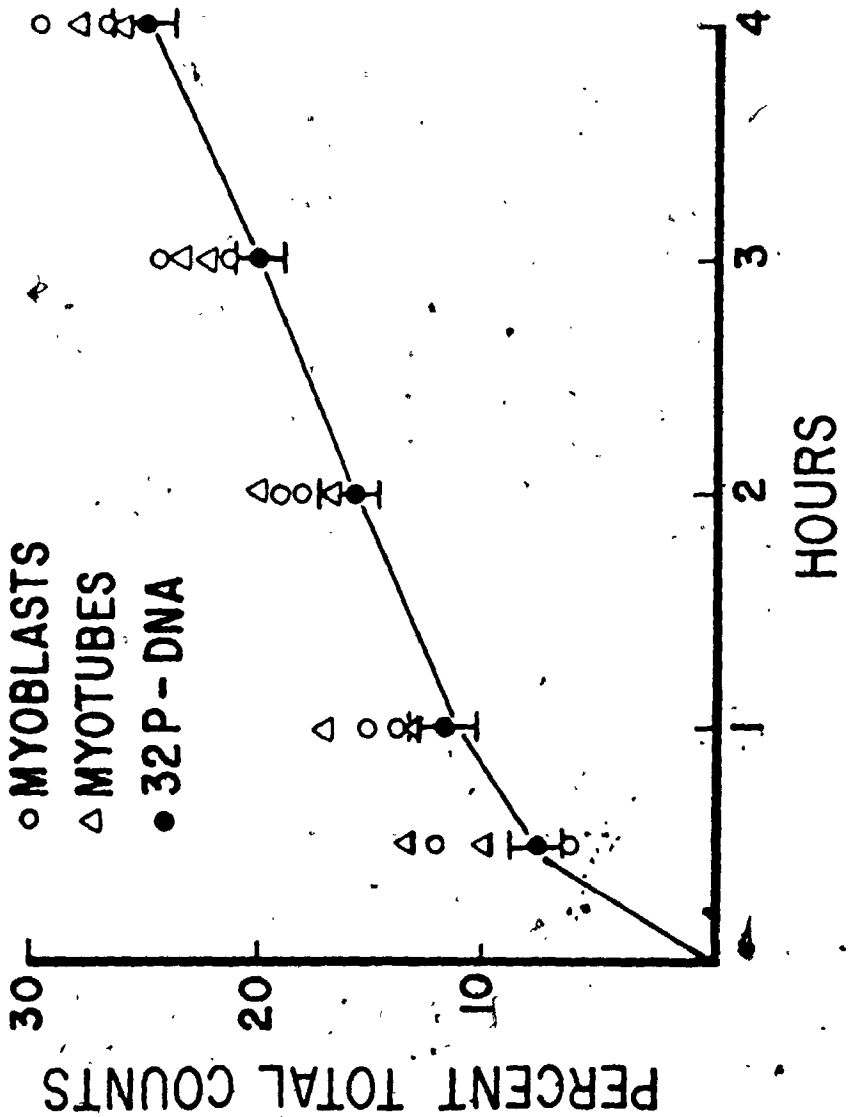
One explanation for the lower specific activity obtained with the myotubes was that the labelled thymidine was diluted by a larger nucleotide pool in the myotube. This possibility was checked by following the repair process with an additional label, [^{32}P]. Assuming [^{32}P] labelled all four nucleotide triphosphates uniformly, the [^{3}H]:[^{32}P] ratio in the repaired DNA would be directly proportional to the specific activity of [^{3}H]-thymidine after it was equilibrated with the intracellular nucleotide pool. When the experiment was done (see materials and methods section) the [^{3}H]:[^{32}P] ratios for myoblasts and myotubes were found to be 2.60 and 2.26 respectively. That is, the dilution of [^{3}H]-thymidine, by itself, cannot account for the difference in repair activity observed.

An error in the measurement, however, could result from the presence of replicating DNA molecules which have

not completed making a new strand at the time of addition of [^{3}H]-thymidine. End-addition of radioactive nucleotides to pre-existing DNA would give exaggerated values for repair synthesis. Although the probability that the parental DNA fraction did contain some of these molecules was reduced by the incubation with BrdUrd before 4NQO treatment, possible contamination must be evaluated. Therefore, the rate at which radioactive nucleotides were released from parental DNA by snake venom phosphodiesterase was examined. Parental DNA fractions obtained from the 4NQO-treated cultures were denatured by heat before digestion. For comparison, the digestion mixture also contained DNA that was uniformly labelled with [^{32}P]. Fig. 8 shows the time course of release of acid-soluble radioactivity. Clearly, the nucleotides incorporated by the 4NQO-treated cells were not more susceptible to the exonucleolytic attack than those in uniformly labelled [^{32}P]-DNA. This indicates that the labelled nucleotides incorporated after 4NQO treatment were located internally rather than at the ends of DNA strands. Thus, the repair activity in the myoblasts is unlikely an artefact effected by end-addition.

The preceding results indicate that the initial rate of repair synthesis in myotubes was about one half of that in myoblasts, but it seemed possible that in myotubes the repair process might persist for a longer

Fig. 8. Release of acid-soluble radioactivity after phosphodiesterase digestion of uniformly labelled [^{32}P]-DNA, and repaired [^{3}H]-DNA from myoblasts and myotubes. Treatment of cells and digestion by phosphodiesterase were as described in the methods section. Duplicated samples were used for repaired DNA. The curve obtained with [^{32}P]-DNA represents the mean of four samples, with variations included in the error bars. Total [^{32}P] per sample = 3,500 cpm; total [^{3}H] per sample = 400-800 cpm. The enzyme: substrate ratio by weight was about 4:1 in all samples.


PERCENT TOTAL COUNTS
30
20
10
HOURS
1
2
3
4
○ MYOBLASTS
△ MYOTUBES
● 32P-DNA

period of time and eventually give the same total extent of repair. This possibility was investigated through a study of the kinetics of incorporation of [^{3}H]-thymidine into the DNA of 4NQO-treated myotubes and myoblasts in the presence of hydroxyurea (Fig. 9). The use of hydroxyurea has effectively inhibited semi-conservative DNA synthesis as judged by the slight incorporation of [^{3}H]-thymidine in the control cells. Treatment with 4NQO has stimulated a considerable further incorporation. The previous results indicated that this incorporation was due to repair synthesis. It is apparent from the results shown in Fig. 9 that the initial rate of repair synthesis was greater in the myoblasts. In addition, at the end of an eight hour post-treatment incubation, when the incorporation was reaching a plateau, the response to 4NQO by the myotubes remained at about 50% of that of the myoblasts. The fusion of myoblasts has led to a reduction in both the rate and the total amount of repair synthesis.

That repair synthesis is influenced by the state of differentiation of a cell is further illustrated by the experiments with human lymphocytes. Fig. 10 shows the results obtained in this case. The preparation of unstimulated lymphocytes showed no DNA synthesis but after the PHA treatment, DNA synthesis was evident in the culture not

Fig. 9. Kinetics of 4NQO stimulated incorporation of [^{3}H]-thymidine into DNA of myoblasts and myotubes in the presence of hydroxyurea. The number of cells in the fused cultures was estimated separately from confluent plates just before fusion occurred. Each point in the figure is the average of results obtained from duplicated samples.

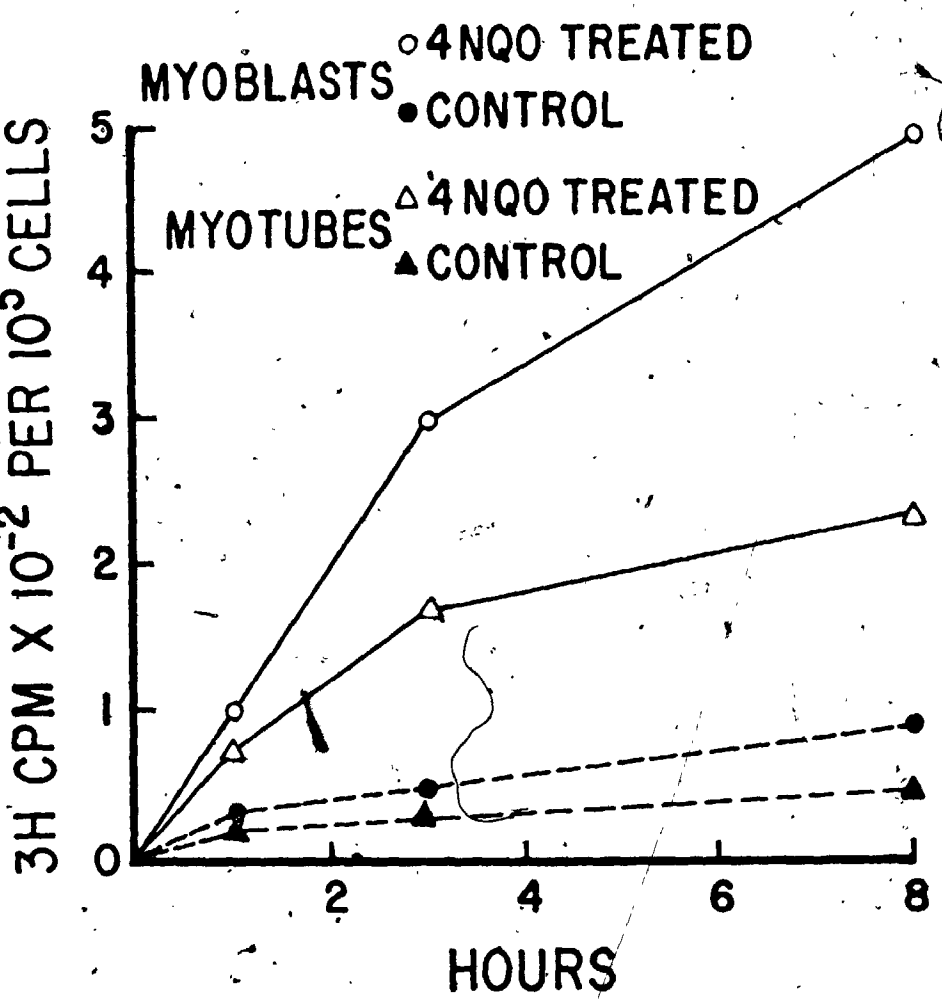
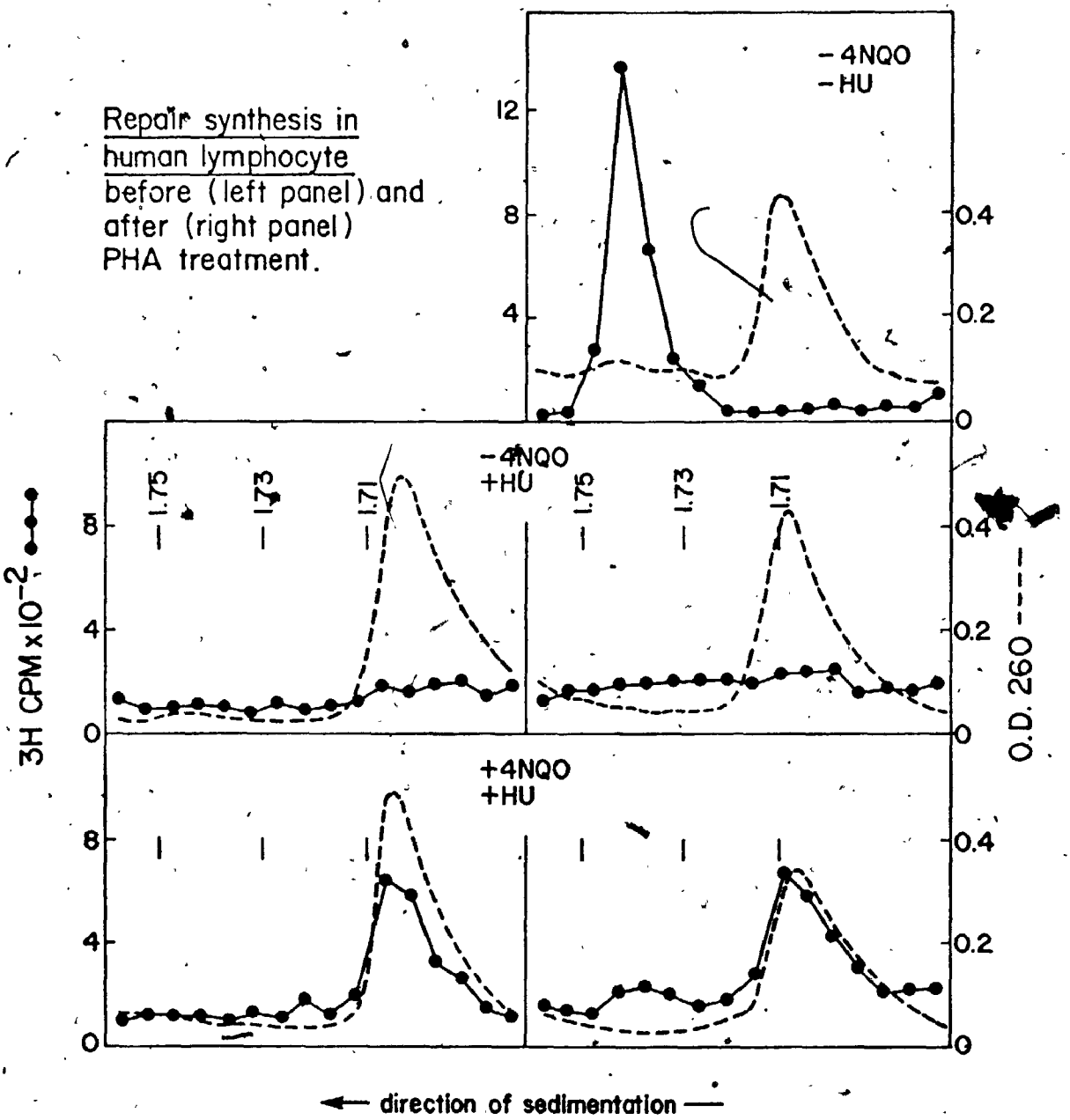

MYOBLASTS
○ 4NQO TREATED
● CONTROL
MYOTUBES
△ 4NQO TREATED
▲ CONTROL
3H CPM X 10⁻² PER 10⁵ CELLS
0
1
2
3
4
5
2
4
6
8
HOURS

Fig. 10. Repair synthesis in human lymphocyte. Isolation of lymphocytes and treatment with phytohemagglutinin (PHA) were as described in section 2.2. Measurement of repair synthesis followed the procedures described for myoblasts and myotubes, except that suspension cultures were used in this case.



Repair synthesis in human lymphocyte before (left panel) and after (right panel) PHA treatment.

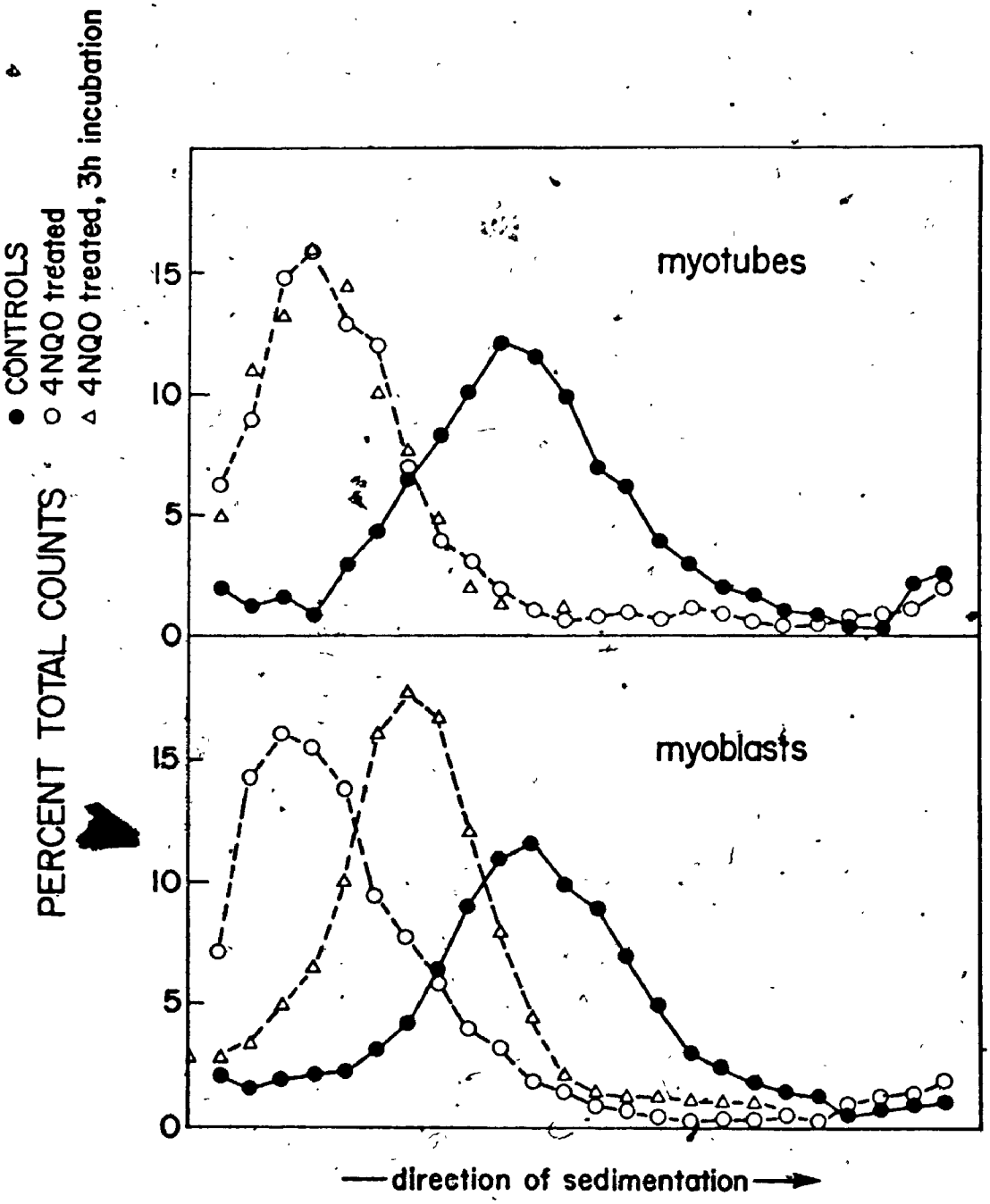
treated with hydroxyurea, resulting in the formation of bromouracil-hybrid DNA which has a buoyant density of close to 1.75 g/cc. (upper right panel of Fig. 10). Suppression of this replication reveals the 4NQO-induced incorporation of radioactive thymidine into unreplicated DNA (middle and lower panels), and as shown in Table II, the specific activity in this fraction was elevated almost three-fold by the PHA treatment. This observation reciprocates that of the myogenic system. A logical explanation would be that PHA has prompted the production of some repair enzyme which is present only at a low level in normal resting lymphocytes. Accordingly, it seems possible the reduction in repair synthesis during the fusion of myoblasts is due to a decrease of the level of enzyme which is required for the repair process.

As a further means of characterizing the DNA-damage and repair process in myoblasts and myotubes the technique of sedimentation in an alkaline sucrose gradient was employed. It had previously been shown with this technique (Walker & Sridhar, 1976) that 4NQO treatment of HeLa and L-cells led to the appearance of single-strand breaks in the DNA from these cells. This damage was seen to be repaired by HeLa but not L-cells. The sedimentation patterns for the DNA from the 4NQO treated myoblasts and myotubes are shown in Fig. 11. The effect of the 4NQO

Cells	Repair Synthesis (cpm/μg DNA)	
	Controls	4NQO Treated
Rat L_6 Myoblasts:		
non fused	2.6	112.0
fused	8.8	66.5
Human Lymphocyte:		
resting	8.1	44.0
PHA-stimulated	12.5	114.0

TABLE II. Effects of fusion in myoblast and of PHA stimulation in human lymphocytes on repair synthesis. The parental DNA fractions from Fig. 7 and 10 were dialysed against standard saline citrate before their specific activities were measured. DNA contents of the dialysed fractions were calculated from absorbance measurements at 260 nm and radioactivity was assayed by scintillation counting.

Fig. 11. Sedimentation patterns of DNA in alkaline sucrose gradients after 4NQO treatment. The 4NQO (1×10^{-5} M) treatment of prelabelled cells was carried out for 1 hour, and medium was replaced for further incubations. The controls received no 4NQO. Each gradient contained $10\text{-}14 \times 10^{3}$ cpm.


● CONTROLS
○ 4NQO treated
△ 4NQO treated, 3h incubation
PERCENT TOTAL COUNTS
myotubes
myoblasts
15
10
5
0
direction of sedimentation

treatment was the same for both cell types, namely, a reduction in the sedimentation coefficient of the DNA from a control value of approximately 160 S to a value of approximately 30 S. When the cells were incubated for 3 hours in fresh medium after the 4NQO treatment, and then layered onto the gradient for lysis and centrifugation, the DNA of the myoblasts had increased in size, whereas that of the myotubes remained unchanged. An additional 2 hours post-treatment incubation produced no further change in either case. Thus, after myoblasts have fused, there is a complete loss of the ability to repair a form of damage that is seen as a single-strand break in alkali and yet there is only partial loss of ability for repair synthesis. One explanation of these findings is that the repair synthesis observed is incomplete and the rejoining step is left undone by the myotubes, but the internal location of the nucleotides incorporated during repair (Fig. 8) disproves the presence of open-ended repaired regions in the DNA. Rather, it seems that the nature of the breaks induced by 4NQO is the underlying cause of our observation. That a further 2 hours post-treatment incubation of myoblasts did not alter the sizes of the DNA indicates that some breaks were never rejoined.

To further investigate the rejoining step, X-irradiated cells were examined. X-rays can directly induce single-strand breaks which are thought to be

repaired by the insertion of only one or a few bases per lesion followed by rejoining (Painter & Young, 1972; Fox & Fox, 1973; Regan & Setlow, 1974). The use of X-rays then, in effect, allows the rejoining step to be viewed by itself. The sedimentation patterns in Fig. 12 were obtained with X-irradiated myoblasts and myotubes before and after a 5 hour recovery period. A decrease in the size of the DNA after irradiation signalled the induction of breaks. These were rejoined after post-treatment recovery, leading to a restitution of the size of DNA comparable to that of the control cells. More important is that the myotubes are also able to repair X-ray induced breaks. It appears that they do possess the enzyme or cofactors required for rejoining. This observation, besides providing additional evidence against an incomplete repair process, further implies that the lack of rejoining of 4NQO-induced breaks is likely a consequence of some peculiarity associated with 4NQO.

The apparently paradoxical effect of 4NQO was also evident in the subcultured fibroblasts. In response to 4NQO, primary fibroblasts incorporated radioactive thymidine into their parental DNA (Fig. 13). The amount of repair incorporation, assayed as before, decreased as the cells were subcultured (Table III), although a significant amount of repair was still evident after the cells were subcultured seven times. On the other hand, the

Fig. 12. Repair of X-ray induced breaks. Prelabelled cells were irradiated with a dose of 6 krad and their DNA was analysed before and after a 5h recovery in fresh medium. The control cultures were not irradiated. Each gradient contained 10-15 x 10^3 cpm.

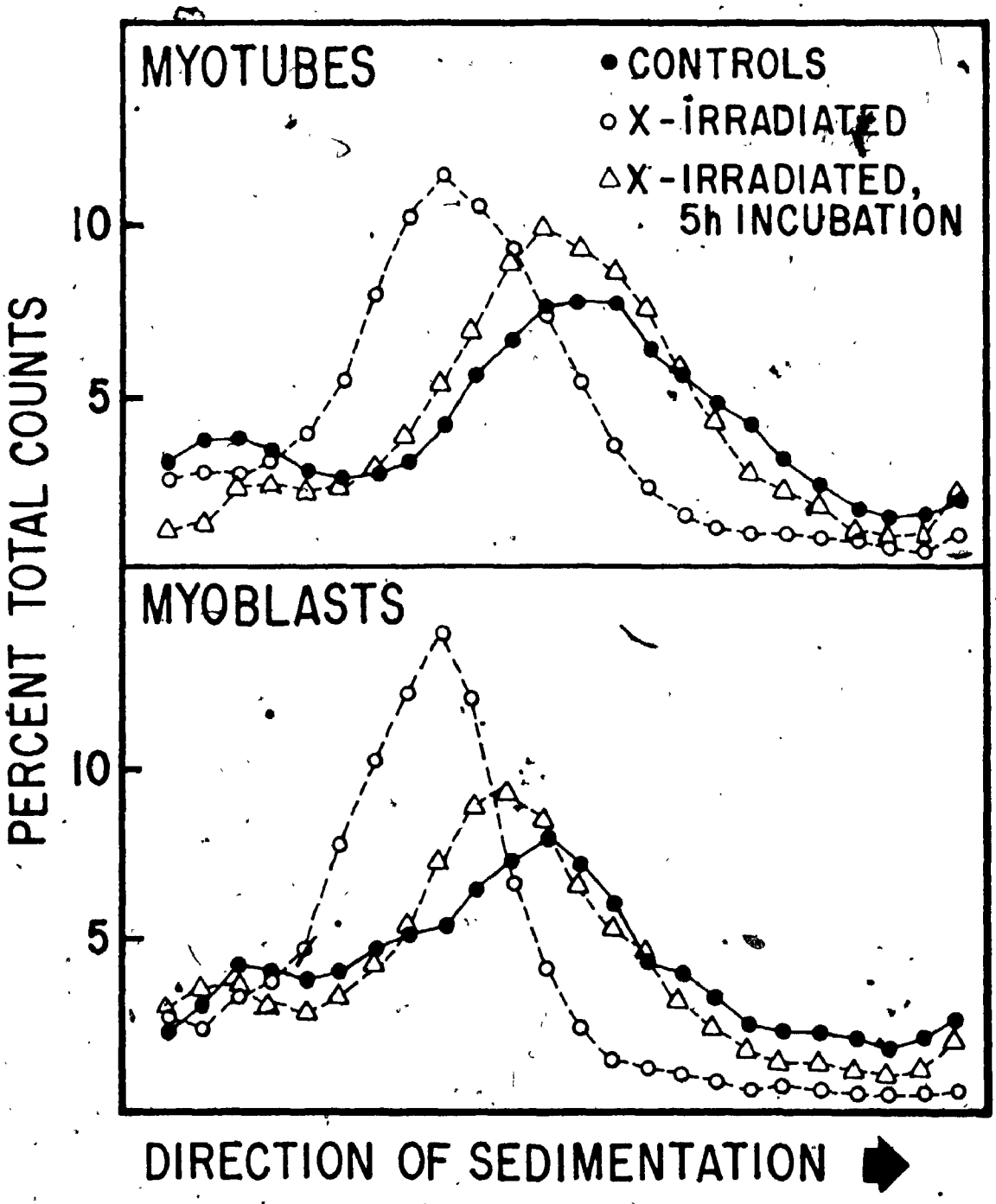
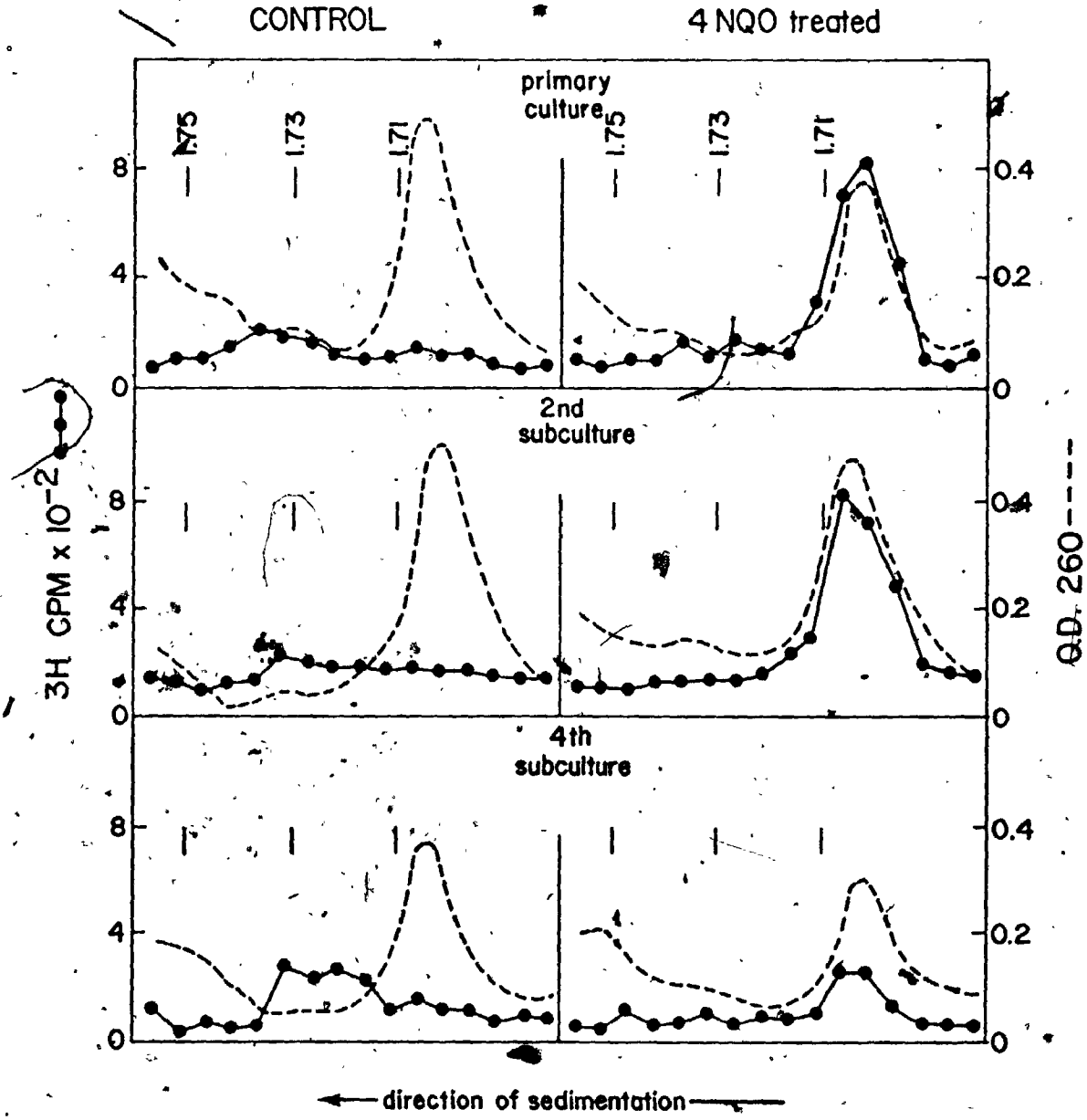

MYOTUBES
● CONTROLS
○ X-IRRADIATED
△ X-IRRADIATED, 5h INCUBATION
10
5
MYOBLASTS
10
5
PERCENT TOTAL COUNTS
DIRECTION OF SEDIMENTATION

Fig. 13. Repair synthesis in rat fibroblasts. Neutral CsCl gradient profiles of DNA isolated from the primary culture (top panel), the second subculture (middle panel), and the fourth subculture (bottom panel) with or without 4NQO treatment and repair. Hydroxyurea was present in all cultures for the duration of the experiment. Isolation of DNA and centrifugation procedure were identical to that described for myoblasts and myotubes.


CONTROL
4 NQO treated
primary culture
2nd subculture
4th subculture
1.75
1.73
1.71
3H CPM x 10⁻²
O.D. 260
direction of sedimentation

Cells	Repair Synthesis (cpm/μg DNA) Controls	4NQO Treated
Rat Fibroblasts:		
Primary	1.9	89.1
2nd subculture	3.5	91.0
4th subculture	7.7	31.2
7th subculture	5.9	14.2

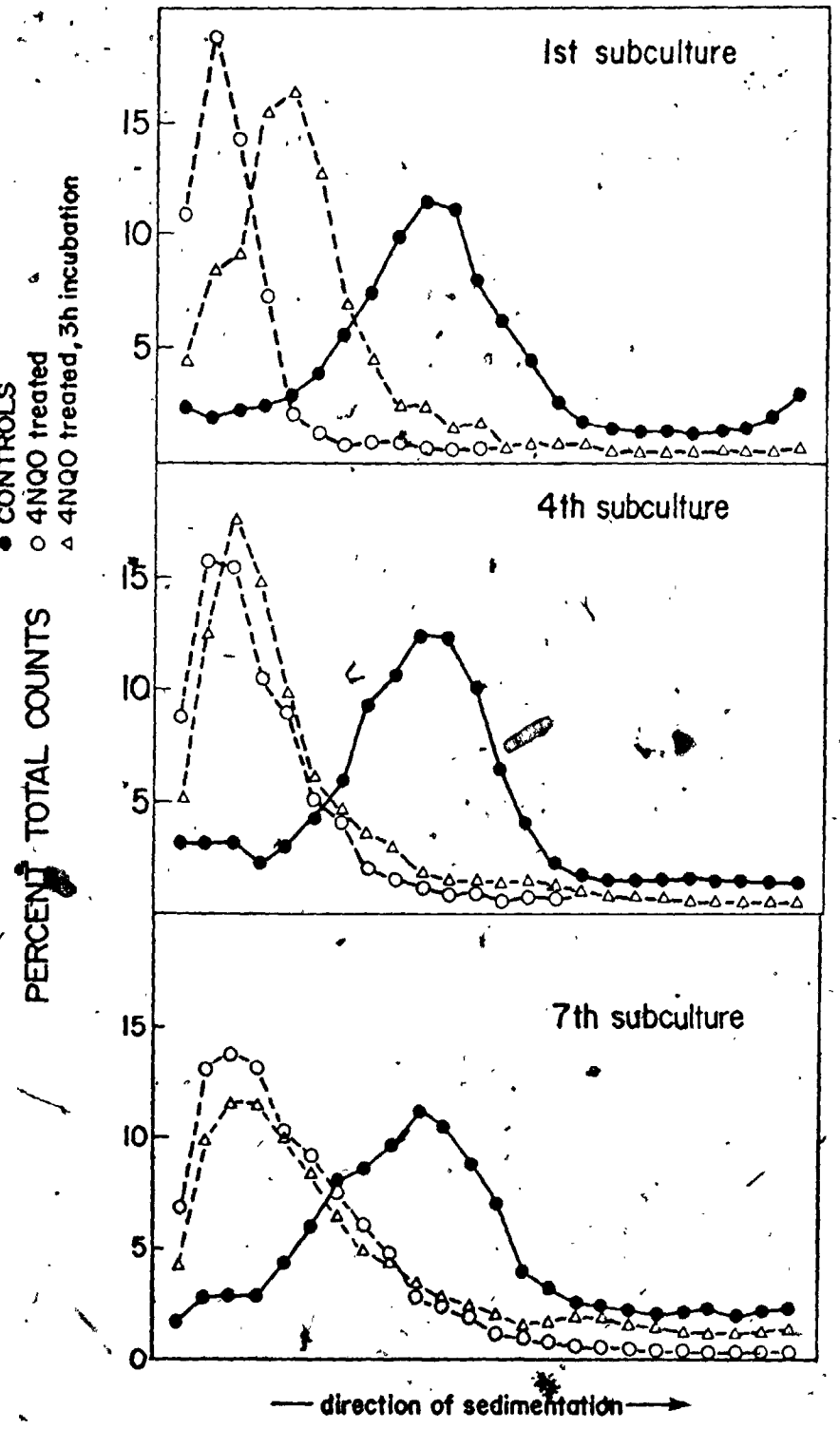
TABLE III. Effect of subculturing on repair synthesis in primary rat fibroblasts. The specific activities of parental DNA (Fig. 13) were assayed as described in the legend of Table II.

subcultured fibroblasts have apparently lost their ability to rejoin breaks in their DNA. As demonstrated by the sedimentation patterns in Fig. 14, a slow sedimenting DNA resulted after 4NQO treatment. A post-treatment incubation effected an increase in the molecular weight of this DNA from the first subculture, but the same procedure did not alter the sedimentation patterns of the 4NQO-treated DNA from later subcultures. This result resembles that obtained with the myotubes; the ability of the subcultured cells to perform repair synthesis is not reflected by their ability to rejoin strand breaks, although repair includes a rejoining step. It is, however, likely that the 4NQO-induced decrease in molecular weight of DNA is not the result of a directly produced single-strand break, but is the result of the cleavage of an alkali-labile modification. 4NQO modified DNA could have been acted upon by alkali when the cells were lysed before centrifugation.

3.4 DISCUSSION

This study has shown that the permanent line of myoblasts, L_6, behaves like primary myoblast cells in its loss of capacity for repair synthesis after fusion to myotubes has occurred. The loss represented a reduction in both the rate and the extent of repair synthesis.

Fig. 14. Sedimentation patterns of rat fibroblast DNA in alkaline sucrose gradients. Prelabelled cells (●) were treated with 1×10^{-5}M 4NQO for 1h (○) and the medium was replaced for post-treatment incubation (△). Each gradient contained 8 to 9 x 10^3 cpm.


1st subculture
4th subculture
7th subculture
● CONTROLS
○ 4NQO treated
△ 4NQO treated, 3h incubation
PERCENT TOTAL COUNTS
15
10
5
0
direction of sedimentation

In contrast there is a marked increase in repair synthesis in lymphocytes stimulated by PHA. Thus, differentiation is concomitant with a reduction in DNA repair. Admittedly, however, the quantitation of repair synthesis by incorporation of radioactive thymidine is not without possible fault. Intracellular nucleotide pools inevitably dilute the specific activity of the label supplied, and the amount of repair incorporation assayed is consequently affected, especially if the pool size alters during differentiation. This source of error had not been eliminated in earlier studies where differentiated cells were compared with their counterparts (Hahn et al., 1971; Stockdale & O'Neill, 1972). Our attempt to evaluate this effect with the double-label experiment shows that the $[^3H]:[^{32}P]$ ratio is approximately 15% higher in the myoblasts. This observation indicates either that the nucleotide pool is about 15% greater in the myotube or that there is a slight preference for thymidine over BrdUrd after fusion has occurred. The latter, however, was minimized by the use of FdUrd and a relatively large supply of BrdUrd (Walker & Ewart, 1973a). Notwithstanding, the dilution effect is small compared to the change in repair synthesis. For lymphocytes, Scudiero et al. (1976) reported a change in the thymidine pool size during Concanavalin A stimulation, but even after correcting for this change, DNA repair, as measured by BND-cellulose (benzoylated naphthoylated DEAE cellulose)

chromatography, was ten times higher in the stimulated cells. Thus, it seems reasonable to conclude that although our results may not be absolutely quantitative, there is a real reduction in repair synthesis in differentiated cells.

Our data also indicate that the reduction in repair is due to a limitation of repair enzymes. In this respect, the myotubes and the lymphocytes are similar to XP cells and other non-dividing systems in which repair is absent or greatly delayed (Cleaver, 1971; Robbins & Kraemer, 1972; Nicoll et al., 1972; Goth & Pajewsky, 1974; Byfield et al., 1974). From the sucrose gradient sedimentation study, it appeared that the fused muscle cells were no longer able to rejoin single-strand breaks induced in their DNA by 4NQO, although they were still capable of rejoining single-strand breaks induced by X-irradiation. This result can be interpreted to mean that the myotubes lack an enzyme that is required for the repair of 4NQO damage but not for the repair of X-ray damage, possibly an endonuclease. The question which needs answering then is: why is there still a significant level of repair synthesis present in the myotubes? It should be recalled that the action of 4NQO on mammalian cells mimics that of UV-light rather than X-irradiation (Stich & San, 1973) and it is likely therefore that 4NQO does not induce the formation of single-strand breaks in vivo. Rather, the breaks seen

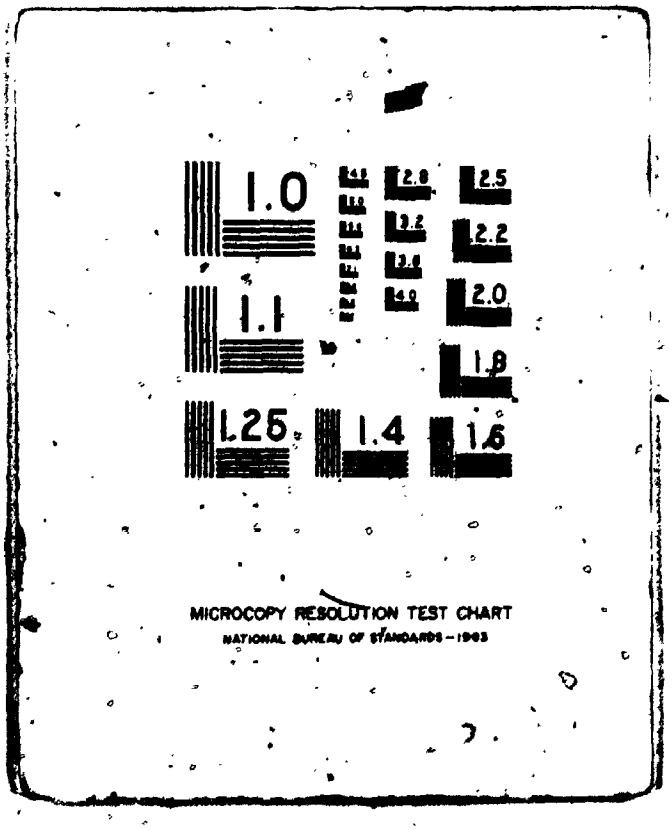
are the result of the action of alkali on the 4NQO modified DNA. Our results can be explained as follows: 4NQO induces at least 2 kinds of modification in DNA which can be removed by an enzymatic repair process. When the modifications are not removed, they show up as single-strand breaks during alkaline sucrose sedimentation. The two modifications are recognized by separate endonucleases; myoblasts contain both but myotubes contain only one. Thus, in the myotubes, repair synthesis is reduced and the unrepaired modification is observed as a persisting single-strand break in alkali. 4NQO also induces a third modification which is observed as an irreparable strand break in alkali; its presence accounts for the incomplete rejoining seen in the myoblasts. A scheme of this model is shown in Fig. 15.

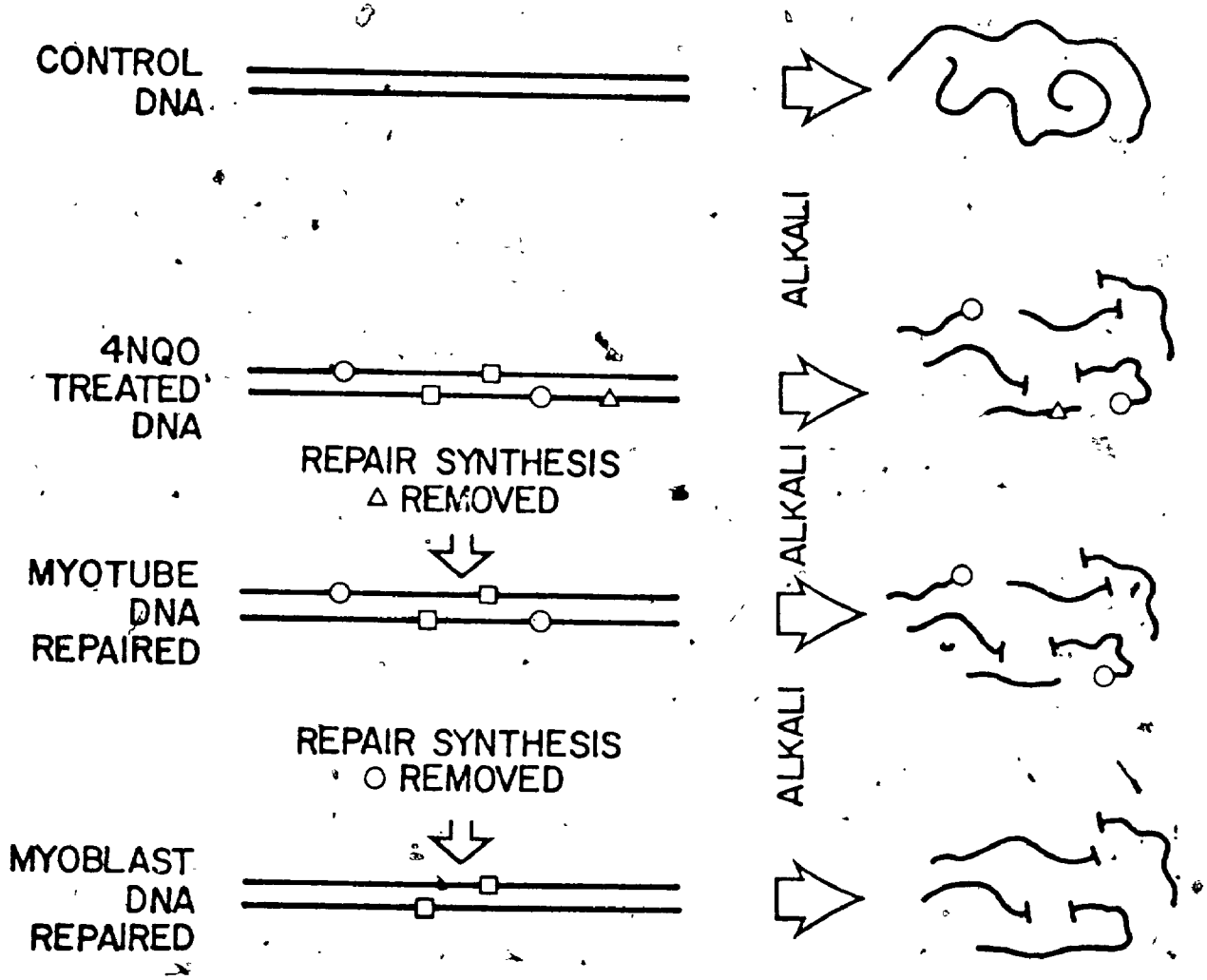
Evidence in favour of the above interpretation is again provided by the experiments with rat fibroblasts. Initially, primary fibroblasts were studied to determine if subculturing has any effect on DNA repair in the rat cells, and the results indicated that in this respect they behaved more like mouse cells than human cells. The ability to perform repair synthesis and the ability to remove 4NQO modifications from their DNA were both diminished in the subcultured fibroblasts. For the diploid human fibroblast WI-38, a decline in repair was only observed in aged cultures just before cell death or

Fig. 15. Schematic representation of 4NQO damage and its repair. 4NQO causes 3 kinds of modifications in DNA. The first modification (Δ) is repaired by the cell before and after fusion, and is apparently alkali-resistant. The second modification (o) is not repaired by the myotubes probably because of the loss of an enzyme. The unremoved damage leads to strand breakage in alkali. The third modification (□) is irreparable and alkali labile.

2 2

OF/DE


1.0
1.1
1.25
1.4
1.6
2.0
2.2
2.5
MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS - 1963


CONTROL DNA
4NQO TREATED DNA
REPAIR SYNTHESIS △ REMOVED
MYOTUBE DNA REPAIRED
REPAIR SYNTHESIS ○ REMOVED
MYOBLAST DNA REPAIRED
ALKALI
ALKALI
ALKALI

senescence occurred (Painter et al., 1973; Clarkson & Painter, 1974; Mattern & Cerutti, 1975). Data presented here, however, are not likely a consequence of cell death; the decline in repair was evident prior to the deceleration of cell growth. When Ben-Ishai and Peleg (1975) measured thymine dimer excision in mouse cells, and noticed a cessation of the activity in the subcultures, they suggested that DNA repair was one of the properties that was lost or altered during passage in culture. One might even speculate that this alteration prevailed during the emergence of a cell line, resulting in poor repair performance in permanently cultured mouse cells (Klimek, 1966; Walker & Ewart, 1973a). Nevertheless, in view of the similarity between subcultured and differentiated rat cells, the loss of some repair enzyme during these processes is a logical conclusion. It is not known, however, whether the loss represents a determined repression or just a chance occurrence.

In summary, this study reiterates and validates the notion that cellular differentiation is correlated with a reduction in DNA excision repair. Furthermore, it shows a parallel between differentiation and cellular aging in culture. Based on the centrifugation analyses, a model is proposed by which the repair of 4NQO-modified DNA in the myogenic cells could be explained; the lack of an endonuclease in the myotubes seems justified. Definite

proof, however, must await the identification and purification of the responsible enzymes.

REFERENCES

Baumuck, C. N. and D. L. Friedman (1971) Cancer Res. 31: 1930-1935.

Ben-Hur, E. and R. Ben-Ishai (1971) Photochem. Photobiol. 13: 337-

Ben-Ishai, R. and L. Peleg (1975) Molecular Mechanism for Repair of DNA; Basic Life Sciences, Vol. 5, part B; P. C. Hanawalt & R. B. Setlow, editors. Plenum Press, New York, 607-610.

Bootsma, D., M. P. Mulder, F. Pot and J. A. Cohen (1970) Mutation Res. 9: 507-516.

Boyce, P. R. and P. Howard-Flanders (1964) Proc. Natl. Acad. Sci. U.S. 51: 293-300.

Boyum, A. (1968) Scand. J. Clin. Lab. Invest. 21: 77-89.

Brandt, W. N., W. G. Flamm and N. J. Bernhein (1972) Chem.-Biol. Interection 5: 327-329.

Bridges, B. A. and S. G. Sedgwick (1974) J. Bact. 117: 1077-1081.

Buhl, S. N., R. B. Setlow and J. D. Regan (1972) Intl. J. Radiat. Biol. 22: 417-424.

Buhl, S. N. and J. D. Regan (1973) Nature 246: 484.

Byfield, J. E., Y. C. Lee, I. Klisak and J. Z. Finklestein (1974) Biochem. Biophys. Res. Commun. 630: 730-735.

Carrier, W. and R. Setlow (1970) J. Bact. 102: 178-186.

Cheevers, W. P., J. Kawalski and K. K. Yu (1972) J. Mol. Biol. 65: 347-364.

Chiu, S. F. N. and A. M. Rauth (1972) Biochim. Biophs. Acta 259: 164-174.

Clarkson, J. M. and H. J. Evans (1972) Mutation Res. 14: 413-430.

Clarkson, J. M. and R. B. Painter (1974) Mutation Res. 23: 107-112.

Cleaver, J. E. (1968) Nature 218: 652-656.

Cleaver, J. E. (1969) Radiation Res. 37: 334-348.

Cleaver, J. E. (1974) Adv. Radiation Biol. 4: 1-76.

Cleaver, J. E. and G. H. Thomas (1969) Biochem. Biophys. Res. Commun. 36: 203-208.

Cleaver, J. E. and J. E. Trosko (1970) Photochem. Photobiol. 11 547-550.

Coleman, J. R. and A. W. Coleman (1968) J. Cell Physiol. 72 Supp. 1: 19-34.

Conard, A. H. and F. H. Ruddle (1972) J. Cell Sci. 10: 471-486.

Cook, J. S. (1970) Photophysiol. 5: 191-233.

Cook, J. S. (1972) John Hopkins Med. J., Supp. 1: 79-94.

Cooper, P. K. and P. C. Hanawalt (1972) J. Mol. Biol. 67: 1-10.

Coyle, M. B. and B. Strauss (1970) Cancer Res. 30: 2314-2319.

Darzynkiewicz, Z. (1971) Expl. Cell Res. 69: 356-360.

Djordjevic, B. and L. J. Tolmach (1967) Radiation Res. 32: 327-346.

Douglas, S. D. (1972) Transplant. Rev. 11: 39-59.

Edenberg, H. and P. Hanawalt (1972) Biochim. Biophys. Acta 272: 361-372.

Edenberg, H. J. and P. C. Hanawalt (1973) Biochim. Biophys. Acta 324: 206-217.

Favour, C. B. (1964) Immunological Methods; J. R. Ackroyd, editor. Blackwell Scientific Publ., Oxford 195-223.

Flamm, W. G., M. L. Birnstiel and P. M. B. Walker (1969) Subcellular Components: Preparation and Fractionation; G. Birnie and S. M. Fox, editors. Butterworth & Co., London. 125-155.

Fox, M. and B. W. Fox (1973) Intl. J. Radiat. Biol. 23: 333-358.

Fox, R. M., J. Mendelsohn, E. Barbosa and M. Goulian (1973) Nature New Biol. 245: 234-237.

Ganesan, A. (1974) J. Mol. Biol. 87: 103-119.

Gautschi, J. R., B. R. Young and J. E. Cleaver (1973) Expl. Cell Res. 76: 87-94.

Goldstein, N. O. and R. J. Rutman (1973) Nature New Biol. 224: 267-269.

Goth, R. and F. Pajewsky (1974) Proc. Natl. Acad. Sci. U.S. 71: 1969-1973.

Grossman, L. (1974) Adv. Radiation Biol. 4: 77-129.

Hahn, G. M., D. King and S. J. Yang (1971) Nature 230: 242-244.

Hand, R. and I. Tamm (1973) J. Cell Biol. 58: 410-418.

Hariharan, P. and P. Cerutti (1972) J. Mol. Biol. 66: 65-81.

Hartman, K. U. and C. Heidelberger (1961b) J. Biol. Chem. 236: 3006-3013.

Howard-Flanders, P. (1968) Ann. Rev. Biochem. 37: 175-200.

Howard-Flanders, P., W. D. Rupp, B. M. Wilkins and R. S. Cole (1968) Cold Spring Harbour Sym. Quan. Biol. 33: 195-209.

Iyer, V. N. and W. D. Rupp (1971) Biochim. Biophys. Acta 228: 117-126.

Jacobs, A. J., R. L. O'Brien, J. W. Parker and P. Paolilli (1972) Intl. J. Cancer 10: 118-127.

Kaplan, J., S. Kushner and L. Grossman (1971) Biochem. 10: 3315-3324.

Kelly, R. B., M. R. Atkinson, J. A. Huberman and J. A. Kornberg (1969) Nature 224: 495-501.

Kidwell, W. R. and G. C. Mueller (1969) Biochem. Biophys. Res. Commun. 36: 756-763.

Kleijer, W. J., P. H. M. Lohman, M. P. Mulder and D. Bootsma (1970) Mutation Res. 9: 517-523.

Klimek, M. (1966) Photochem. Photobiol. 5: 603-607.

Kushner, S. R., J. C. Kaplan, H. Ono and L. Grossman (1971) Biochem. 10: 3325-3334.

Lawley, P. and P. Brooks (1963) Biochem. J. 89: 127-138.

Lehmann, A. R. (1972) J. Mol. Biol. 66: 319-337.

Lett, J. T., I. Cadwell, C. J. Dean and P. Alexander (1967) Nature 214: 790-792.

Lett, J., E. Klucis and C. Sun (1970) Biophys. J. 10: 277-292.

Ley, R. D. (1973) Photochem. Photobiol. 18: 87-95.

Lieberman, M. W., R. N. Baney, R. E. Lee, S. Sell and E. Farber (1971) Cancer Res. 32: 1855-1860.

Magnusson, G., F. Pigiet, E. L. Winnacker, R. Abrams and P. Reichard (1970) Proc. Natl. Acad. Sci. U.S. 70: 412-415.

Mattern, M. R. and P. A. Cerutti (1975) Nature 254: 450-452.

Meyn, R. E. and R. M. Humphrey (1971) Biophys. J. 11: 295-301.

McGrath, R. A. and R. W. Williams (1966) Nature 212: 534-535.

Mukherjee, K. L. and C. Heidelberger (1962) Cancer Res. 22: 815-812.

Nicoll, J., P. Swann and A. Pegg (1972) Nature 254: 261-262.

Nuzzo, F., A. Berga and A. Faláschi (1970) Proc. Natl. Acad. Sci. U.S. 65: 1017-1024.

Olivera, B. M., K. G. Lark, R. Herrmann and Bonhoeffer (1973) in DNA Synthesis In Vitro. R. D. Wells and R. B. Inman editors. University Park Press, Baltimore, 220.

Painter, R. B. and J. E. Cleaver (1961) Radiat. Res. 37: 451-466.

Painter, R. B. and B. R. Young (1972) Mutation Res. 14: 225-235.

Painter, R. B., J. M. Clarkson and B. R. Young (1973) Radiat. Res. 56: 560-564.

Palcic, B. and L. D. Skarsgard (1972) Intl. J. Radiat. Biol. 21: 417-433.

Paterson, M. C. and R. B. Setlow (1972) Proc. Natl. Acad. Sci. U.S. 69: 2927-2931.

Paterson, M. C., P. H. M. Lohman and M. C. Sluyter (1973) Mutation Res. 19: 245-256.

Pauli, R. M. and B. S. Strauss (1973) Expl. Cell Res. 82: 357-366.

Pettijohn, D. and P. Hanawalt (1964) J. Mol. Biol. 9: 395-410.

Pigiet, F., E. L. Winnacker, R. Eliasson and P. Reichard (1973) Nature, New Biol. 245: 203-205.

Powell, W. F. (1962) Biochim. Biophys. Acta 55: 979-986.

Radman, M. and M. Errera (1970) Mutation Res. 9: 553-560.

Rasmussen, R. and R. Painter (1964) Nature 203: 1360-1362.

Rasmussen, R. E., B. L. Reisner and R. B. Painter (1970) Intl. J. Radiat. Biol. 17: 285-290.

Regan, J. D., J. E. Trosko and W. L. Carrier (1968) Biophys. J. 8: 319-325.

Regan, J. D., R. B. Setlow and R. D. Ley (1971) Proc. Natl. Acad. Sci. U.S. 68: 708-712.

Regan, J. and R. Setlow (1973) Chemical Mutagens 3: 151-170.

Regan, J. D. and R. B. Setlow (1974) Cancer Res. 34: 3318-3325.

Richler, C. and D. Yaffe (1970) Develop. Biol. 23: 1-22.

Robbins, J. H. and K. H. Kraemer (1972) Biochim. Biophys. Acta 277: 7-14.

Roberts, J. J., A. R. Crathorn and T. P. Brent (1968) Nature 218: 970-972.

Rupp, W. D. and P. Howard-Flanders (1968) J. Mol. Biol. 31: 291-304.

Rupp, W. D., C. E. Wilde III, D. L. Reno and P. Howard-Flanders (1971) J. Mol. Biol. 61: 25-44.

Sadoff, R. B. and W. P. Cheevers (1973) Biochem. Biophys. Res. Commun. 53: 818-823.

Santi, D. V., C. S. McHenry and Hans Sommer (1974) Biochem. 13: 471-480.

Schandl, E. K. and J. H. Taylor (1969) Biochem. Biophys. Res. Commun. 34: 291-300.

Scudiero, D. and B. Strauss (1974) J. Mol. Biol. 83: 17-34.

Scudiero, D., A. Norin, P. Karran and B. Strauss (1976) Cancer Res. 36: 1397-1403.

Segev, N., C. Miller, R. Sharon and R. Ben-Ishai (1973) Biochem. Biophys. Res. Commun. 52: 1241-1245.

Setlow, R. B. and W. L. Carrier (1964) Proc. Natl. Acad. Sci. U.S. 51: 226-231.

Shainberg, A., G. Yagil and D. Yaffe (1971) Develop. Biol. 25: 1-29.

Slor, H. (1973) Mutation Res. 19: 231-235.

Smith, K. C. and D. H. C. Meun (1970) J. Mol. Biol. 51: 459-472.

Spiegler, P. and A. Norman (1969) Radiat. Res. 39: 400-410.

Stich, H. F., R. H. C. San and Y. Kawazoe (1973) Mutation Res. 17: 127-139.

Stockdale, F. E. (1971) Science 171: 1145-1147.

Stockdale, F. E. and M. C. O'Neill (1972) J. Cell Biol. 52: 589-597.

Strauss, B. S. and M. Robbins (1968) Biochim. Biophys. Acta 161: 68-75.

Studier, F. W. (1965) J. Mol. Biol. 11: 373-390.

Sugino, A., S. Hirose and R. Okazaki (1972) Proc. Natl. Acad. Sci. U.S. 69: 1863-1867.

Sutherland, B., M. Chamberlin and J. Sutherland (1972) J. Biol. Chem. 248: 4200-4205.

Tada, M. and M. Tada (1971) Chem.-Biol. Interaction 3: 225-229.

Tanooka, H., M. Tada and M. Tada (1975) Chem.-Biol. Interaction 10: 11-18.

Tattersall, M. N. N. and K. R. Harrop (1973) Cancer Res. 33: 3086-3090.

Till, J. E., G. F. Whitmore and S. Gulyas (1963) Biochim. Biophys. Acta 72: 277-289.

Trosko, J. E., E. H. Y. Chu and W. L. Carrier (1965) Radiat. Res. 24: 667-672.

Trosko, J. E. and E. H. Y. Chu (1973) Chem.-Biol. Interaction 6: 317-332.

Walker, I. G. and D. F. Ewart (1973a) Can. J. Biochem. 51: 148-157.

Walker, I. G. and D. F. Ewart (1973b) Mutation Res. 19: 331-341.

Walker, I. G., D. F. Ewart and A. Chan (1973) Can. J. Biochem. 51: 7-10.

Walker, I. G. and R. Sridhar (1976) Chem.-Biol. Interaction 12: 229-239.

Wise, G. E. and D. M. Prescott (1973) Proc. Natl. Acad. Sci. U.S. 70: 714-717.

Yaffe, D. (1968) Proc. Natl. Acad. Sci. U.S. 61: 477-483.

Young, C. W., G. Schochetman and D. A. Karnofsky (1967) Cancer Res. 27: 526-534.

Amaldi, F., F. Carnevali, L. Leoni and D. Mariotti (1972) Expl. Cell Res. 74: 367-374.

Meneghini, R. and P. Hanawalt (1976) Biochim. Biophys. Acta 425: 428-437.

Sutherland, B. M. (1974) Nature 248: 109-112,

Sutherland, B. M., M. Rice and E. K. Wagner (1975) Proc. Natl. Acad. Sci. U.S. 72: 103-107.

Sutherland, B. M., R. Oliver, C. O. Fuselier and J. C. Sutherland (1976) Biochemistry 15: 402-406.