Reports

Effects of 3-Methylcholanthrene and Phenobarbital on Amino Acid Incorporation into Protein

Abstract. Treatment of rats with methylcholanthrene and phenobarbital, two compounds known to enhance microsomal enzyme activity, stimulates the incorporation of free or soluble ribonucleic acid bound amino acid into proteins of cell-free liver preparations. Mitochondria, microsomes, and cell sap all contribute to the methylcholanthrene effect on the incorporation of free amino acid.

A number of compounds have recently aroused considerable interest because of their profound influence on several liver microsomal enzyme systems (*1–8*). Richardson *et al.* (*1*) observed that 3-methylcholanthrene (MC), when administered at low levels in the diet, markedly inhibited tumor formation in the livers of rats fed aminoazo dyes. Miller *et al.* (*2*) subsequently explained this effect by finding that the simultaneous feeding of this compound prevents the loss of microsomal dye-metabolizing enzymes which usually accompanies feeding with aminoazo dyes. Other investigations have shown that treatment in vivo with methylcholanthrene or benzpyrene increases a number of microsomal enzymatic activities assayed in vitro. These include N-demethylation (*3*), ring hydroxylation (*4*), aminoazo dye reduction (*3*), reduced triphosphopyridine nucleotide–cytochrome *c* diaphorase activity (*5*), formation of protein-bound aminoazo dye (*6*), and the incorporation of leucine-C^{14} into microsomal protein (*5*). The effect of treatment with methylcholanthrene, however, is not general to all microsomal enzymes, since some activities have been found to be either unaffected or decreased (*7*).

Many of the enzyme systems enhanced by these compounds are involved in drug metabolism. Conney and Burns (*8*) have shown that their enhanced activity has functional significance as indicated by an increased rate of drug metabolism in vivo and a shortened duration of drug action. For example, treatment of rats with 3-methylcholanthrene reduces zoxazolamine paralysis from 730 to 12 min, and phenobarbital, which was also found to enhance the activity of some microsomal enzyme systems (*8*), reduces the duration of meprobamate paralysis from 1356 to 175 min.

It has been suggested that the increased activities of the microsomal enzymes reflect an increase in their rates of synthesis (*3, 4, 6*). In order to examine this possibility further, a study was undertaken to determine the effects of two of these compounds, methylcholanthrene and phenobarbital, on the in vitro rate of amino acid incorporation into protein. Two cell-free amino acid incorporating systems were used. One consisted of mitochondria, microsomes, and cell sap, in which adenosine triphosphate (ATP) was generated by oxidative phosphorylation (system I). In the other, mitochondria and oxidizable substrate were replaced by a creatine phosphate-ATP generating system (system II). All experiments were performed on matched groups of control rats and rats treated with methylcholanthrene or phenobarbital. Each group consisted of four Sprague-Dawley male rats weighing 40 to 50 g. The methylcholanthrene treatment consisted of a single intraperitoneal injection of 1 mg of compound in 0.25 ml of corn oil. The phenobarbital treatment consisted of three daily intraperitoneal injections of 2.0 mg of sodium phenobarbital in 0.25 ml of water. Control groups were given equivalent amounts of solvent alone. In the experiments with methylcholanthrene the animals were killed 20 hours after injection; in the experiments with phenobarbital the animals were killed 1 day after the last dose. Animals were fasted for 20 hours before they were killed. The rats were decapitated, and the livers were removed and homogenized individually in 5.0 ml of ice-cold 0.25*M* sucrose per gram of liver. The homogenates from each group of rats were pooled, and mitochondria, microsomes, and supernatant fractions were separated by differential centrifugation (*9*). In system I the mitochondria and microsomes were suspended in 0.25*M* sucrose and mixed with supernatant fluid in proportions yielding mitochondria and microsomes equivalent to 200 mg and supernatant fluid equivalent to 67 mg of fresh liver per 0.9 ml, the aliquot of the mixture added per flask. In system II microsomes and supernatant fluid only were used, and these were so mixed that 0.6 ml, the aliquot of mixture per flask, contained microsomes equivalent to 200 mg and supernatant fluid equivalent to 67 mg of

Table 1. The effects of treatment with methylcholanthrene (MC) or phenobarbital on amino acid incorporation in vitro.

Treatment	Precursor	Specific activity	Effect (%)*
	System I†		
Control	DL-Leu-C^{14}	53.5	
MC	DL-Leu-C^{14}	85.9	+ 61 (8)
Control	DL-Leu-C^{14}	50.1	
Phenobarbital	DL-Leu-C^{14}	132.0	+ 163 (2)
	System II		
Control	DL-Leu-C^{14}	48.4	
MC	DL-Leu-C^{14}	64.1	+ 32 (2)
Control	sRNA-Val-C^{14}	56.6	
MC	sRNA-Val-C^{14}	64.9	+ 15 (1)
Control	sRNA-Pro-C^{14}	17.7	
MC	sRNA-Pro-C^{14}	21.6	+ 22 (2)

* The numbers in parentheses indicate the number of experiments.

† In system I each flask contained 20 μmole of potassium phosphate buffer, *p*H 7.4; 50 μmole of potassium α-ketoglutarate; 10 μmole of $MgCl_2$; 270 μmole of sucrose, 0.8 μmole DL-leucine-1-C^{14} (specific activity 5.47 μc/μmole), mitochondria and microsomes each equivalent to 200 mg of rat liver and supernatant fluid equivalent to 67 mg of rat liver. In system II each flask contained 20 μmole of potassium phosphate, *p*H 7.4; 270 μmole of sucrose; 40 μmole of creatine phosphate; 5.0 μmole of adenosine 5′-monophosphate; 0.5 μmole of guanosine 5′-triphosphate; 10 μmole of $MgCl_2$; 0.25 mg of creatine phosphokinase; microsomes equivalent to 200 mg of rat liver; and supernatant fluid equivalent to 67 mg of rat liver. The radioactive precursors in this system were either 0.8 μmole of DL-leucine-1-C^{14} (specific activity 5.47 μc/μmole), sRNA-L-proline-U-C^{14} (5.3 OD_{260} units containing 1262 count/min) or sRNA-L-valine-U-C^{14} (24 OD_{260} units containing 1063 count/min). The sRNA was prepared by the method of Cantoni (*13*), and the sRNA-amino acid was prepared by incubating sRNA with the appropriate C^{14} amino acid and activating enzyme (*14*). All flasks were prepared in duplicate and had a final volume of 1.7 ml. Incubations were at 37°C for 20 min. Reactions were stopped with 1.7 ml of 12 percent trichloroacetic acid, and the proteins were washed, plated, and counted as previously described (*15*).

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Type manuscripts double-spaced and submit one ribbon copy and one carbon copy.

Limit the report proper to the equivalent of 1200 words. This space includes that occupied by illustrative material as well as by the references and notes.

Limit illustrative material to *one* 2-column figure (that is, a figure whose width equals two columns of text) or to *one* 2-column table or to *two* 1-column illustrations, which may consist of two figures or two tables or one of each.

For further details see "Suggestions to contributors" [*Science* **125**, 16 (1957)].

Table 2. The amino acid incorporating activity of various recombinations of fractions from normal rats and rats treated with methylcholanthrene (MC).

Source of homogenate fraction			% of Control*
Mitochondria	Microsomes	Supernatant	
Normal	Normal	Normal	100
MC	Normal	Normal	122
Normal	MC	Normal	112
Normal	Normal	MC	125
MC	MC	Normal	150
MC	Normal	MC	152
Normal	MC	MC	144
MC	MC	MC	166

* The activity of the control flask containing all normal components was arbitrarily set at 100. Represents average of two experiments. Experimental conditions are the same as system I described in Table 1.

fresh liver. Incubation procedures are described in Table 1.

In the series of experiments with the system containing mitochondria (system I), administration of methylcholanthrene or phenobarbital resulted in average increases of 61 percent (eight experiments) and 163 percent (two experiments), respectively, in the rate of amino acid incorporation into protein (Table 1). In experiments with the microsomal-supernatant system (system II), treatment with methylcholanthrene resulted in an average stimulation of 32 percent (Table 1).

Although all the enzyme systems previously reported to be enhanced by administration of methylcholanthrene are microsomal, the effect on amino acid incorporation is not wholly accounted for by a difference in the microsomes. As is shown in Table 2, each of the homogenate fractions contributed to the increased incorporation rate observed in the preparations from the rats treated with methylcholanthrene. Thus amino acid incorporation was stimulated 12 to 25 percent when only one of the three fractions used was from the treated rats, 46 to 55 percent when two of the three were from treated rats, and 66 percent when all three were from treated rats. Conversely, the effect was reduced but not completely removed by the replacement of any one or two of the fractions from treated rats by the corresponding fractions from normal rats.

One of the later stages of protein synthesis involves the transfer of soluble ribonucleic acid bound amino acid (sRNA-AA) to microsomal protein (*10*). As shown in Table 1, this is at least one of the steps stimulated by treatment with methylcholanthrene.

The results of the present study demonstrate that treatment with methylcholanthrene stimulates amino acid incorporation into protein whether the amino acid is added free or bound to soluble ribonucleic acid (sRNA). These results, therefore, suggest that this treatment accelerates the rate of protein biosynthesis. Such an action is consistent with the hypothesis that the enhanced enzyme activities previously observed after treatment with methylcholanthrene result from an increased rate of enzyme synthesis. It is noteworthy that ethionine, which is known to inhibit protein synthesis (*11*), has also been observed to inhibit the stimulatory effect of methylcholanthrene on the enhancement of certain enzyme activities (*3*, *6*).

Confirmation of this hypothesis requires a demonstration of a net increase in newly synthesized enzymes. In the absence of such definitive evidence, alternative explanations of the effect of methylcholanthrene should be considered. It is possible that the increased rate of amino acid incorporation may not be causal to the increased enzymatic activities, but rather another example of a microsomal enzyme system enhanced by methylcholanthrene. A possible mechanism is an increased microsomal membrane permeability resulting in a greater accessibility of microsomal enzyme sites (*12*).

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References and Notes

1. H. L. Richardson, A. R. Stier, E. Borsos-Nachtnebel, *Cancer Research* **12**, 356 (1952).
2. E. C. Miller, J. A. Miller, R. R. Brown, J. C. MacDonald, *ibid.* **18**, 469 (1958).
3. A. H. Conney, E. C. Miller, J. A. Miller, *ibid.* **16**, 450 (1956).
4. ———, *J. Biol. Chem.* **228**, 753 (1957).
5. A. Von Der Decken and T. Hultin, *Arch. Biochem. Biophys.* **90**, 201 (1960).
6. H. V. Gelboin, J. A. Miller, E. C. Miller, *Cancer Research* **19**, 975 (1959).
7. A. H. Conney, J. R. Gillette, J. K. Inscoe, E. R. Trams, H. S. Posner, *Science* **130**, 1478 (1959).
8. A. H. Conney and J. J. Burns, *Ann. N.Y. Acad. Sci.* **86**, 167 (1960).
9. W. C. Schneider, *J. Biol. Chem.* **176**, 259 (1948).
10. P. C. Zamecnik, Harvey Lectures 256 (1958–59).
11. M. V. Simpson, E. Farber, H. Tarver, *J. Biol. Chem.* **182**, 81 (1950).
12. The excellent technical assistance of John Cason is gratefully acknowledged.
13. G. L. Cantoni, personal communication.
14. H. V. Gelboin and G. L. Cantoni, in preparation.
15. L. Sokoloff and S. Kaufman, *Science* **129**, 569 (1959).

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Nonlinear Property of the Visual System at Fusion

Abstract. The response of the visual system to intermittent stimulation at rates above the fusion point is generally considered to be independent of frequency, that is, linear with respect to time-average luminance. However, trains of 1000 light pulses per second alternated successively with trains of 500 light pulses per second may be perceived as flickering even though the time-average luminance is the same in each train.

Recent experiments (*1*) have indicated that the traditional critical flicker frequency (CFF) is at best a special case of a much more general class of visual phenomena relating to temporal visual acuity. A perceptually fused train of light pulses may be reduced to flicker by shortening the duration of every other "on-off" time in the train, an operation which preserves duty ratio and keeps average energy constant. Such an effect suggests that the nature of the visual response may be further illuminated by more extended use of this type of stimulation.

The research reported here involved trains of pulses such as shown schematically in Fig. 1. In this figure, the typical CFF configuration is represented by the top line; square wave pulses are presented successively, with "on" times equal to "off" times. The remaining three lines represent the type of stimulation used in the experiment; two different frequencies of light pulses (standard and variable) presented successively in a continuous sequence. The figure, drawn to scale and pictured as the stimuli appear on an oscilloscope, illustrates the effect obtained with one setting of the standard for three frequencies of the variable train of pulses. Note that, in all cases, each "off" time equals the preceding "on" time.

The standard and variable trains of pulses were generated by two independent circuits, each providing "on" and "off" triggers with continuously variable frequency. The circuits were designed so that duty ratio (proportion of "on" time) remained invariant at 0.5 with changes of frequency. Alternation of the standard and variable trains of pulses was accomplished by switching back and forth between the two circuits. Gating times were controlled by two cascaded time-delay units. The standard train of pulses was generated for a duration determined by the setting of one of the time-delay