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PROGRESS REPORT (May 1971 - May 1972)

Supplement No. 1:

Mutagenic Action, Loss of Transforming Activity, and Inhibition of DNA Template Activity In Vitro Caused by Chemical Linkage of Carcinogenic Polycyclic Hydrocarbons to DNA. V. M. Maher, S. A. Lesko, Jr., P. A. Straat and P. O. P. Ts'o. J. Bacteriol. 108, 202 (1971).

Supplement No. 2:

Interaction and Linkage of Polycyclic Hydrocarbons to Nucleic Acids. S. A. Lesko, Jr., H. D. Hoffmann, P. O. P. Ts'o, and V. M. Maher. Progress in Molecular and Subcellular Biology, Vol. 2, ed. F. E. Hahn (Springer-Verlag, 1971).

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PROGRESS REPORT (May 1971 - May 1972)

During the past twelve months significant progress has been made in this research program. Two reprints are attached to this report as supplements.

1. <u>Improvement of isolation procedures of DNA-B(a)P product from the reaction mixture</u>.

Our early procedure in isolating the DNA-B(a)P complex from the chemical reaction mixture involved repeated precipitation of the DNA followed by numerous washings of the precipitate with ethanol. This method resulted in certain physical degradations of the DNA with the concomitant lowering of biological activities as template for polymerase and as transforming principle. Considerable effort has been made during the last year to devise a procedure through which DNA-B(a)P complex can be freed from the undesirable B(a)P reaction products with minimal physical damage. Dialysis with alcohol, continuous liquid-extraction with benzene or cyclohexane have been tried and both were found to be unsatisfactory. Now we have developed a very gentle and efficient procedure which involves extraction of the reaction mixture 4-5 times with aqueous buffer-saturated phenol (pH 7). This method was shown to remove 99.99% of the unreacted products with little degradation of DNA as assayed by analytical ultracentrifuge.

The general procedure for the reaction of ^{3}H-6-OH B(a)P with DNA is as follows. The DNA in 10^{-2} or 2×10^{-2} M phosphate buffer was carefully diluted with an equal volume of 95% ETOH. The final concentration was generally 2.5 OD/ml. The desired amount of 6-OH B(a)P was dissolved in a small volume of ETOH or acetone, and added to the DNA solution which was allowed to

stand at room temperature for 2 days. After this period, the volume of solution was increased 50% with buffer and the solution centrifuged at 5,000 rpm to pellet precipitated quinones. The supernatent was dialyzed vs. buffer to remove ethanol and extracted with buffered aqueous phenol a total of 4-5 times which removed all radioactive B(a)P derivatives not covalently bound to the DNA. Ether extraction was followed by dialysis into an appropriate aqueous buffer and the ratio of B(a)P moieties to nucleotides was determined by measurement of the radioactivity and ultraviolet absorption.

For the reaction of B(a)P with DNA induced by iodine, the following procedure was adopted. DNA, B(a)P, and iodine dissolved in ethanol, were incubated for a period of 1/2 hour to 1 hour in ethanol-buffer 1:1 solvent system at room temperature. After incubation, the reaction mixture was extracted 4-5 times with chloroform to remove the iodine. Non-bound B(a)P reaction products are then extracted 4-5 times with phenol and the DNA solution is dialyzed against buffer to remove the residual phenol. This process can then be repeated again for a higher yield. Normally DNA (particularly that from a mammalian source) went through the iodine-induced reaction 2-3 times to yield 1 B(a)P per 300-600 bases as the final product.

During the course of this study we also found that the amount of benzene present in solution also has a large effect on the yield of the iodine-induced B(a)P-DNA reaction. In the presence of 0.4 M benzene, the ratio of B(a)P to nucleotides in the iodine-induced reaction was decreased about 4-fold. This was presumably due to the complex formation between B(a)P and iodine which may have reduced the availability of iodine to the reaction.

2. The reaction of 6-OH B(a)P with DNA.

The investigation of this reaction based on the procedure described in the above section provides the following specific observations.

(a) The amount of B(a)P residue covalently linked to DNA (expressed as hydrocarbon:nucleotide ratio) appears to be dependent linearly on the amount of 6-OH B(a)P introduced to the reaction. This has led to binding ratios as high as 1/80 with *B. subtilis* and T_7 DNA in a small reaction mixture. However, only a small fraction of 6-OH B(a)P molecules reacted with the DNA in solution. The bulk of the 6-OH B(a)P is converted to the 3,6; 1,6; and 6,12 quinones based on chromatographic, uv spectral, and fluorescent data. Clearly there are competing reactions of the reactive 6-OH B(a)P in its reaction with DNA. For reasons not very clear at the moment, the binding ratio of the reaction with thymus and *E. coli* DNA appears to be only 1/4 of that of *B. subtilis* and T_7 DNA.

(b) Upon solution of 6-OH B(a)P in benzene, a radical which yields well-defined hyperfine spectra was observed, with the concentration possibly as high as 10-15%. Currently, the reactivity of this radical in a variety of compounds is being investigated by ESR-quenching procedure.

(c) Transforming DNA from *B. subtilis* (SB_{19}) was reacted in a 6-OH B(a)P solution to give 1 hydrocarbon/80 nucleotides. In this sample, the percentage of survival of the DNA biological activity was reduced to about 10% and the mutation of the sample was increased 20-fold over the control. The biological properties of this system are now being further investigated.

(d) T_7 DNA has been reacted with 6-OH B(a)P to the

extent of B(a)P base ratio of 1/420, 1/1240, and 1/3040. The transcription of this B(a)P-phage DNA has been studied by the use of purified E. coli RNA polymerase. The conditions employed *in vitro* only allow for specific initiation of transcription of one set of genes of the T_7 genome (the early genes), which is one RNA product equivalent to 12% of the total genome. To insure that RNA polymerase is initiating on specific promotor sites and not on locally denatured regions of the DNA created by the B(a)P lesion, RNA synthesis was initiated by first forming a rifampicin-resistant preinitiation site on the genome (Bautz and Bautz, Cold Spring Harbor Symposium, 1970). This assay selects for RNA synthesis resulting only from initiation on specific binding sites as opposed to unspecific initiation at free ends or single-stranded regions of the genome created at B(a)P lesions. In this type of assay, a dramatic inhibition of RNA synthesis by covalently bound B(a)P was found which could only be attributed to blockage of the movement of the polymerase as it transcribed the template. The extent of RNA synthesis and the size of the RNA product was generally inversely proportional to the dose of B(a)P. The initiation of RNA synthesis is not affected except at high doses of B(a)P where the probability of modification of specific initiation sites is increased.

3. Enzymatic conversion of 6-acetoxy B(a)P to 6-hydroxy B(a)P.

Animal testing of 6-OH B(a)P has shown it not to be carcinogenic and this has been used as an argument against this and other metabolic products being involved in carcinogenesis. However, the observed reactivity of 6-OH B(a)P suggests that the compound would decompose too rapidly to be effective when applied in the manner used for testing live animals. The reactivity of the compound might well rule out other important experiments in

which it is used.

On the other hand, 6-OAc B(a)P is a quite stable compound under conditions where 6-OH B(a)P is not. Enzymatic, *in vivo*, hydrolysis of 6-OAc B(a)P to 6-OH B(a)P by esterases might very likely take place. Furthermore, in preliminary experiments using ultraviolet spectroscopy for analysis, it has been observed that 6-OAc B(a)P is hydrolyzed, *in vitro*, using α-chymotrypsin, to 6-OH B(a)P. This demonstrates the potential for using 6-OAc B(a)P as a precursor for *in situ* 6-OH B(a)P formation in biological systems where introduction of undecomposed 6-OH B(a)P might not otherwise be possible and experiments are being carried out using 6-OAc B(a)P. 6OAc B(a)P has been given to two National Cancer Institute laboratories (Drs. Gelboin and DiPaolo) for testing its tumorigenicity.

4. Products of reaction of B(a)P and 6-OH B(a)P with DNA, poly A and poly G.

The first objective of this research was to determine whether the product of the iodine-induced reaction of B(a)P with DNA is the same as that of the reaction between 6-OH B(a)P and DNA. Preparations of poly A-^{3}H-6-OH B(a)P and poly G-^{3}H-6-OH B(a)P were made in a similar manner as for DNA. The reaction products of poly G with 1 6-OH B(a)P/400 bases and of poly A with 1 6-OH B(a)P/1200 bases were obtained. In addition, Chinese hamster DNA and bacterial DNA containing approximately 1 B(a)P/1300 bases obtained from iodine-induced reaction were used for the study. Preliminary data indicate that at least certain products from the iodine-induced reaction of B(a)P with DNA are not the same as those of the 6-OH B(a)P with DNA. For instance, after depurination of the DNA by mild acid hydrolysis, the liberated purines were examined in butanol/NH_3/H_2O system.

The analysis revealed that 75-80% of the radioactivity remained at the origin from samples derived from iodine-induced B(a)P-DNA. The remainder of the counts move approximately the same as adenine and guanine. On the other hand, in samples from acid-treated 6-OH B(a)P-DNA, 85% of the radioactivity moves as a single peak in the chromatogram. However, we have no knowledge at present whether these chromatographic spots containing most of the radioactivity from B(a)P contain purine bases from the DNA.

Previous experiments have shown that the radioactivity remains with the DNA after a moderate alkaline treatment on samples of B(a)P-DNA induced by iodine, and from DNA reacted with 6-OH B(a)P. For this reason, poly G and poly A reacted with B(a)P were hydrolyzed by alkaline digestion and examined by paper electrophoresis at pH 7.5. In the case of poly G-B(a)P (iodine-induced) and 6-OH B(a)P-poly G, similar electrophoretic patterns were obtained in which a component containing 50% radioactivity was detected which migrates at a mobility of 0.42 relative to the GMP marker. The remaining portion of the radioactivity was found at origin. However, under acidic conditions, the radioactivity remained with poly G for the B(a)P-poly G sample (iodine-induced) while the radioactivity was removed from the poly G in the sample of 6-OH B(a)P-poly G in dialysis experiments. Another difference can be observed from the alkaline treatment with the poly A-B(a)P chemical complex. Examination of the poly A-B(a)P products after alkaline hydrolysis shows a product (Re = 0.50) containing approximately 50% of the radioactivity. Again, counts are present at the origin. In the case of poly A-6-OH B(a)P, most of the recovered radioactivity failed to migrate by electrophoresis. From these results,

therefore, we tentatively conclude that at least certain products of the B(a)P-nucleic acid induced by iodine may be different from those of 6-OH B(a)P-nucleic acid.

5. Uptake of exogenous DNA by Syrian hamster fibroblasts.

Investigation of the fate of B(a)P-DNA in mammalian cells cannot be studied without the prior knowledge of the fate of unreacted DNA inside the cell. T_7 DNA was chosen as an example. On the one hand, the possible expression of this viral genome in mammalian cells could be examined; on the other hand, this well-defined viral DNA can serve well as the experimental model. Culture plates containing about 1×10^6 cells in fetal calf serum are exposed to about 25 μg. ^{32}P or ^3H labeled T_7 DNA for various lengths of time. It was found that pre-treatment with 10 μg. DEAE dextran for 15 minutes (afterwards the DEAE dextran is removed by washing) greatly stimulated the uptake of DNA (20-fold). In an hour, 10^5 molecules of T_7 became bound to a single cell, which is equivalent to about 10-20% of the amount of the original host cell DNA. Approximately 10% (or 10^4) T_7 DNA may be transported to the nuclei after further incubation when the external source has been removed. This conclusion is based on the fact that these portions of the DNA now become resistant to DNase. Furthermore, 90% of the bound T_7 DNA continues to have large molecular weights as examined by sucrose gradient sedimentation even after the cell has grown for two divisional cycles. The expression of the T_7 genome in the mammalian cell will be studied by hybridization technique and our biochemical assay will be supplemented by radioautographic analysis.

Intact T_7 phage particles also bind to hamster cells. This binding is not dependent on DEAE dextran pre-treatment although

DEAE dextran pre-treatment stimulates two-fold. Approximately 1/3 of the bound virus are resistant to release from the cells by trypsinization which may suggest that T_7 phage particles are taken intracellularly.

6. <u>Transformation studies with B(a)P</u>.

In an attempt to examine the <u>in vitro</u> transformation event induced by B(a)P, primary or secondary mixed embryo cell cultures of PAGE (Philadelphia Albino-Gey Rat strain embryo) and GSHE (normal Golden Syrian hamster embryo) cells were each treated at various seeding levels ranging from 5×10^4 to 5×10^6 cells per 60 mm culture dish, with varying concentrations of the carcinogenic hydrocarbon ranging from 1 μg B(a)P/ml medium to 20 μg B(a)P/ml medium. Appropriate controls accompanied all test runs; these included cells seeded at the same densities cultivated in medium alone or in medium containing the solvent (acetone or dimethylsulfoxide) at the same concentration used to dissolve the hydrocarbon. Treatment was begun 24 hours after seeding, thereby allowing for cell attachment and adaptation to the culture vessel. Transformation was assayed for by the agar suspension assay and by direct microscopic observation of fixed and stained colonies.

A. Effects of the carcinogen on PAGE rat cells.

With respect to the PAGE cell strain, the results obtained by both determinations indicate that (1) 10 μg B(a)P/ml medium was required to transform the rat cells, and (2) cell density plays an important role in allowing expression of the transformation event. If during the treatment period the seeded cell population reached confluency before the experiment was terminated, transformation was not recognizable at the cellular level, by either soft agar assay or direct observation.

However, if at the outset of treatment the seeding level was light enough to avoid the inhibitory action of confluency, then isolated morphologically aberrant cells could be detected. Although less than 1% of the cells could be characterized as atypical deviants from the normal counterparts, these all displayed certain morphological variations typical of known transformants:

1. Bizarre shapes with multiply-branched stellate projections.
2. Bi- and tri-nucleated giant cell types with 2,3 and 4 nucleoli per nucleus.
3. Small isolated colonies of piled-up cells.
4. Small clusters of bizarre cells displaying disorganized random growth patterns.

Whether or not these morphological variants actually represent malignant transformants is yet to be established. Further confirmation may be derived from survival of these altered cells in factor-free medium, and finally, from the inoculation of cloned populations of these morphologically modified cells into isologous hosts.

B. Effects of the carcinogen on GSHE hamster cells.

When these techniques were extended to the study of B(a)P-treated primary and secondary mixed embryo cell cultures (GSHE), it was found that unlike the rat embryo cells which remained as isolated single cells or small cell clusters after treatment with the carcinogen, the hamster cells were able to form individual colonies at low cell density seeding levels. The colonies were judged to be cloned from single parental types since (1) microscopic examination of the cultures after cells had been planted revealed that at the low cell densities used, only single cell isolates attached to the plastic Petri dish substrate, and (2) no colony was comprised of more than

one cell type (although the stock cultures from which these clones were derived included both epitheleoid and fibroblastic cells).

Even though the cloning efficiency of the GSHE cells was relatively low, a small percentage of these clones (approximately 1%) did display morphological deviations when observed after appropriate fixation and staining. "Dense" (contiguous) and "light" (semi-contiguous) clones were observable in which organized growth patterns had been lost; member cells of such clones were no longer contact-inhibited and arranged themselves into small mounds of piled-up cells. All appropriate controls proved to be negative. It should be noted that similar results have also come from other laboratories such as that of J. A. DiPaolo *et al*. (J. Nat. Cancer Inst. 42: 867, 1969). Attempts to isolate and cultivate such morphologically transformed clones into larger populations are presently underway in order to determine their oncogenicity *in vivo*. In addition, other *in vitro* tests are being performed on these altered clones, such as the agar suspension assay and the factor-free medium growth test.

7. Search for improved procedure for detection and measurement of cell transformation.

A. Development of soft agar assay of MacPherson and Montagnier (Virology 23: 291, 1964).

This procedure was used for screening of viral-transformed cells and could provide easily retrievable viable transformed clones. It is hoped that this procedure will be developed for chemical carcinogenesis studies.

In order to determine the sensitivity of the soft agar assay, a test was developed to detect W-319 rat fibrosarcoma

cells (Philadelphia Albino-Walker rat strain, *in vivo* tumor of spontaneous origin) among normal rat embryo fibroblasts, PAGE. For mixed populations of 1×10^5 cells containing the neoplastic rodent cells ranging in percent concentration from 10^2 to 10^{-2}, W-319 cells were reproducibly detectable when they comprised as few as 1% of the total cell sample. At lower concentrations of W-319 (e.g. 0.1% and 0.01%), detection was inconsistent.

At the present time, the soft agar assay is being tested on Syrian hamster cells transformed by various polycyclic carcinogenic hydrocarbons as well as established human neoplastic cell lines. The possibility of increased sensitivity of this assay is also being explored.

B. Selectivity of incomplete growth medium and altered atmospheric conditions.

In an attempt to apply more restrictive *in vitro* growth conditions such that we might distinguish between the environmental requirements of neoplastic cells and normal early passage embryo cells, the cytotopic effects of both an anaerobic atmosphere and a serum-free growth medium were studied. Normal Golden Syrian hamster embryo cells cultivated at $37^{o}C$ in F_{12} nutrient medium (Gibco) supplemented with 10% fetal calf serum (Gibco) and exposed to a humidified atmosphere of 100% argon continued to double in number until 3 days of exposure to these conditions, after which no further cell multiplication could be detected. On the other hand, GSHE cells incubated in serum-free F_{12} medium (SFM) in the same gaseous environment showed a steady decline in number with more than 75% cell loss occurring within the first 24 hours of treatment.

In order to establish whether this cytotopic effect was

attributable to the atmospheric conditions, to the absence of serum, or both, GSHE cells were cultivated with or without the serum supplement in a humidified atmosphere of either 100% argon or 5% CO_2 and 95% air. It was determined that those cells receiving the serum supplement displayed growth curves that paralleled one another, regardless of the gaseous environment, and that their generation times approximated each other as well. Similarly, this held true for the hamster embryo cells incubated in SFM, although in this case more than 91% of the cells were killed within 24 hours of the onset of treatment. pH measurements of the incubation media were taken immediately before cells were counted; all were pH 6.8. It was therefore concluded that lack of serum in the growth medium was apparently more restrictive to *in vitro* survival of GSHE cells than was an atmosphere approaching anaerobiosis.

Next, the survival of a neoplastic cell line was determined under conditions of anaerobiosis and serum starvation. Unfortunately, at this time a tumorigenic hamster cell line was unavailable. Therefore, the W-319 rat fibrosarcoma line was employed instead. This time, the transformed cell line proved to be able to survive in an atmosphere of 100% argon, as well as in the absence of serum, whether the gaseous state was composed of 5% CO_2 + 95% air or 100% argon. However, the generation time of these cells in serum-free medium and in each of these atmospheres slowed to 141 and 179 hours respectively.

At the present time, similar tests are being carried out on transformed hamster cell lines as well as on those of human origin. It is felt that a differential survival of normal and neoplastic cells in which the latter is favored will fulfill the selective requirements for an assay to detect induced transformation.

C. Selectivity of "factor-free" medium.

At the time this last series of experiments was being carried out, Scher and Todaro (Exptl. Cell Res. 68: 479, 1971) reported on the growth selectivity of a culture medium lacking serum growth factor. We therefore tested this system for its ability to selectively support the growth of transformed cells as compared to normal rat and hamster fibroblasts. Using the cell lines available at that time, i.e. W-319, PAGE, GSHE, and HeLa (wild type), we proceeded to repeat the Scher and Todaro study. Each cell line was seeded in factor-free medium (FFM), which consisted of Eagle's MEM (Gibco) + 10% factor-free γ-globulin-free newborn calf serum (FF-GGF-NBC) (Gibco) + 100 units penicillin + 100 μg/ml streptomycin (PS). The FF-GGF-NBC was rendered "factor-free" by heating GGF-NBC to 70°C for 30 min. and rapidly cooling in an icebath before addition to the MEM. Twenty-four hours later half the culture dishes of each cell line had their fluids replaced with complete medium (Eagle's MEM + 10% fetal calf serum + PS) and the remainder received the equivalent volume of fresh FFM. Media were changed twice each week throughout the course of the experiment, 13 days. It was found that all four cell types grew well, as they should, in complete medium. Of the four, only the two transformed lines, i.e. W-319 and HeLa, survived the restrictive growth conditions imparted by the FFM, and, in fact, were able to increase their numbers, although at a slower rate than the controls. The normal cell strains (PAGE and GSHE) showed a gradual but steady decline in number below the number planted.

Presently, this assay is being carried out using a methylcholanthrene-transformed hamster cell line and a normal human

neonatal fibroblast strain, thereby constituting two additional appropriate controls. We are hopeful that the selectivity of the factor-free medium for transformed cells will allow the growth of suspected aberrant cells, thereby allowing us another parameter by which to recognize the transformation event.