Biochemical Pharmacology, Vol. 19, pp. 2211–2220. Pergamon Press. 1970. Printed in Great Britain

DRUG EFFECTS ON CATALASE ACTIVITY IN THE MAMMALIAN CELL

THE ROLE OF THE CELL IN DRUG ACTION*

C. DE LUCA, G. B. VALLS and J. A. MASON

Department of Oral Biology, School of Dentistry,
The State University of New York, Buffalo, N.Y., U.S.A.

(*Received* 10 *September* 1969; *accepted* 23 *December* 1969)

Abstract—Porphyria-inducing derivatives of allylisopropylacetic acid depress catalase activity in rat liver *in vivo*. The availability of the mammalian cell culture technique has made it possible to investigate the action of these drugs on catalase activity of cells of a single type isolated *in vitro* from the influence of cells of other types. This report describes the results of studies of the effect of allylisopropylacetylcarbamide (AIC) and allylisopropylacetamide (AIA) on two mammalian cell lines derived from carcinomas of widely differing tissues: RPMI 2402, from the intestine in the hamster, and H4-II-E-C3 cells, derived from a minimal-deviation hepatoma in the rat. It has been shown that $7{\cdot}10 \times 10^{-3}$ M AIA, sufficient to inhibit the growth of both these cell lines by 40–50 per cent, exerted two different effects on their catalase activity. In the case of the 2402 cells, it provoked a continued increase in the specific activity of this enzyme over 4 days of growth to a value up to 70 per cent greater than that of the first day. With the H4 cells, however, the presence of the drug caused a progressive decrease in the specific activity, as it did in the rat *in vivo*, to a mean level that was 65 per cent of the control by the fourth or fifth day.

These observations reflect the multiplicity of enzyme patterns seen in the studies of Potter *et al.* and of Rechcigl *et al.* with a series of minimal-deviation hepatomas. They support the hypothesis that the specificity of drug action may reside in part in the cell receptor and may not be entirely a property of the agent itself.

SCHMID *et al.*[1] have shown that, simultaneous with the induction of acute porphyrinuria in rats, allylisopropylacetylcarbamide (AIC) caused a dramatic decrease in catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) activity in rat liver. Most interestingly, this action of the drug appeared to be specific for catalase, and essentially only for hepatic catalase. No effect was shown on other porphyrin-containing proteins: cytochrome c content of the liver did not differ appreciably from that of control animals; cytochrome oxidase and succinate dehydrogenase were not affected at all over an extended period of treatment. While catalase activity in the liver decreased to 10 per cent or less of the original in 3 days, it was only slightly affected in the kidney and was not at all affected in the erythrocyte, even over a period of 1 month.

Goldberg and Rimington[2] and also Talman *et al.*[3] tested a wide variety of congeners of AIC and showed a derivative, allylisopropylacetamide (AIA), to be even more effective at inducing porphyria. Rechcigl and his associates later showed this latter

* This study was initiated at the Roswell Park Memorial Institute, Buffalo, N.Y.

compound to have the ability also to decrease catalase levels in rat liver and kidney and used it in their elegant studies on the kinetics of turnover of this enzyme *in vivo*.[4,5]

The availability in our laboratory of mammalian cell lines maintaining relatively high levels of catalase activity *in vitro* presented an opportunity to determine the effects of these porphyria-inducing agents on cells isolated from the influence of other cell types. Extension of earlier studies to a system of this kind should contribute to our knowledge, and possibly to our understanding, of the tissue specificity of action apparent for these drugs. Furthermore, it well documented and, indeed, may be a general phenomenon, that the specific activity of a variety of enzymes fluctuates in a regular and periodic manner throughout each single growth cycle of populations of mammalian cells *in vitro*.[6] It appeared that agents like AIA or AIC could be employed to elucidate, as a corollary, the mechanism(s) underlying changes apparent in levels of catalase[6,7] and, perhaps by extension, other enzymes in mammalian cell culture in general.

With this in mind, appropriate experiments were designed first of all to determine the the effects of these two agents on growing cultures of mammalian cells and then to ascertain whether they could influence catalase levels as they did in the steady state situation which obtained in the studies *in vivo*. We wish to report the results of these experiments here. The preliminary data obtained suggest that the specificity of action observed for both AIA and AIC may depend in part on the receptor cell and not entirely on the drugs themselves.

MATERIALS AND METHODS

Cell culture methods. Two established lines of mammalian cells were employed in these studies. The earlier studies were carried out with suspension cultures of RPMI 2402, derived from a carcinoma of the small intestine in the hamster. This line was adapted to grow in suspension in Medium 906 supplemented with 5% calf serum.[8] The more recent studies were performed with cell line H4-11-E-C3, which was derived about 8 years ago from the minimal Reuber hepatoma in the rat.[9] This cell line was obtained through the generosity of Dr. Van R. Potter at the McArdle Laboratory. It is maintained in this laboratory in monolayer form in roller bottle and stationary cultures in Medium S-20 (personal communication), containing 20% fetal calf serum.

Stock cultures of RPMI 2402 were maintained by dilution of cell suspensions with fresh medium at 3–4 day intervals and by periodic transfer to new spinner flasks. Monolayer cultures of the hepatoma-derived line were maintained by serial transfer with trypsin at weekly intervals. Mediums 906 and S-20 and the calf serum were prepared by the Tissue Culture Medium Service at Roswell Park Memorial Institute (Buffalo, N.Y.). Fetal calf serum was purchased from Grand Island Biological Co. (Grand Island, N.Y.).

Full details for studies employing the suspension technique have been published elsewhere.[8] For studies employing the monolayer technique, replicate 250-ml centrifuge bottles, each containing 10 ml of appropriate medium equilibrated to room temperature, were inoculated with a suspension of cells trypsinized from late-logarithmic phase cultures. The new cultures were then immediately adjusted to pH 7·2 to 7·4 with a 5% CO_2/95% air mixture and incubated at 37° in drums rotating at $\frac{1}{5}$ rpm. Thereafter the pH was not readjusted, and the medium was not replenished, throughout the remainder of the experiment. Strict aseptic conditions were employed and

maintained in all cell culture techniques; only data derived from cultures shown to be free of common contaminating organisms and *Mycoplasma* were considered acceptable.

Cell-free preparations. Methods used for the preparation of lysates of 2402 cells from suspension culture have been published.[7,8] For the preparation of cell-free extracts of H4 cells, the medium was decanted from an appropriate number of bottles, and the cell layer was rinsed twice with about 4 ml of normal saline and once with trypsin; the cells were then released by treatment with 0·20× trypsin (Difco, 1:250) for 15 min at 37°. After release of the cells, the trypsin was diluted and the cells were pooled by transfer to a centrifuge tube in three 5- to 10-ml washings with ice-cold saline. The cells were collected by centrifugation at 700 *g*, 5°, transferred to a conical bottom homogenizer tube in three washings with cold saline, and then recentrifuged. The washed pellet of cells was finally suspended in cold 0·005 M phosphate buffer, pH 7·2, and frozen at −14°. This suspension was thawed only at the time of assay and homogenized with a tight-fitting glass pestle. The homogenate was centrifuged at 700 *g* as before; the supernatant fluid obtained by this procedure was used immediately in assays for catalase activity. Finally, this preparation was assayed for its protein content.

Assay procedures. Catalase activity was determined by the spectrophotometric method of Price *et al.*[4] The instrumentation and protocol for the assay of whole lysates derived from suspension cultures were exactly as previously described.[7] For the recent work with homogenate-supernatants derived from monolayer cultures, the Gilford 2400 spectrophotometer was employed. Assay cuvettes contained: 160 μmoles phosphate buffer, pH 7·2; 44·5 μmoles hydrogen peroxide; 0·02 to 0·05 ml supernatant (containing 50–100 μg protein) and water in a total volume of 3·0 ml. The reaction was started by addition of the enzyme preparation. Catalase activity was determined by the rate of decrease in absorbance at 230 mμ at 25° relative to a blank cuvette which contained all the ingredients of the assay mixture except H_2O_2. It was found that, with the protocol given above, the rate of change in absorbance, measured between 12 and 60 sec, was a linear function of the amount of supernatant preparation used. The amount of enzyme which was capable of decreasing the absorbance by 0·001 unit per min, under the conditions specified, was taken as the unit of catalase activity.

6-Phosphogluconate dehydrogenase was determined spectrophotometrically from the rate of reduction of nicotinamide-adenine dinucleotide phosphate in the presence of an excess of the coenzyme, magnesium chloride, and 6-phosphogluconate in Tris (hydroxymethyl) aminomethane buffer, pH 8·6. The unit of this enzyme was based on the rate of increase in absorbance at 340 mμ.

Protein concentrations were determined by the method of Lowry *et al.*;[10] bovine plasma albumin (Cohn Fraction V, Pentex Corp., Kankakee, Ill.) was used as standard. Specific activity for each enzyme was calculated from the units determined per milligram of protein in each preparation.

Growth was measured by direct count of cells in suspension cultures using a dye exclusion technique.[11] With the monolayer cultures, growth was expressed on the basis of protein recovered in the supernatant used for enzyme assay. It had previously been shown that this was essentially a constant fraction of the total protein in each culture.

Replicates of each experiment were run. All spectrophotometric assays and protein determinations were done at least in duplicate; average values were calculated using only results agreeing within 10 per cent of one another.

Statistical analyses. To determine the variation inherent in the regimen adopted for starting and randomizing cultures, sampling, etc., two separate preliminary experiments were performed. (1) Suspension cultures were set up in duplicate and sampled daily over a 4-day period and assayed; then the percentage difference between duplicate samples taken on each given day were subjected to statistical analyses. (2) Replicate monolayer cultures were allowed to grow for 4 days, then all cultures were harvested simultaneously and assayed; the mean of all results and standard deviation were then calculated. Statistical analysis of the data obtained from suspension cultures showed that the mean difference between any two samples taken simultaneously was 10·2 per cent with a standard deviation of 5·9 per cent throughout the entire growth cycle. Analysis of the data derived from monolayer cultures indicated that replicate samples harvested simultaneously show a standard deviation that is 7·6 per cent of the calculated mean.

Due to the nature of the procedures necessary for a study of the type described in this presentation, it was not feasible to perform replicate experiments simultaneously. It was necessary, therefore, to repeat experiments sequentially from week to week. Absolute values obtained for a given series of cultures, control or experimental, were thus subject to more variation than would be expected from replicate experiments all done simultaneously; overall specific activity profiles, however, were reproducible. Accordingly, each experiment was designed to be self-contained with both control and treated cultures run concurrently. In this manner each drug-treated culture could be compared directly with its respective control. Statistical analyses[12] were then carried out on the differences observed between these two for any given day. The means and standard deviations were calculated from these differences for corresponding days from among replicate experiments.

Reagents. Allylisopropylacetylcarbamide and allylisopropylacetamide were gifts of Hoffman-LaRoche, Inc. and were obtained through the courtesy of Dr. W. E. Scott. Hydrogen peroxide (30%), Superoxol, was the Fisher certified reagent. All other chemicals employed were the highest purity available.

RESULTS

Observations on variations in the specific activity of catalase throughout a single growth cycle of suspensions of RPMI 2402 cells have been published.[7] Normally, the profile of activity displayed a decay from a maximum value on the first day after transfer to approximately one-half that value by the fourth day. Under the influence of constant aeration, however, catalase activity appeared to be induced and the specific activity did not decline but, rather, continued to increase. Under these conditions, the specific activity reached a maximum value, around the third or fourth day of growth, that was routinely observed to be three to five times that of the unaerated culture. It would appear that the aerated system would be the most logical one to employ for studying the effect of an inhibitor of the synthesis of the enzyme.

The effects of AIC on the basic activity profile was, therefore, examined at a level which inhibited growth by approximately 50 per cent. Table 1 presents data from three experiments in which 2402 cells were grown in the presence of AIC with continuous aeration. It may be seen that catalase continued to increase in specific activity, despite the presence of the drug, to an average of 2·53-fold over the value for day 1. This observed increase in activity took place during the time when cellular protein continued

TABLE 1. CATALASE ACTIVITY OF AERATED CULTURES OF 2402 CELLS TREATED WITH ALLYLISOPROPYLACETYLCARBAMIDE (AIC)

Experiment* no.	Relative specific activity† Day 1	2	3	4
1	1·00	1·55	1·76	2·47
2	1·00	1·00	2·00	2·23
3	1·00	1·36	2·24	2·88
Average	1·00	1·30	2·00	2·53

* Cultures in all three experiments were treated with AIC at a concentration of $5{\cdot}43 \times 10^{-3}$ M.

† Calculated from the ratio of the specific activity for any given day to that for the first day.

to increase. These data agree in both their qualitative and quantitative aspects with those previously observed with this system in the absence of any drug.

Although the unaerated system did not appear to be suited for the study of an inhibitor of this particular enzyme, it was employed in an attempt to ascertain whether AIA could further influence the normally observed diminution of catalase activity. If AIA affected the biosynthesis of catalase, then one should expect to see an even greater lowering of the specific activity of this enzyme in the face of increasing protein during active growth. Representative results of these experiments are shown in Fig. 1. The left panel (a) of this figure illustrates typical growth characteristics and the relative inhibition seen with AIA in this system. Despite the fact that the cell density attained in the presence of drug was only about one-half that seen in its absence, the viability of the cells was still in the range of 95–100 per cent. The right panel (b) of Fig. 1 illustrates

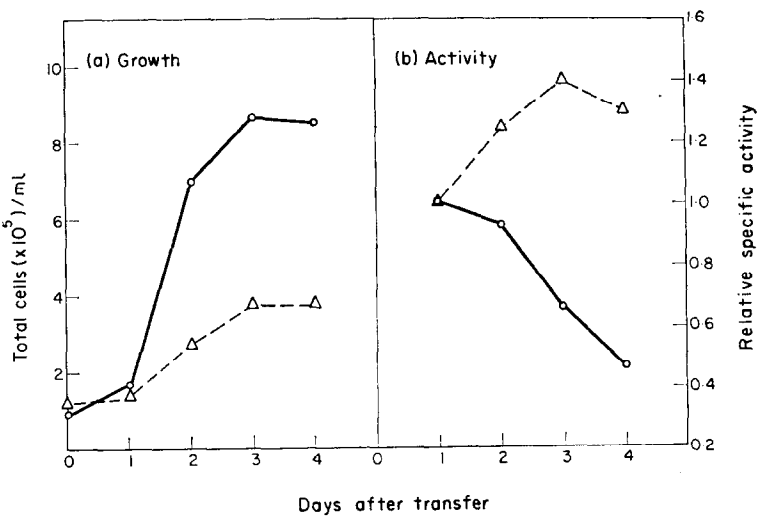

FIG. 1. Effects of AIA on growth and catalase activity of RPMI 2402 cells. Growth was determined by direct cell count on aliquots of suspension cultures. Enzyme activity was determined on cell-free lysates prepared from aliquots removed daily; relative specific activity was calculated from the ratio of the specific activity for any given day to that for the first day. Control cultures are denoted by the circles (-○-); the triangles (-△-) signify cultures treated with AIA at a concentration of $7{\cdot}10 \times 10^{-3}$ M.

the typical decay in specific activity for catalase normally exhibited by control cultures. It may be seen that the AIA-treated cultures do not show any further reduction of activity. In fact, the specific activity usually continued to increase beyond the level of the first day to a maximum around day 3 or 4. If a comparison is made between day 1 and day 4 only (for the sake of simplicity), then the mean percentage increase in activity between these 2 days for a series of five AIC-treated cultures is 33·4 per cent with a standard deviation of 28·6. For a series of four AIA-treated cultures analyzed in the same way, the mean increase was 33·8 per cent with a standard deviation of 35·1. Despite the wide variation seen in these experiments, the results show that catalase activity in treated cultures of 2402 cells ranges from values not significantly different from that of day 1 to values that may be 62–70 per cent greater than that for day 1.

In an effort to explain this apparent inability of AIC and of AIA to depress the level of catalase in this cell line and reconcile these results with those obtained in whole animal studies, it was hypothesized that the effect of these agents noted *in vivo* might be mediated specifically by the liver parenchymal cell. It was decided therefore to test this hypothesis with cultures of cells derived from a minimal-deviation hepatoma. Although the line chosen, H4-11-E-C3, had been in culture for an extended period of time, it still exhibited some of the characteristics of the liver cell and of the original cell line. For example, it has maintained the ability to respond to steroid hormones for the induction of tyrosine transaminase activity[13,14] (C. De Luca and R. P. Gioeli, unpublished results); it has retained also the ability to induce hepatoma-like tumors in rats *in vivo* (C. De Luca and J. A. Mason, unpublished results).

Interestingly, when the effect of AIA on the catalase activity of growing cells of the H4 line was examined, entirely different results were obtained. Shown in Fig. 2 are

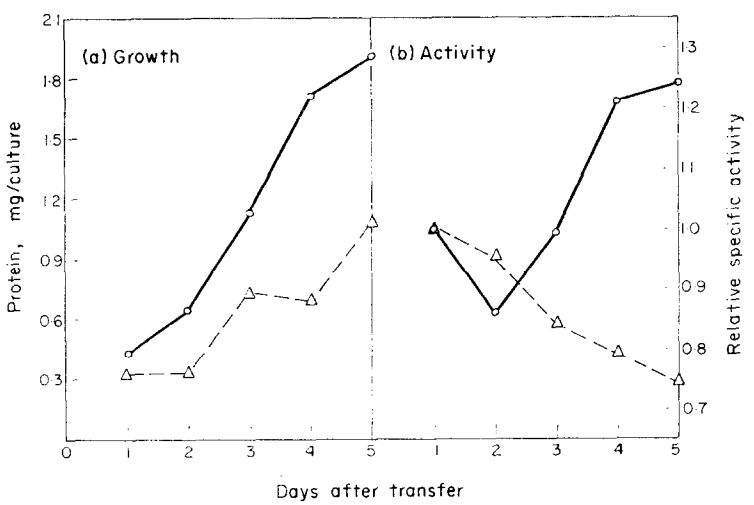

Fig. 2. Effects of AIA on growth and catalase activity of H4-II-E-C3 cells. Growth is based on the protein content of homogenate-supernatants prepared from monolayer cultures terminated daily. Enzyme activity was determined on the same supernatant preparations. Relative specific activity was calculated from the ratio of the specific activity for any given day to that for the first day. Control cultures are signified by the circles (-○-); the triangles (-△)- represent cultures treated with AIA at a concentration of $7{\cdot}10 \times 10^{-3}$ M.

illustrative data typical for this system. Again the left panel (a) of the figure shows growth curves for treated and untreated cultures respectively. It may be seen that amounts of AIA comparable to that used with the 2402 line inhibited overall growth of the hepatoma cells to the extent of about 40 per cent of the controls. The peculiar stepwise increase shown here for the AIA-treated culture was highly reproducible and is reminiscent of growth curves obtained for cells in synchrony. The right panel (b) of Fig. 2 shows the catalase activity. From this section several points should be evident. (1) The profile of variation of the specific activity of catalase for control cultures is entirely different from that typically observed for the 2402 line grown in suspension. This is not surprising in view of earlier findings[8] on the influence of various factors on patterns of variation in enzyme activity in mammalian cells. As shown here, it is characteristics for the specific activity of catalase in H4 cells to rise to a maximum value at about the fourth or fifth day of growth after a lag or a decrease between the first and second or third day. (2) It may be seen, further, that AIA clearly influenced the synthesis of catalase in these cells. In the presence of this drug, the specific activity showed a continued decay to a level of enzyme which was about 75 per cent that of the original. This occurs even though overall cellular protein increased better than 3-fold. Shown in Table 2 are results derived from several experiments which illustrate the

TABLE 2. OBSERVATIONS ON THE DIFFERENCES IN CATALASE ACTIVITY APPARENT BETWEEN CONTROL AND ALLYLISOPROPYLACETAMIDE (AIA)-TREATED CULTURES OF H4-II-E-C3 CELLS

Day	Differences in specific activity* (Control minus experimental)
1	0·14 ± 0·92
2	0·37 ± 0·54
3	1·02 ± 0·71
4	1·24 ± 0·34
5	1·66 ± 0·56

* The values shown are the means ± standard deviations of differences in specific activity observed each day between AIA-treated cultures and their respective controls. Each mean and standard deviation was calculated from the results of 4 experiments. Experimental cultures were treated with $7{\cdot}10 \times 10^{-3}$ M AIA.

magnitude of differences in enzyme between control and treated cultures on any given day of the growth cycle. This shows, in agreement with the effect seen on growth (Fig. 2), that AIA has an early influence on this cell. Further, it shows the effect of this drug to be a progressive one, which becomes very much pronounced by the fourth or fifth day, when the overall difference reached an average of 1·66 specific activity units. This represents an average difference of 35·2 per cent in absolute terms. Repeated injections, twice daily, of AIA have been shown[4] to reduce catalase activity of the liver *in vivo* by 75 per cent.

In order to ascertain the specificity of action of AIA or AIC in this system, the activities of certain other enzymes not related to catalase were measured. It was found

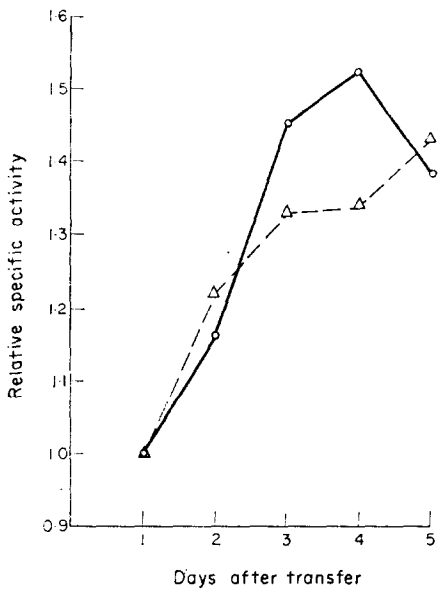

Fig. 3. Effect of AIA on 6-phosphogluconate dehydrogenase. Enzyme activity was determined on homogenate-supernatants prepared from monolayer cultures of H4-II-E-C3 cells terminated daily. Relative specific activity was calculated from the ratio of the specific activity for any given day to that for the first day. Control cultures are denoted by the circles (-○-); the triangles (-△-) represent cultures treated with AIA at a concentration of $7{\cdot}10 \times 10^{-3}$ M. The values shown are the average of two experiments.

that, in cultures of 2402 cells, the drug had no effect on lactate or glucose 6-phosphate dehydrogenase, just as it had no effect on catalase. With the H4 line only the enzyme, 6-phosphogluconate dehydrogenase was used to determine specificity. It was found that the drug had no significant effect on either the profile of variation or the amount of activity of this enzyme throughout the growth cycle. Average values from two experiments are presented in Fig. 3. The values observed for this enzyme in treated cultures vary at most between 8 and 12 per cent from those of control cultures.

DISCUSSION

The mammalian cell cultured *in vitro* offers much to the investigator as a model system for the elucidation of a variety of problems of major interest today. Eagle[15] has summarized certain of these relating to control mechanisms in the mammalian cell. Perlman[16] more recently has described in detail the use of this system especially for pharmacologic studies. He has clearly stated the central problem involved in the screening of pharmacologic agents and has pointed out the need to be concerned about molecular effects of drugs, which has come to be recognized from the lack of correlation between effects observed *in vitro* and those observed *in vivo*. That we cannot rely merely on gross parameters such as growth or overall protein synthesis in the evaluation of drug efficacy is now being more fully appreciated. Indeed, more sensitive, specific parameters need to be employed in order that more meaningful interpretation and extrapolation of data may result from studies on the action of drugs

in a biologic system. It follows from this that it would be important to recognize whether such parameters to be employed normally undergo characteristic changes. It is well documented, for example, that specific activities of a wide variety of enzymes fluctuate in a regular periodic mode throughout the growth cycle of cultured mammalian cells.

It was the uncertainty about the mechanism of this fluctuation in activity that led us to pursue earlier studies.[17] The activity of an enzyme may change because of an altered rate of synthesis; it may also depend on the formation of a holo-oligomeric structure from subunits. If the rate of formation of an enzyme is not affected, its stabilization or its activity, or both, might be influenced by the availability of cofactors or by the presence of effectors acting competitively at the site of enzyme action or allosterically by influencing conformational change.

It was desirable to take advantage of an agent interfering specifically with the synthesis of catalase in an effort to answer this question and gain information about control mechanisms operative in the mammalian cell. The results obtained, however, were seemingly paradoxical. From the studies with unaerated cultures of 2402 cells, on the one hand, an unexplained increase in specific activity of catalase occurred in the presence of AIA even while cellular protein was increasing. This is in contrast to the effect of the drug cited previously[1,4,5] for the whole animal. This effect could also result from an agent preventing endogenous catabolism or decay of the enzyme. There is no evidence for an action of this type for AIA. The tracer studies of Schmid *et al.*[1] and of Rechcigl and Price[5] have shown that AIA could almost completely block the synthesis of new catalase. In order to reconcile the conflicting results found between the whole animal studies and those utilizing the 2402 cells, it was necessary to seek an explanation in the cell itself. This was supported by studies with the H4 line, cells maintaining the biochemical semblance of mammalian liver. Studies with these cells did indeed indicate a decrease in synthesis of catalase in the presence of AIA. Examined in broader perspective, then, the results presented here are most encouraging. They corroborate the belief that the cultured cell may indeed be usefully employed to explain the mechanism of specific drug action.

The differences observed in the effect on catalase between the two cultured cell lines cannot be explained by differential uptake or stability of the drug, since it exhibited both an early and sustained effect on growth with each cell. The lack of effect of AIA on the catalase activity of 2402 cells might have been due to differences in the two mediums employed or possibly in the mode of culture. It has been shown in a wide variety of systems that the environment may have a tremendous influence on the modulation of cellular activity. (See reference 18 for a brief summary.) This point can very readily be tested and is presently being pursued in this laboratory.

The results described here reflect the disparity described by Perlman[16] in comparative studies involving whole animals and isolated cells of a given type. Interesting differences have been found for catalase also in resting, non-dividing cells (C. De Luca and J. A. Mason, in preparation). The data presented are not sufficient by themselves to uphold, but are consistent with, the hypothesis that the specificity of action of a pharmacologic agent may lie partially in its receptor site and not entirely in the agent itself. That is to say, a drug may exert its peculiar effect only after interaction in some specific manner with a particular cell type. It is of interest that, while this manuscript was in preparation, a report appeared by Rechcigl *et al.*[19] showing an analogous response of catalase

to AIA treatment in a series of hepatomas *in vivo*. These authors reported: first, that catalase activity did not respond to the same degree in all hepatomas as it did in the liver of the host; and second, that when a series of hepatomas, including the more common minimal-deviation hepatomas, were studied, a wide range of response to AIA was encountered. Some of these tumors showed even a lack of response to this agent. These findings concurred with those of Potter and Watanabe[20] with an overlapping series of minimal-deviation hepatomas. In a very thorough series of studies, these latter investigators have discovered a multiplicity of response of certain other enzymes to environmental conditions. This has emphasized the biochemical diversity of, and may lead to the essential characterization of, a series of neoplastic tissues all classified as minimal-deviation hepatomas. It is most tempting to inquire whether this diversity is seen as a result merely of phenotypic response or whether it is a manifestation of actual genotypic change.

Acknowledgement—This work was supported in part by Institutional Grant 1N-54E-3 of the American Cancer Society, GRS Grant 5-S01-FR-05330-07 of the United States Public Health Service, and Grant P-513 of the American Cancer Society.

REFERENCES

1. R. Schmid, J. F. Figen and S. Swartz, *J. biol. Chem.* **217**, 263 (1955).
2. A. Goldberg and C. Rimington, *Proc. R. Soc.* **B. 143**, 257 (1955).
3. E. L. Talman, R. F. Labbe and R. A. Aldrich, *Archs Biochem. Biophys.* **66**, 289 (1957).
4. V. E. Price, W. R. Sterling, V. A. Tarantola, R. W. Hartley, Jr. and M. Rechcigl, Jr., *J. biol. Chem.* **237**, 3468 (1962).
5. M. Rechcigl, Jr. and V. E. Price, *Progr. exp. Tumor Res.* **10**, 112 (1968).
6. Y. L. Pan and R. S. Krooth, *J. cell. Comp. Physiol.* **71**, 151 (1968).
7. C. De Luca, *Expl Cell Res.* **44**, 403 (1966).
8. C. De Luca, *Expl Cell Res.* **43**, 39 (1966).
9. H. C. Pitot, C. Peraino, P. A. Morse, Jr. and V. R. Potter, *Natn. Cancer Inst. Monogr.* **13**, 229 (1964).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
11. C. De Luca, P. F. Rakowski and G. L. Tritsch, *Biochim. biophys. Acta* **86**, 346 (1964).
12. G. W. Snedecor, *Statistical Methods*, 5th edn, p. 36. Iowa State College Press, Ames, Iowa (1956).
13. V. R. Potter, M. Watanabe, J. E. Becker and H. C. Pitot, *Adv. Enzyme Regulat.* **5**, 303 (1968).
14. J. R. Reel and F. T. Kenney, *Proc. natn. Acad. Sci. U.S.A.* **61**, 200 (1968).
15. H. Eagle, *Science, N.Y.* **148**, 42 (1965).
16. D. Perlman, *Science, N.Y.* **160**, 42 (1968).
17. C. De Luca and H. M. Nitowsky, *Biochim. biophys. Acta* **89**, 208 (1964).
18. C. De Luca and A. V. Thomas, *Life Sci.* **8**, 1145 (1969).
19. M. Rechcigl, Jr., Z. Hruban and H. P. Morris, *Enzym. biol. clin.* **10**, 161 (1969).
20. V. R. Potter and M. Watanabe, in *Proc. Int. Conf. Leukemia-Lymphoma* (Ed. C. J. D. Zarafonetis), p. 33. Lea & Febiger, Philadelphia (1968).