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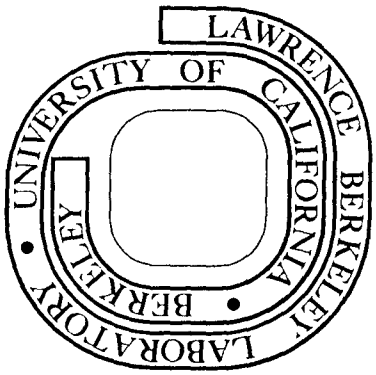

DISCLAIMER

ARYL HYDROCARBON HYDROXYLASE INDUCTION IN MOUSE LIVER CELLS - RELATIONSHIP TO POSITION IN THE CELL CYCLE[1]

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Key Words: Benzo[a]pyrene, aryl hydrocarbon hydroxylase, epithelial cells, induction, cell cycle

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[2]Present address: Department of Physics, California State University, Fullerton, California 92634.

[3]The abbreviations used in this manuscript are: BaP, benzo[a]pyrene; AHH, aryl hydrocarbon hydroxylase; DMSO, dimethyl sulfoxide.

[4]Pearlman, A.L., manuscript in preparation.

[5]Bartholomew, J.C., Pearlman, A.L., Landolph, J.R., and Straub, K., manuscript in preparation.

SUMMARY

The inducibility of aryl hydrocarbon hydroxylase (AHH) by benzo[a]-pyrene (BaP) has been studied in synchronously grown cultures of mouse liver cells. These cells (NMuLi cl 8) have low basal levels of AHH which can be induced greater than 100-fold by BaP. Cells were synchronized in $G_1(G_0)$ by serum starvation, and in S by release from serum starvation in combination with excess thymidine. When released from $G_1(G_0)$ by replating at lower cell density in fresh medium with 20% serum, cells began entering S with a lag of 12 hr. Addition of BaP (1 ug/ml) 8 hr before serum stimulation, at the time of stimulation, or 8 hr after stimulation all gave similar induction kinetics: the AHH activity peaked as the cells began entering S regardless of when the BaP was added. Cells blocked in various parts of S by excess thymidine were inducible for AHH activity as efficiently as cells moving through S and into G_2. These results indicate that the inducibility of AHH is greater when cells are actively proliferating and may be a contributing factor to why growing cells are more sensitive to mutagenesis and transformation than quiescent cells.

INTRODUCTION

The growth state of cells has a large influence on the response of cells to carcinogens (1-9). In general, cells are more susceptible to chemical mutation(10) or transformation(11,12) when they are actively growing. This increased sensitivity has been seen with carcinogens that need metabolic activation as well as with compounds that are already activated. Some studies with activated compounds have indicated that particular phases of the cell cycle are more sensitive to transformation than others(13-18). Cell cycle studies have not been possible with compounds that need metabolic activation because of the long time needed to induce the enzymes required for metabolism and the relatively slow turnover of the substrates. This report describes our experiments to determine the effect of cell cycle position on the inducibility of AHH[3], the enzyme responsible for the first steps in the metabolism of polycyclic aromatic hydrocarbons to carcinogenic derivatives(19,20). Our results indicate that maximal AHH induction requires cells to be stimulated from density dependent inhibition of growth. The induced activity in density inhibited cells is greatly reduced when compared with cells in other phases of the cell cycle. Enzyme activity is high when the cells are making DNA. It follows from these studies that cells are exposed to high levels of activated derivatives of polycyclic aromatic hydrocarbons at the time when they are most sensitive to transformation.

MATERIALS AND METHODS

Cell Culture Techniques

The cells used in this study (NMuLi cl 8) were derived from NMuLi(21,22) mouse liver epithelial cells by the cloning technique of Puck et al.(23). The sensitivity of NMuLi cl 8 to BaP cytotoxicity was first demonstrated using a clonal assay. Cytotoxicity was shown to correlate with the presence of a highly inducible AHH activity(24). The cells were cultured in plastic dishes (Falcon, Oxnard, Calif.) and incubated at 37°C in a 5% CO_2 incubator.

The medium used to grow the cells was Eagle's minimal medium(25) (GIBCO, Grand Island, N.Y.) containing 10% donor calf serum (Flow Laboratories, Rockville, Md) and 10 ug/ml insulin (Schwarz/Mann, Orangeburg, N.Y.). The cells were judged free of mycoplasma by incorporation of ^{3}H-thymidine (20.1 Ci/mM; New England Nuclear, Boston, Mass.) into the nucleus of the cells and not the cytoplasm(26). Stock cultures were maintained by subculturing the cells twice weekly at a cell density of 1×10^{4} per cm^{2}. BaP was dissolved in DMSO (Matheson, Coleman, and Bell, Los Angeles, Calif.) immediately before addition to the cultures. The final DMSO concentration in all experiments was 0.5%.

Synchronized cell cultures were obtained by allowing the cells to grow to saturation density in 100 mm plates. The cells were stimulated to move synchronously into the G_1 phase of the cell cycle by replating them at 1/4 the saturation density in the presence of 20% calf serum. The thymidine block was accomplished by the addition of 5 mM excess thymidine to the medium.

Cell Cycle Analysis

To determine the position in the cell cycle of individual cells of a population, the cells were stained with propidium iodide using the technique described by Crissman and Steinkamp(27). The DNA content of the stained cells was analyzed using a flow cytometer as described previously(28). Analysis of the resulting histograms was carried out using a modification[4] of a program described by Fried et al.(29).

Aryl Hydrocarbon Hydroxylase Induction and Assay

AHH induction and assay was as described previously(24). The assay is essentially that of Nebert and Gelboin(30) as modified by Nebert and Gielen(31).

RESULTS

AHH Induction After Serum Stimulation of "Quiescent" Cells

Previous experiments in our laboratory had indicated that when NMuLi cl 8 cells had reached saturation density the induction of AHH was greatly reduced relative to cells actively growing(24). When the "quiescent" cells are replated at lower cell density in fresh medium containing elevated levels of calf serum, cells progress through the cell cycle with a dispersion that depends on the experimental conditions[5]. Figure 1 demonstrates how the induction of AHH relates to the addition of BaP before and after serum stimulation of cell cycle traverse. In this experiment BaP was added to some cells at the time of replating and serum stimulation. To other cells the BaP was added 8 hr prior to replating and serum stimulation. (BaP was also present in the replated cells.) Maximal AHH enzyme activity occurred approximately 12 hr after the cells were stimulated by excess serum. This peak in enzyme activity occurred at the same time after serum stimulation regardless of when the BaP was added to the cells. It was determined from the DNA histograms of the populations that the peak of enzyme activity occurred when the cells were near the G_1-S boundary. Even when BaP was added 7 hr after serum stimulation, as shown in Figure 2, the peak in induced activity occurred at essentially the same time after serum stimulation. The activity increased more sharply when BaP was added at 7 hr after stimulation compared to the case when it was added at or before stimulation. This indicates that little induction takes place during the "quiescent" phase of the cycle which occurs before serum stimulation, and that it takes some time (10-12 hr) after stimulation for the activity to induce maximally. It should be noted that the level of maximum enzyme activity is somewhat variable in these experiments and depends on such things as the previous history of the cell cultures.

The BaP concentration (1 μg/ml) used to induce enzyme activity is not rate limiting. Changing the medium regularly to maintain the BaP concentration has been shown to have no effect on the induction process(24).

The results of a detailed cell cycle analysis of the DNA histograms are shown in Figure 3. The increase in induced AHH activity (with BaP added at 7.5 hr after serum stimulation) is shown in the lower half of the figure and is correlated in this case with the increase in the fraction of the total cell population present in the S-phase of the cell cycle.

AHH Induction in "S"

In addition to using serum stimulation to produce synchronously growing cell cultures, excess thymidine was also used to block cells in various parts of S-phase. Cells were blocked in early S-phase by growing them in the presence of excess thymidine for 17 hr following serum stimulation. The DNA Histograms for these cells at 17, 19, 21, and 23 hr after stimulation are shown in the upper half of Figure 4. The cells are essentially arrested in early S. BaP was added to these cells at 17 hr after stimulation and the level of induced AHH activity is shown in Figure 5. A rapid rise in activity can be seen.

To obtain cells arrested in the middle of S-phase, the thymidine block was removed for 2 hr at 17 hr after serum stimulation, and replaced at 19 hr. BaP was also added at this time. The DNA histograms for these cells are shown in the lower half of Figure 4 and the levels of induced AHH activity are shown in Figure 5. The induction of enzyme activity is similar in cells arrested in early S and middle S phase of the cell cycle. Cells arrested in S phase apparently are still capable of inducing AHH enzyme activity, even in the presence of excess thymidine.

AHH Induction in G_2+M and Early G_1

When cells were released from excess thymidine block at 29 hr after serum stimulation and allowed to progress from S into G_2+M and on into G_1

(Figure 6), the time course of enzyme induction (Figure 7) was essentially the same as that for cells progressing from late G_1 into S (Figure 3) and for cells blocked in S (Figure 5).

DISCUSSION

Essentially no induction of AHH enzyme activity occurs in the "quiescent" phase of the cell cycle before release from density dependent growth inhibition. Presumably, either the initial RNA synthesis or the subsequent continued protein synthesis required for AHH induction(32) does not take place during this "quiescent" phase. Induction does take place in early and middle S phase whether the cells remain blocked in S by excess thymidine or are allowed to progress through S. The excess thymidine block has no apparent effect on AHH enzyme induction or activity, and induction continues as cells progress through S, G_2+M, and into early G_1. Maximum induced activity occurs 4 to 7 hr after addition of BaP, except when BaP is added during the "quiescent" phase. High levels of induced enzyme activity are seen during S-phase, just when the cells are involved with replicating their DNA. Thus, activated metabolites of BaP are present in the cell following the "quiescent" phase and may account for the increased sensitivity of growing cells to chemical mutation and transformation.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Fig. 1. Induction of aryl hydrocarbon hydroxylase activity in NMuLi Cl 8 mouse liver cells as a function of the length of time in the presence of BaP. The induction process was started by the addition of BaP either 8 hrs before, or at the time the cells were stimulated to move synchronously through the cell cycle.

Fig. 2 Induction of AHH activity in synchronous culture of NMuLi Cl 8 cells with BaP added at 7 hrs after the cells were stimulated to move synchronously through the cell cycle.

Fig. 3. A. The fraction of NMuLi Cl 8 cells in G_1, S, and G_2+M phases of the cell cycle as a function of time after the cells were stimulated to move synchronously through the cell cycle. BaP was added 7.5 hrs after stimulation.

B. Induction of AHH activity in the same cells as in A above.

Fig. 4. DNA histograms of NMuLi Cl 8 cells in the presence of excess thymidine. Excess thymidine was used to block the cells in early S phase (17-17 through 17-23) and in mid S phase (19-19 through 19-25). The first number in the label for each histogram indicates the time after serum stimulation when the BaP was added. The second number in the label refers to the time the cells were harvested. Induced AHH activity was assayed in these cells and the results are shown in Fig. 5.

Fig. 5. Induction of AHH activity in NMuLi Cl 8 cells in the presence of excess thymidine. BaP added 17 and 19 hrs after stimulation.

Fig. 6. DNA histograms of NMuLi Cl 8 cells after release from excess thymidine block. At 29 hrs after stimulation, BaP was added to the cells and the excess thymidine was removed. The second number in the label refers to the time the cells were harvested. Induced AHH activity was assayed in these cells and the results are shown in Fig. 7.

Fig. 7. Induction of AHH activity in NMuLi Cl 8 cells after release from excess thymidine block. BaP was added 29 hrs after stimulation.

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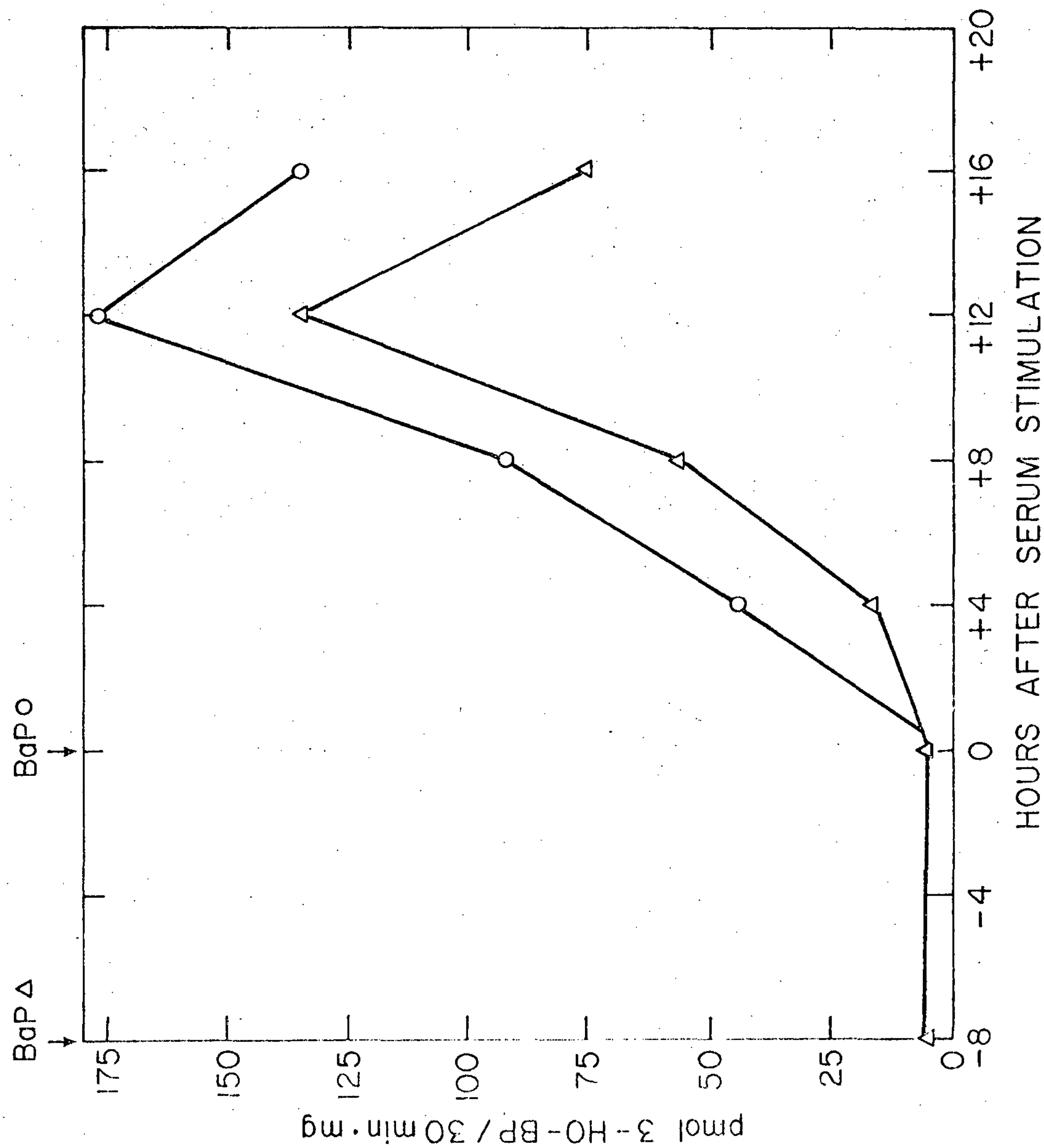

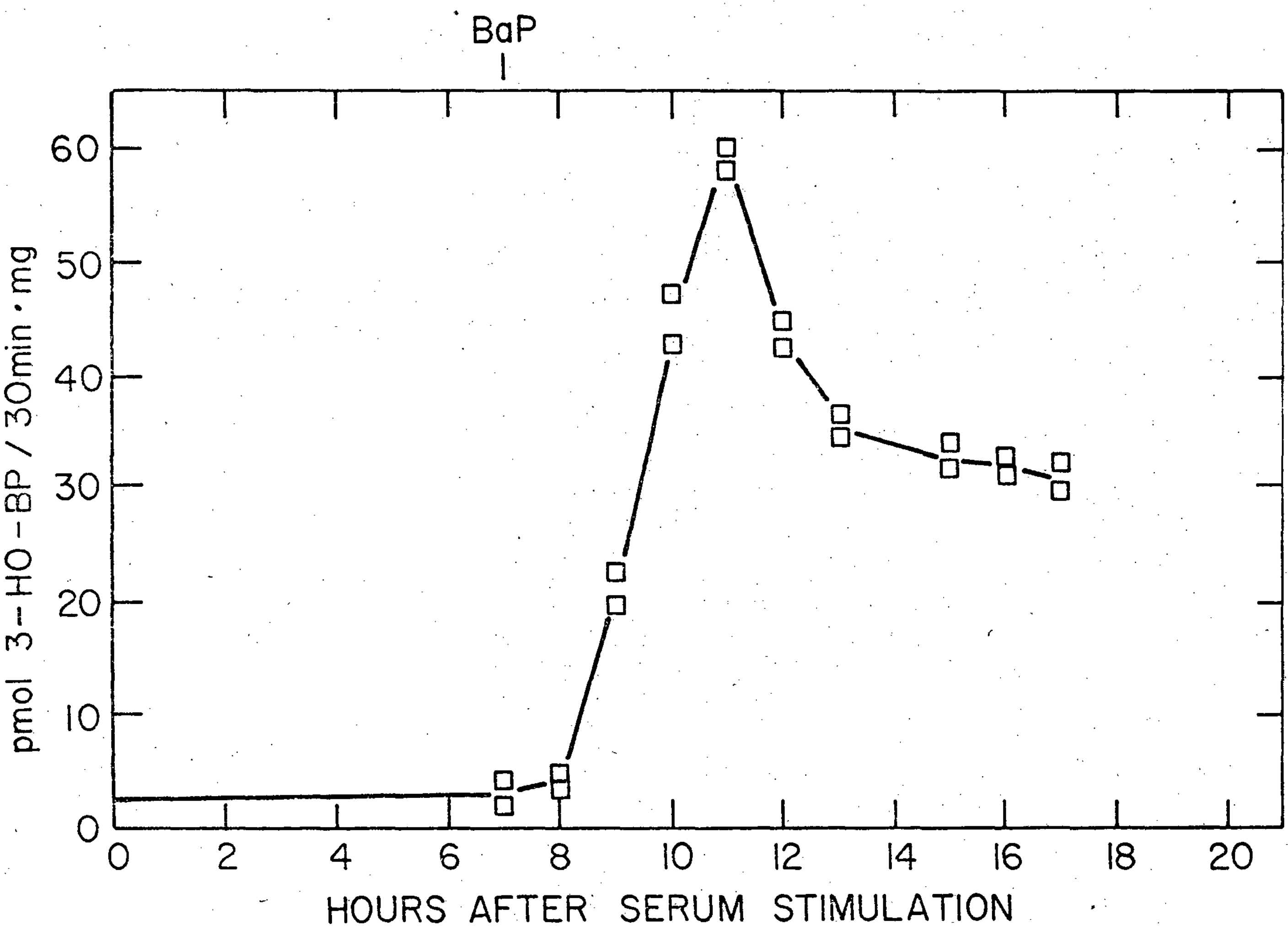
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60
50
40
30
20
10
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HOURS AFTER SERUM STIMULATION

3

Fig. 2

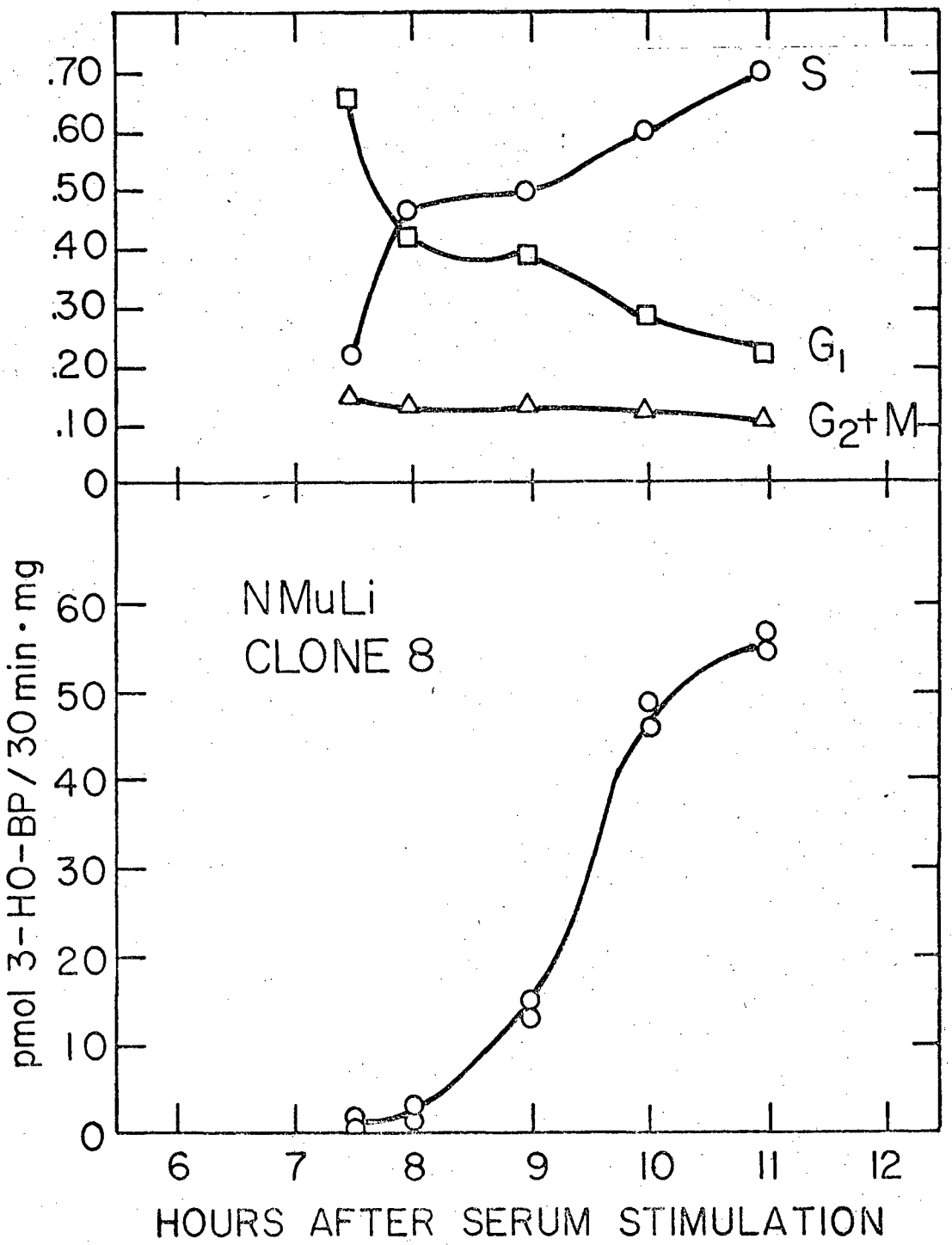
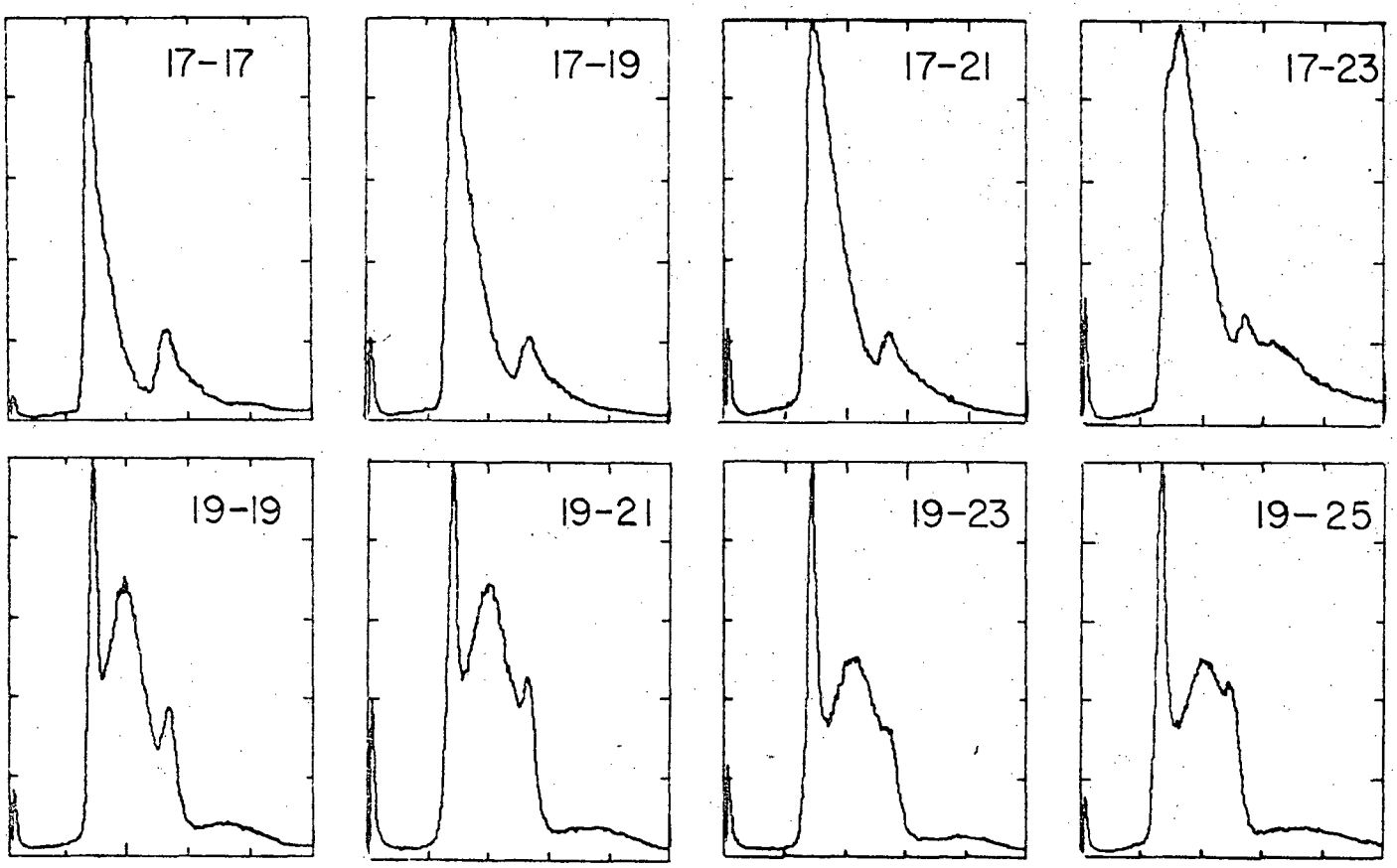


Fig 3 Parker & Bartholomew



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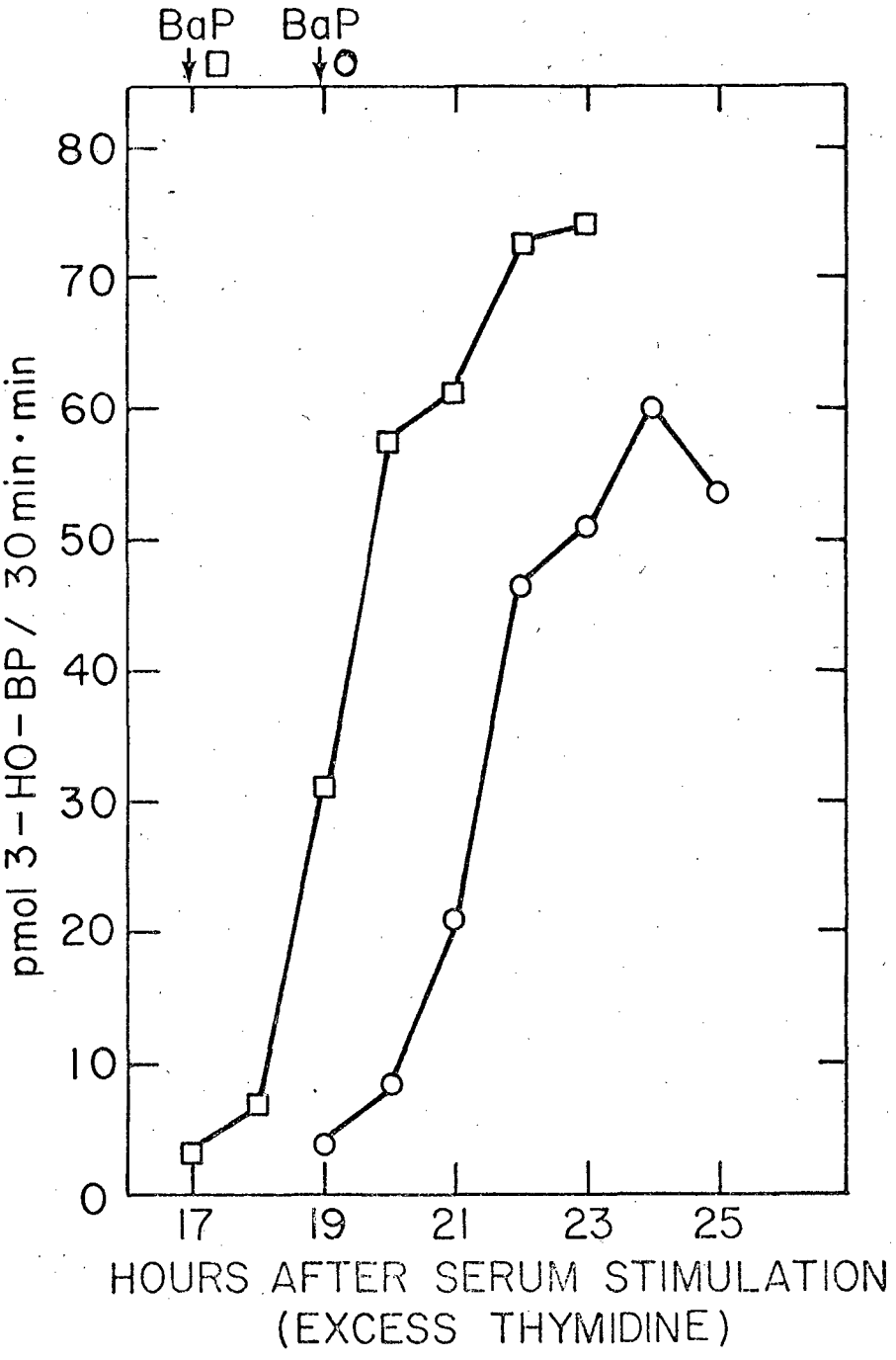

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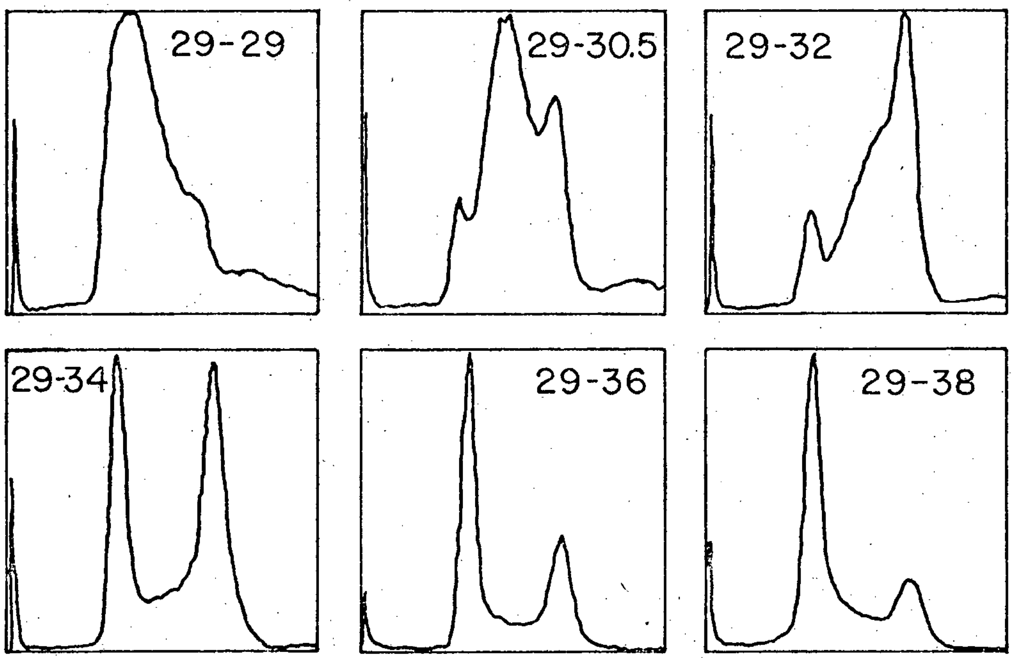

Fig. 6 Dooley & Bartholomew

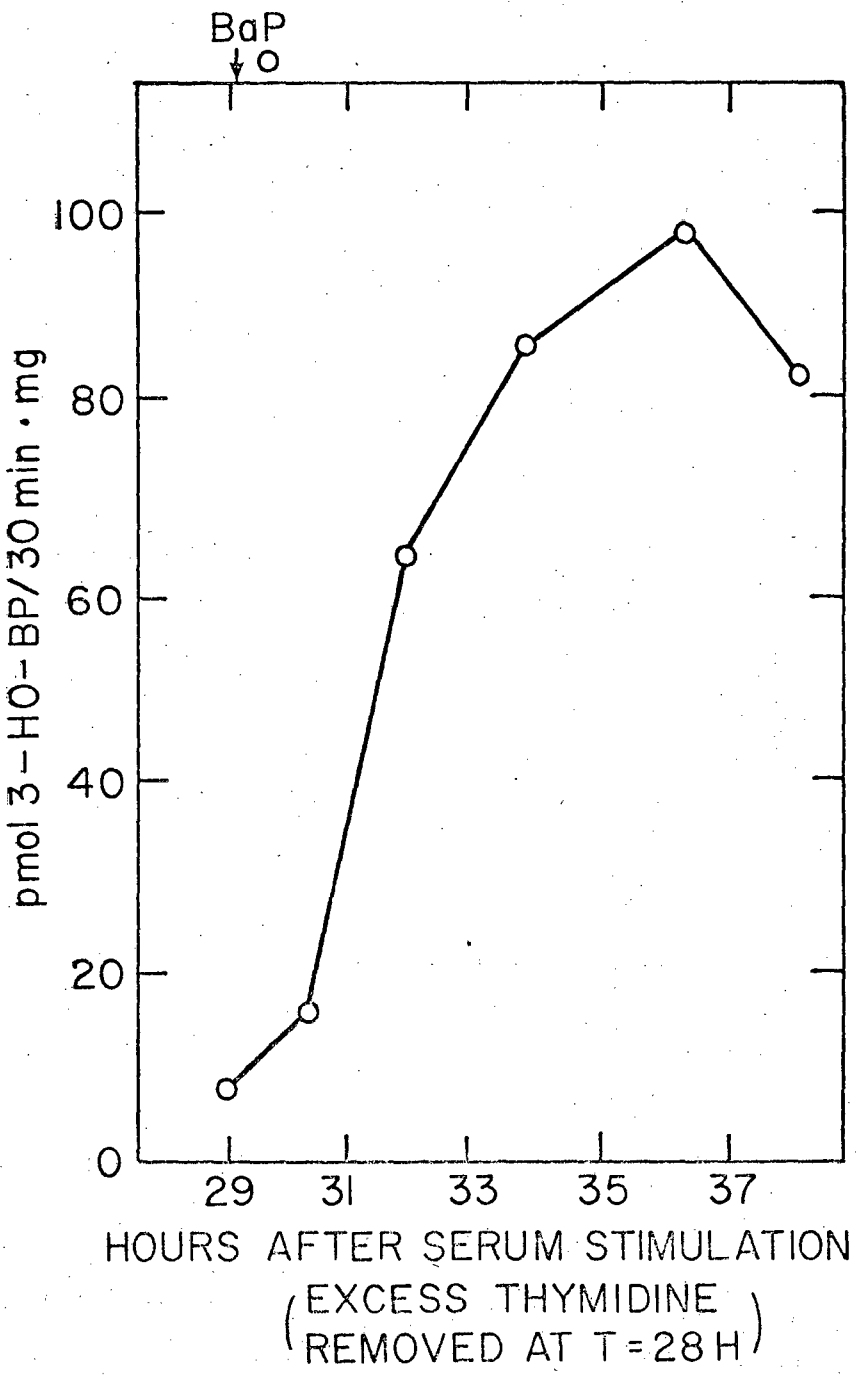

Fig 7 Baker & Bechtel