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BIOSYNTHESIS OF CHOLINESTERASE IN RABBIT BONE MARROW CELLS IN CULTURE

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Abstract—To gain insight into biochemical mechanism(s) involved in cholinesterase (ChE) synthesis, rabbit bone marrow cells in an active state of erythropoiesis induced by pretreatment of animals with phenylhydrazine were dispersed in minimum essential medium (MEM) and an aliquot was inhibited with pinacolyl methylphosphonofluoridate (soman), an irreversible inhibitor of ChE. Both normal and soman-inhibited cells were placed in tissue culture for 24 hr, harvested, and assayed for ChE activity. Although the initial enzyme activity of soman-pretreated cells was less than 5 per cent of normal, the net increase in ChE activity during the 24-hr incubation period was comparable to that observed in normal cells. With soman-pretreated cells there was an increase in the acetylcholine hydrolysis rate from 9·3 to 165·0 nmoles hydrolyzed/hr/mg of protein. Addition of 10^{-3} M N^6,O^2-dibutyryl adenosine 3′,5′-cyclic monophosphate (DBcAMP) at the beginning of the incubation period resulted in a further increase in the hydrolysis rate to 317·0. Actinomycin D severely reduced the renewal of enzyme activity of soman-inhibited cells and completely abolished the stimulatory effect by DBcAMP on enzyme formation The results suggest that the return of ChE activity is related to synthesis of new enzyme protein and that DBcAMP and possibly endogenous cAMP may be involved in the regulation of ChE synthesis. ^{14}C-L-leucine incorporation and ^{3}H-methyl-thymidine uptake were not markedly altered by pretreatment with soman but were inhibited by actinomycin D.

RECENTLY, the effects of tissue explants and nerve homogenates on cholinesterase (ChE) activity of muscle of the newt in organ culture were examined.[1] These studies required incubation periods of from 7 to 14 days to show pronounced changes in ChE activity. In an extension of the work with the newt, Lentz[2] showed that adenosine 3′,5′-cyclic monophosphate (cAMP) was important in maintaining the ChE activity of the motor end plate of denervated muscle and that this activity was partially restored in tissue inactivated with diisopropyl fluorophosphate. The mechanism(s) by which the cyclic nucleotide exerted its effect on the ChE enzyme is not known. It is known that erythroid cells in culture undergo profound morphologic and biochemical changes, some of which have been studied thoroughly.[3,4] While much has been learned about the nature of the biochemical phenomena involved, especially in hemoglobin synthesis,[5] little is known concerning the mechanisms that are responsible for the synthesis and regulation of other proteins, as for example acetylcholinesterase (AChE). In studying the effects of drugs on enzyme regulation at the cellular level, we felt that a tissue culture system capable of "rapid" ChE formation would be of utmost importance; many drugs are unstable in cell cultures for prolonged periods of time.

Initial studies indicated that bone marrow cultures would meet the criterion. Thus, marrow cultures were chosen as the system of choice for obtaining basic information on the biochemistry of ChE formation.

In order to provide sensitivity in the assay for the production of new enzyme, it was necessary to inactivate the existing ChE permanently and almost completely. For this purpose, the organophosphorus inhibitor, pinacolyl methylphosphonofluoridate (soman), was used.[6] Soman is a typical organophosphorus compound in that it reacts rapidly and covalently with the enzyme to produce an inactive, phosphorylated enzyme.[7] Inhibition of the ChE by most phosphorus anticholinesterases results in slow spontaneous reactivation, which would cause obvious difficulty in studying formation of new enzyme protein.[8,9] However, soman is rather unusual; because of the rapid "aging" reaction of the resultant inhibited enzyme, no spontaneous reactivation occurs.[10]

This paper describes a tissue culture system, composed of bone marrow cells, which is capable of producing significant quantities of ChE in 24 hr. The results show that the return of ChE activity after soman pretreatment is dependent on the synthesis of native enzyme and that it is markedly enhanced by N^6,O^2-dibutyryl adenosine 3′,5′-cyclic monophosphate (DBcAMP).

MATERIALS AND METHODS

Materials. Adenosine 3′,5′-cyclic monophosphate and DBcAMP were purchased from Calbiochem, San Diego, Calif. Erythropoietin (step 3) was purchased from Connaught Medical Research Laboratories, Willowdale, Ontario, Canada. ^{14}C-L-leucine (U) (300 mCi/m-mole), ^{3}H-methyl thymidine (19·5 Ci/m-mole) and 1-^{14}C-acetylcholine (1·4 mCi/m-mole) were obtained from New England Nuclear Corp., Boston, Mass. Actinomycin D (AMD) was a product of Merck, Sharp & Dohme, Rahway, N.J. Disposo-trays, model FB16-24TC, were purchased from Linbro Chemical Co., New Haven, Conn. L-Glutamine (100X) was a product of Microbiological Associates, Bethesda, Md. Minnimum essential medium (Eagle) with Hank's salts was purchased from Grand Island Biological Co., Grand Island, N.Y. and Rehatuin fetal serum was a product of Reheis Chemical Co., Chicago, Ill. Triton X-100 was a product of Packard Instrument Co., Inc., Downers Grove, Ill. NCS reagent was purchased from Nuclear-Chicago, Des Plaines, Ill., and BW 284C51 was obtained from the Burroughs Welcome Co., Tuckahoe, N.Y.

Radiometric assay for ChE activity. The method is based on the adsorption of unhydrolyzed 1-^{14}C-acetylcholine (ACh) on Amberlite CG 120 resin suspended in dioxane. The supernatant solution containing the product of hydrolysis, 1-^{14}C-acetic acid, is counted in a liquid scintillation spectrometer. Details concerning preparation of resin and the scintillation mixture (fluor) are given by Siakotos *et al.*[11]

Red blood cells (RBCs) and bone marrow cells (BMCs) were obtained from normal and phenylhydrazine (PHZ)-treated rabbits. The cells were washed in minimum essential medium (MEM), counted in a Coulter counter, diluted in cold saline to a concentration of 5×10^6 cells/ml. and centrifuged. The pellet was lysed in 0·5 ml distilled water. One-tenth ml of 3×10^{-4} M ACh was added to 0·1-ml aliquots of the lysed cells in an equal volume of 0·1 M sodium phosphate buffer at pH 7·8 containing 0·3 M NaCl and 1% (w/v) Lubrol WX (lubrol buffer) at 37°. The mixture was allowed to incubate for varying lengths of time. Hydrolysis of the substrate was stopped by the addition of dioxane/resin.[11] Parallel blanks for individual time intervals were always run without cell lysate in this and other assays described. Experimental readings were corrected for the blank values. A 20-min incubation period was selected

for all assays for ChE activity because we found that, even with the most active bone marrow cell preparations, the extent of hydrolysis of ACh was linearly proportional to time beyond 20 min.

Inducing erythropoiesis in rabbits. Rabbits of both sexes, weighing between 1·8 and 2·2 kg, were given a subcutaneous injection of 0·4 ml/kg of 2·5% neutralized PHZ solution once each day for 4 days. Blood samples were taken from the ear vein just before dosing and on the day the animal was sacrificed for marrow. Blood was transferred to heparinized capillary tubes and centrifuged in an International microcapillary centrifuge, model MB, to determine the hematocrit. The percentage of reticulocytes in the blood was also determined.

Removal and culturing of bone marrow cells. Four days after the last dose of PHZ, the hind limbs of anesthetized rabbits were perfused by infusing saline into each femoral artery after first cutting the femoral vein. Details for removal and processing the marrow cells from the tibias are described elsewhere.[12] The cells then were dispersed with a pipette in 10 ml MEM, divided, and one of the portions was treated with 2×10^{-7} M soman for 15 min to inhibit existing ChE. After excess inhibitor had been removed by washing the cells in MEM, both the normal and the soman-treated cells were reconstituted in MEM. Aliquots from each were diluted and counted on a Coulter counter or laser cytograf. Five $\times 10^6$ cells/ml were cultured in a medium composed of 10% fetal calf serum, 2 mM L-glutamine; in MEM alone; and also in medium containing either 1 μCi ^{14}C-L-leucine or 2·5 μCi ^{3}H-methyl thymidine. One-ml aliquots of the seeded media then were placed into individual Disposo-tray cups. Untreated controls were treated with 0·2 ml of culture medium alone. To others, the same amount of culture medium containing either DBcAMP, cAMP, erythropoietin, AMD or some combination of drugs was added. Zero time cell samples were removed and the cells collected by centrifugation. The cell pellet was washed twice with 10 ml of cold saline per wash to remove traces of culture medium and then lysed in 0·5 ml deionized water. One-tenth-ml aliquots were taken for assays of enzyme activity and protein content.[13] The bone marrow cultures then were incubated on a rocking platform for various periods of time, usually 24 hr, at 37° in a moist atmosphere containing 83% N_2, 7% O_2 and 10% CO_2. After the indicated incubation period, the cells were scraped loose from the trays with a plastic scraper and removed. Where duplicate or triplicate samples were cultured, one sample from each was removed, counted and sized on a laser cytograf. Those samples to be assayed for ChE activity were harvested and processed as described above.

^{14}C-L-leucine incorporation into marrow cells. The reactions were stopped with an equal volume of ice-cold 20% (w/v) trichloroacetic acid (TCA) containing unlabeled 4×10^{-3} M DL-leucine. The precipitated proteins were further processed according to Fleisher and Harris.[14] The residue was then digested in 0·2 ml NCS at 37° until the pellet was completely dissolved. Five ml dioxane was added to each tube with thorough mixing and the entire solution transferred to a scintillation vial containing 12 ml fluor and counted.

^{3}H-methyl-thymidine uptake into marrow cells. The cells were pipetted onto a wet 0·45 μ Millipore filter, rinsed with saline and with cold 5% TCA. The TCA-precipitated proteins were washed with saline and rinsed with distilled water. The filters were allowed to dry overnight and placed in a scintillation mixture consisting of 10 ml Triton X-100–toluene (1:2), 0·1 g 1,4-bis-[2-(5-phenyloxazolyl)]-benzene

(POPOP) and 5·0 g 2,5-diphenyloxazole (PPO) per 1·5 l. and counted in a Nuclear-Chicago scintillation counter. Correction for quench was performed by the channels ratio method.

RESULTS

Effects of PHZ pretreatment on blood and marrow ChE activity. The marked reticulocytosis (56% reticulocytes) and reduction of the hematocrit after PHZ administration indicate that the marrow preparations used in these studies were in an active state of erythropoiesis. Microscopic examination of the marrow cells indicated that the per cent erythroid precursor cells in the marrow from PHZ-treated animals varied from 60 to 80 per cent, while control marrow contained about 40 per cent erythroid precursor cells.*

TABLE 1. CHOLINESTERASE ACTIVITY OF BONE MARROW AND RED BLOOD CELLS FROM NORMAL AND PHENYLHYDRAZINE-PRETREATED RABBITS

Tissue	Cholinesterase activity* (nmoles ^{14}C-ACh hyd/hr/mg protein) Normal	Phenylhydrazine†
RBCs	124·0 ± 10·7	130·7 ± 28·6
BMCs	282·7 ± 90·0	395·0 ± 51·9

* Values based on four or more separate experiments; mean ± S.D.

† Tissues were removed 4 days after the last dose of phenylhydrazine.

The comparison (Table 1) of the ChE activities per mg of protein between RBCs and BMCs from normal and PHZ-treated rabbits demonstrates that circulating erythrocytes (largely reticulocytes) have roughly the same amount of enzyme per mg of protein as mature erythrocytes from normal rabbits. Bone marrow cells, whether measured per mg of protein or per cell (not shown), have approximately three times the enzyme activity of peripheral blood cells. In addition, PHZ marrow cells show an even higher level of enzyme activity than cells from control marrow.

Cholinesterase formation in rabbit marrow cultures. In initial studies, ChE activity was determined on marrow cells at 3, 6, 12 and 24 hr after beginning cultivation. While it was easy to detect changes in enzyme activity (the linear rate of enzyme synthesis diminished after 12 hr) in soman-pretreated cells in culture, the large initial activity in unpoisoned cells made it difficult to measure significant increases in activity at time intervals of 12 hr or less. For this reason and because of the convenience of time, we chose to measure routinely the ChE activity of cells before and after 24 hr in culture. The data in Table 2 show that cultivation of marrow cells resulted in a significant increase in enzyme activity. Although the initial ChE activity of soman-treated cells was very low, the increase in ChE in both untreated and soman-treated cells was about the same.

Effects of DBcAMP, cAMP, erythropoietin and AMD (alone and together with DBcAMP) on ChE activity in marrow cultures. Table 3 shows that DBcAMP caused a marked increase in enzyme activity in both control and soman-treated cells; the

* J. P. Petrali and K. R. Mills, unpublished observations

TABLE 2. CHANGE IN CHOLINESTERASE ACTIVITY IN RABBIT MARROW CULTURES

Treatment*	ChE activity† (nmoles ^{14}C-ACh hyd/hr/mg protein) 0 time	24 hr	Ratio ChE act.-24 hr: ChE act.-0 hr
None	372·0 (341·0–403·0)	576·0 (504·3–647·7)	1·6
Soman	9·3 (7·4– 11·2)	165·0 (145·6–184·4)	17·7

* Marrow cells were inhibited with 2×10^{-7} M soman and excess inhibitor was removed by washing cells in MEM prior to seeding.

† Values are means with 95% confidence limits in parentheses.

TABLE 3. EFFECTS OF VARIOUS DRUGS ON CHOLINESTERASE ACTIVITY IN CONTROL AND SOMAN-PRETREATED CELLS IN CULTURE

Treatment*	Cholinesterase activity† (nmoles ^{14}C-ACh hyd/hr/mg protein)
Normal cells	
None	510·0 ± 73·0
AMD (10^{-7} M)	469·8 ± 51·8
DBcAMP (10^{-3} M)	640·0 ± 55·6
DBcAMP(10^{-3} M) + AMD(10^{-7} M)	469·0 ± 23·4
cAMP (10^{-3} M)	515·3 ± 122·0
Erythropoietin (0·35 U)	554·8 ± 129·0
Soman-pretreated cells	
None	165·0 ± 33·7
AMD (10^{-7}M)	36·0 ± 5·2
DBcAMP (10^{-3} M)	317·0 ± 65·0
DBcAMP(10^{-3} M) + AMD(10^{-7} M)	34·6 ± 18·8
cAMP (10^{-3} M)	116·5 ± 37·6
Erythropoeitin (0·35 U)	117·6 ± 41·6

* Drugs were added at the beginning of the incubation period.

† Values are means ± S.D. at 24 hr.

TABLE 4. EFFECTS OF VARIOUS DRUGS ON ^{14}C-L-LEUCINE AND ^{3}H-METHYL THYMIDINE UPTAKE IN CONTROL AND SOMAN-PRETREATED CELLS IN CULTURE

Treatment*	^{14}C-L-leucine incorporation Untreated cells (%)	Soman-pretreated† cells (%)	^{3}H-methyl thymidine uptake Untreated cells (%)	Soman-pretreated† cells (%)
None	100·0	100·0‡	100·0	100·0§
Actinomycin D (AMD)	58·3 ± 2·9	58·4 ± 9·4	42·3 ± 4·4	55·5 ± 12·5
Erythropoietin	116·0 ± 8·1	120·4 ± 13·6	119·0 ± 13·1	131·8 ± 26·9
DBcAMP	106·0 ± 9·9	101·6 ± 8·5	108·0 ± 8·7	133·8 ± 21·2
cAMP	87·4 ± 7·7	85·1 ± 7·1	99·0 ± 11·1	108·7 ± 8·2
Erythropoietin + cAMP	80·6 ± 9·5	79·0 ± 11·3	121·0 ± 21·6	127·7 ± 15·9
DBcAMP + AMD	57·7 ± 13·7		88·7 ± 13·4	

* Actinomycin D (10^{-7} M), erythropoietin (0·35 U), DBcAMP (10^{-3} M) and cAMP (10^{-3} M) were added to the medium at the beginning of the 24-hr incubation period. Values are means ± S.D.

† Cells were pretreated with 2×10^{-7} M soman prior to cultivation

‡ 89·0 ± 9·3 per cent of untreated cell incorporation.

§ 82·2 ± 9·1 per cent of untreated cell uptake.

net increase in enzyme activity amounted to more than 125 nmoles ACh hydrolyzed/hr/mg of protein in each instance. The time course for ChE synthesis in these experiments also was followed initially. Unlike control cells (not grown in the presence of DBcAMP), the rate for ChE formation remained almost the same throughout the 24-hr time period. The new ChE activity observed in the 24-hr studies (even after DBcAMP treatment) was largely due to AChE, because when 10^{-6} M BW284C51, a specific inhibitor of AChE, was added to the enzyme preparations, the hydrolysis of ACh was decreased by more than 95 per cent. This concentration of BW284C51 was shown to inhibit AChE activity completely without affecting pseudocholinesterase activity.[11] Table 3 also shows that AMD inhibited enzyme formation by 83 per cent in soman-treated cells and completely blocked the DBcAMP-enhanced ChE activity in both control and soman-treated cells. Neither cAMP nor erythropoietin affected overall ChE activity in marrow cultures when compared with controls cultivated for the same time.

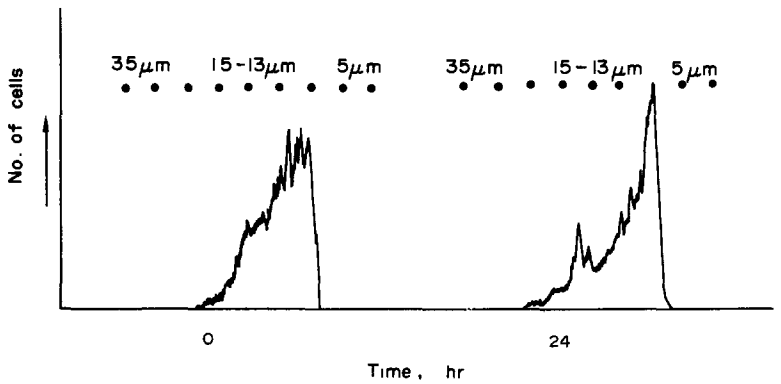

FIG. 1. Size distribution of rabbit marrow cells before and after cultivation as measured by laser cytograf.

Effects of AMD, erythropoietin, cAMP (alone and together with erythropoietin) and DBcAMP (alone and together with AMD) on protein and DNA synthesis in control and soman-pretreated cells in culture. Zero-time marrow cultures were processed for ^{3}H-methyl-thymidine uptake and ^{14}C-L-leucine incorporation, and in each instance contained less than 5 per cent of the 24-hr values. The 24-hr results are shown in Table 4. The percentage for drug-treated cultures is based on values for untreated cultures which were taken as 100 per cent for each experiment (mean values $\pm$S.D. for ^{14}C-L-leucine incorporation and ^{3}H-methyl-thymidine uptake in untreated cells, five separate experiments, are $42{\cdot}5 \pm 2{\cdot}8 \times 10^{3}$ and $64{\cdot}9 \pm 13{\cdot}4 \times 10^{4}$ cpm per 5×10^{6} cells respectively). The data show that neither protein nor DNA synthesis was markedly affected by pretreating the cells with soman prior to culturing. The presence of AMD in marrow cultures markedly inhibited both protein synthesis and labeled thymidine uptake. When DBcAMP was present with AMD in untreated cells, the inhibitory effect of AMD was considerably less in ^{3}H-methyl-thymidine uptake. The addition of erythropoietin caused a small increase in protein synthesis and ^{3}H-methyl-thymidine uptake, but the increase in protein synthesis was abolished by cAMP.

Size distribution of marrow cells before and after cultivation. Just before planting and immediately after harvesting, the cells were thoroughly dispersed with a pipette and

1 ml was slowly added to 9 ml of 10% neutral buffered formalin solution. The fixed cells were then sized and counted in a model 6301 laser cytograf. The results in Fig. 1 show a noticeable change in size distribution of marrow cells cultivated for 24 hr. The difference may reflect a change in the population of different cells in culture, particularly of erythroid cells, since a higher portion of small cells is present after 24 hr. The number of cells in a medium after harvesting, however, was not significantly different from that of preparations at time of seeding (by *t*-test for significance).

DISCUSSION

The data presented indicate that rabbit marrow cultures are capable of forming ChE and that pretreating cells with soman prior to cultivation does not alter the capacity of the cells to increase ChE activity. The greatest difference (on a percentage basis) was obtained in cells treated with 2×10^{-7} M soman to inhibit pre-existing ChE activity. Table 2 shows a 17·7-fold increase in enzyme activity of soman-pretreated cells when compared to zero-time samples. Inasmuch as the initial ChE activity had been virtually eliminated by treatment with the irreversible anticholinesterase, the increase in enzyme activity most probably can be attributed to synthesis of new enzyme protein.

The fact that cAMP was instrumental in maintaining ChE activity in denervated muscle[2] led us to explore the effects of DBcAMP (an analog of cAMP which can readily penetrate cellular membranes[15]) on enzyme formation in marrow cultures. The results presented in Table 3 indicate that incubation with DBcAMP has a pronounced influence on ChE activity in bone marrow cells grown in culture. The effect appears to be preferential for ChE, since overall protein synthesis was not stimulated (Table 4) and was prevented when AMD was added. In contrast, theophylline (an inhibitor of phosphodiesterase, the enzyme which hydrolyzes cAMP to 5′-AMP) alone or with cAMP (not shown) failed to enhance enzyme activity above the 24-hr control values in our marrow system. One possible explanation is that our studies were of short duration when compared to those with organ cultures and, since cAMP does not readily penetrate cellular membranes,[15] the intracellular levels of cAMP may not have reached sufficient concentrations to stimulate enzyme formation. Another possibility is that exogenous cAMP and DBcAMP do not always produce the same response in the same tissue at the same concentration.[16]

Incipient studies with DBcAMP-treated cultures revealed that the rate of enzyme synthesis (in contrast to controls, where the rate of synthesis gradually diminished after 12 hr) was roughly the same throughout the 24-hr period. The mechanism(s) by which DBcAMP enhanced ChE formation in these studies is not known but could be related to a specific effect on transcription or an effect on cell maturation, that is, nuclear expulsion or cell division. For instance, Bottomley *et al.*[17] have shown that DBcAMP increases Δ-aminolevulinic acid (ALA) synthetase activity in bone marrow cells and that this activity is suppressed by inhibitors of RNA and protein synthesis. In our studies, the augmentative effect by DBcAMP on ChE formation was completely inhibited by AMD, an inhibitor of DNA-directed RNA synthesis. Thus, DBcAMP may have stimulated either the transcription of additional messenger RNA for ChE or facilitated its transmission from nucleus to cytoplasm. DBcAMP also has been shown to increase AChE activity in mouse neuroblastoma cells *in vitro*.[18] The authors suggest that an increase in enzyme activity in these cells may

be linked to the inhibition of cell division. In our studies, no effort was made to study cell division. We did count and size the cells before and after harvesting. No sizable change in cell number was detected, but the laser cytograf did detect changes in size distribution of the heterogeneous population of marrow cells during 24 hr in culture (Fig. 1). The presence of DBcAMP in the culture medium failed to change the pattern observed for 24-hr control cells (not shown), but parallel morphologic studies on these cells are necessary to correlate the laser data; only then will it be known whether DBcAMP affects cell maturation.

In initial studies, incubation of normal bone marrow cells produced only marginal net increases in ChE activity. We felt that the increase in ChE observed was due largely to the presence of erythroid cells in the marrow. When we incubated marrow cultures from phenylhydrazine-pretreated animals (rich in erythroid cells), we observed almost a 10-fold increase in ChE formation as compared with control cultures. Marrow cells are composed of undifferentiated stem cells, megakaryoblasts, and infant red and white cells in various stages of maturation and differentiation. Megakaryocytes do not proliferate; instead, platelets are formed from these cells by the peeling and fragmentation of megakaryocytic cytoplasm.[19] Inasmuch as megakaryocytes possess ChE[20] activity but do not proliferate and since the majority of platelets remain in the medium and are removed by washing, their contribution to enzyme formation during the 24-hr culture period may be limited. It should be emphasized, however, that no effort was made in these experiments to determine which cells contributed to ChE formation.

The addition of erythropoietin, a hormone which stimulates sensitive stem cells to differentiate into more mature erythroid cells,[19] produced an increase in protein synthesis as well as in ^{3}H-methyl-thymidine uptake (Table 4). These findings are in agreement with earlier reports on marrow culture which showed an erythropoietin-induced increase in protein synthesis[21] and labeled precursor incorporation into RNA[22] and DNA.[23] The hormone, however, failed to increase ChE activity. This suggests that increased ChE synthesis may not be related to hemoglobin synthesis or other cellular activities incited by the hormone.

The tissue culture system described in this paper offers a "rapid" but sensitive system for studying ChE formation after irreversible inhibition. The data obtained with soman-treated cells suggest that a deficiency of ChE does not affect drug activity on the cell nor does it markedly damage cellular activities associated with DNA and protein synthesis (Table 4). Whether soman affects cell maturation (preliminary studies with the laser cytograf suggest that it does not) or whether cell maturation is related to the DBcAMP effect on ChE synthesis is not presently known, but these possibilities are currently under investigation.

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REFERENCES

1. T. L. LENTZ, *Science* **171,** 187 (1971).
2. T. L. LENTZ, *Nature New Biol.* **238,** 154 (1972).
3. H. BORSOOK, S. JIGGINS and R. T. WILSON, *J. Cell Physiol.* **79,** 267 (1971).
4. S. B. KRANTZ, O. GALLIEN-LARTIQUE and E. GOLDWASSER, *J. biol. Chem.* **238,** 4085 (1963).

5. D. G. Laycock and J. A. Hunt, *Nature, Lond.* **221,** 1118 (1969).
6. J. H. Fleisher, L. W. Harris and E. F. Murtha, *J. Pharmac. exp. Ther.* **156,** 345 (1967).
7. L. W. Harris, J. H. Fleisher, J. Clark and W. J. Cliff, *Science, N.Y.* **154,** 404 (1966).
8. J. H Fleisher, L W. Harris and P. T. Berkowitz, *Biochem Pharmac.* **19,** 421 (1970).
9 L. W. Harris, J. H. Fleisher, T. A. Innerebner, W. J. Cliff and V. M. Sim, *Toxic. appl. Pharmac.* **15,** 216 (1969).
10. L. W. Harris, H. I. Yamamura and J. H. Fleisher, *Biochem Pharmac.* **20,** 2927 (1971).
11. A. N. Siakotos, M. Filbert and R. Hester, *Biochem. Med.* **3,** 1 (1969).
12. A. J. Erslev, *Blood* **24,** 331 (1964).
13. O H. Lowry, N. G. Rosebrough, R J Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951)
14. J. H. Flfisher and L. W Harris. *Biochem. Pharmac.* **14,** 641 (1965)
15 E. Kaukel and H. Hilz, *Biochem biophys. Res. Commun.* **46,** 1011 (1972).
16. H. Hiltz and W. Tarnowski, *Biochem. biophys. Res. Commun.* **40,** 973 (1970)
17. S. S. Bottomley, W. H. Witcomb, G A. Smithee and M Z. Moore, *J. Lab. clin. Med.* **77,** 793 (1971).
18. K. N. Prasad and A. Vernadakis, *Expl. Cell Res.* **70,** 27 (1972).
19. A. J. Erslev, *Am. J. Path.* **65,** 629 (1971).
20. J. Zajicek, *Acta haemat.* **12,** 238 (1954).
21. S. S. Bottomley and G. A. Smithee, *J. Lab. clin. Med.* **74,** 445 (1969).
22. S. B. Krantz and E. Goldwasser, *Biochim. biophys. Acta* **103,** 325 (1965).
23 E Powsner and L. Berman, *Life Sci.* **6,** 1713 (1967)