phrine on smooth muscle. It was suggested that increased production of adenosine triphosphate by epinephrine may stimulate the active extrusion of sodium from the cell, with a resulting hyperpolarization of the membrane.[14] It must still be established whether the metabolic and muscle-relaxing effects of epinephrine have a causal or parallel relationship.

The results of the present study demonstrated relatively high concentrations of an epinephrine-oxidizing enzyme in muscles which are relaxed by epinephrine. An actomyosin ATPase inhibitor was formed as a product of the oxidation of epinephrine by extracts of guinea pig trachea and rabbit ileum (Fig. 1). In studies now in progress, extracts from the uteri of five rats, sacrificed in various stages of the estrous cycle, all showed the capacity to form the ATPase inhibitor from epinephrine.

These findings lend support to our hypothesis of a possible relationship between the oxidation of epinephrine to an actomyosin ATPase inhibitor in certain smooth muscles and the negative inotropic effects of the hormone.[1, 3, 4]

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REFERENCES

1. M. A. INCHIOSA, JR. and N. L. VANDEMARK, *Proc. Soc. exp. Biol. Med.* **97**, 595 (1958).
2. M. A. INCHIOSA, JR., *Fedn Proc.* **17**, 77 (1958).
3. M. A. INCHIOSA, JR., *Biochem. Pharmac.* **16**, 329 (1967).
4. M. A. INCHIOSA, JR. and I. B. RODRIGUEZ, *Biochem. Pharmac.* **18**, 1883 (1969).
5. R. L. TSE and M. J. OESTERLING, *Clinica chim. Acta* **8**, 393 (1963).
6. J. H. GADDUM, in *Methods in Medical Research* (Eds. R. W. GERARD and J. H. GADDUM), p. 116 Year Book Publishers, Chicago (1950).
7. F. ELMADJIAN, in *Methods in Hormone Research* (Ed. R. I. DORFMAN), vol. II, p. 371. Academic Press, New York (1962).
8. J. C. COSTILLO and E. J. DE BEER, *J. Pharmac. exp. Ther.* **90**, 104 (1947).
9. D. F. BOHR, in *Ann. N.Y. Acad. Sci.* **139**, 799 (1967).
10. A. LUND, *Acta pharmac. tox.* **5**, 121 (1949).
11. S. PUSZKIN, S. BERL, ELENA PUSZKIN and D. D. CLARKE, *Science, N.Y.* **161**, 170 (1968).
12. J. AXELROD, *Biochim. biophys. Acta* **85**, 247 (1964).
13. L. LUNDHOLM, *Pharmac. Rev.* **18**, 255 (1966).
14. E. BÜLBRING, in *Adrenergic Mechanisms*, Ciba Found. Symp. (Eds. J. R. VANE, G. E. W. WOLSTENHOLME and M. O'CONNOR), p. 275. Little, Brown, Boston (1960).

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Differential inhibitor sensitivities of thymidine phosphorylases from *E. coli* and mammalian tissues

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THYMIDINE phosphorylase (EC 2.4.2.4) catalyzes the phosphorolytic cleavage of thymine- or uracil-deoxyribonucleosides into free base and deoxyribose-1-phosphate. The enzyme can be easily dis-

tinguished from uridine-deoxyuridine phosphorylase (EC 2.4.2.3)—catalyzing the same reaction—by its insensitivity to pyranoid nucleosides (e.g. 2'-deoxy-β-D-glucopyranosyl-thymine, 2'-deoxy-β-L-ribopyranosyl-thymine).[1, 2] By contrast, these nucleosides inhibit strongly the uridine-deoxyuridine phosphorylase *in vitro*[1, 2] and *in vivo*.[3, 4] The presence of thymidine phosphorylase was demonstrated in certain bacteria as *E. coli* as well as in tissues of man and some animal species. Since the enzyme is responsible for the rapid degradation of the unnatural cytostatic nucleosides 5-fluoro-2'-deoxyuridine, 5-bromo-2'-deoxyuridine and 5-iodo-2'-deoxyuridine in the human body (for ref. see[5]), its inhibition may improve the cancerostatic effectiveness of these nucleosides. For this reason inhibitors of thymidine phosphorylase were developed independently by Baker and his group (e.g.[6–8]) and Langen *et al.*[5] Interestingly enough, the compounds found by both groups to be the most effective inhibitors are C-6 substituted uracils or uracil derivatives. While Langen *et al.* used 6-aminothymine, a compound with quite a small substituent at C-6, Baker and coworkers investigated uracil derivatives with very bulky groups in this position e.g. 6-benzylaminouracil[7] and 6-(2.3-dichloroanilino)-uracil,[8] the latter being the most active compound.

In order to find out the most effective inhibitor (especially with regard to *in vivo* experiments to be carried out in the future) we compared *in vitro* the effectiveness of 6-aminothymine with that of the above-mentioned most active compounds of Baker *et al.* The experiments are described in this paper. Since Baker *et al.* used the enzyme from *E. coli B* while Langen *et al.* used the one from horse liver, the activity of the compounds was checked with enzymes from both sources, and in addition with the enzyme from human spleen.

TABLE 1. INHIBITION OF THYMIDINE PHOSPHORYLASE FROM *E. COLI* B AND HUMAN SPLEEN BY 6-AMINOTHYMINE (AT), 6-(2,3-DICHLOROANILINO)URACIL (DClAU), 6-BENZYLAMINOURACIL (BAU) OR 6-BENZYLAMINOTHYMINE (BAT)

Enzyme source	Inhibitor	Inhibitor concentration (μM)									Concentr. for 50 per cent inhib.(μM)	$\left(\frac{[S]}{[I]}\right)0{\cdot}5$†
		1000	300	100	30	10	3	1	0·3	0·1		
E.coli B	AT	77*	53	41							200	5
	DClAU	insoluble		58	57	56	55	55	44	32	0·5	2000
	BAU	insoluble		63	52	37	20				25	40
	BAT	42	32	24							—	—
Human spleen	AT	76	60	37							180	5·5
	DClAU	insoluble		no inhibition							—	—
	BAU	insoluble		no inhibition							—	—
	BAT	4	1	no inhibition							—	—

Enzyme sources were the 100,000 *g* supernatants of ultrasonically broken *E.coli B* cells (washed cells in 10 volumes 0·01 M phosphate–0·01 M mercaptoethanol buffer pH 7·4, dialyzed against 0·01 M mercaptoethanol) and of a homogenate of human spleen (in 5 vol. 0·9% NaCl–0·01 - mercaptoethanol).

Enzyme assay: The arsenolysis of 1mM fluorodeoxyuridine was followed by estimation of the liberated deoxyribose as described earlier.[5] Substrate cleavage in the control samples was about 45 per cent.

* Numbers correspond to per cent inhibition.

† Ratio of 1mM substrate to inhibitor concentration giving 50 per cent inhibition.

6-Aminothymine[9] was prepared as described earlier.[5, 10] 6-Benzylaminouracil[7, 11–13] was synthesized by the original method of Pfleiderer and Nübel[11] from 6-chlorouracil and benzylamine. The hitherto unknown 6-benzylaminothymine was prepared in the same way starting with 6-chlorothymine. M.p. of 6-benzylaminothymine after recrystallization from AcOH-H_2O 273–275° (Kofler Heizbank, corr.); λ_{max} in 0·1 N HCl : 281 nm, in 0·1 N NaOH : 279 nm; Anal. Calc. for $C_{12}H_{13}N_3O_2$: N, 18·17. Found: N, 18·44. 6-(2,3-dichloroanilino)uracil was prepared as described by Baker and Rzeszotarski:[8] colourless crystals from AcOH, m.p. 328–329° (Kofler Heizbank, corr.), Lit.[8] 322–323° (uncorr.).

The results obtained with the enzyme from *E. coli* and from human spleen, are shown in Table 1.

It can be seen that in accordance with Baker's results 6-benzylaminouracil is a very potent inhibitor of the enzyme isolated from *E. coli*. 6-aminothymine is also a good inhibitor of this enzyme, but if related to the 50 per cent inhibition values, 6-benzylaminouracil is eight times as effective. On the other hand, 6-benzylaminouracil does not inhibit the enzyme from human spleen, while 6-aminothymine inhibits this enzyme to a similar extent, as earlier described for horse liver.[5] 6-(2,3-dichloroanilino)uracil, in accordance with Baker's results, gives 50 per cent inhibition of the *Coli* enzyme at an inhibitor: substrate ratio of 1:2000. However, this compound produced partial inhibition only. When using 1 mM 5-fluoro-2'-deoxyuridine as substrate the maximal value comes to 58 per cent with inhibitor concentrations up to 0·1 mM. The partial inhibition obtained with 6-(2,3-dichloroanilino)uracil may possibly be explained by the assumption that this and similar compounds bearing bulky hydrophobic groups at C-6 complex with some binding site of the enzyme independently of the active center, thereby lowering the enzyme-substrate affinity. However, this process apparently does not completely block the reaction even if the site is saturated with the inhibitor. As 6-benzylaminouracil, 6-(2,3-dichloroanilino)uracil proved to be completely inactive with the human spleen enzyme.

In addition, we checked on the enzymes from both sources the inhibitory properties of 6-benzylaminothymine. With the human spleen enzyme 6-benzylaminothymine proved to be inactive and with the *Coli* enzyme it was much less active than the corresponding uracil derivative. This is conflicting with the fact that thymine is a better inhibitor of the enzyme than uracil;[14] therefore one might reasonably expect that generally C-6 substituted derivatives of thymine are more effective than the corresponding uracil compounds. Actually, 6-aminothymine is much better an inhibitor of the horse liver[5] and the *Coli* enzyme[14] than 6-aminouracil.

Further experiments with the following enzyme preparations also failed to show any significant activity of Baker's compounds on mammalian thymidine phosphorylase: unpurified extracts from horse and mouse liver, dialyzed 45–90% $(NH_4)_2SO_4$ precipitation of the crude extract from horse liver and enzyme from horse liver purified according to Friedkin and Roberts.[15]

The thymidine phosphorylases from E.coli and mammalian tissues, because of their identical substrate specificity[15, 16] and pH-optima, hitherto could be assumed to be very similar if not identical enzymes. However, the different sensitivities to various inhibitors show that there must be great differences in the protein conformation of the enzymes. The results confirm Baker's concept of "active site directed irreversible inhibitors"[18] insofar, that 6-aminothymine (a "classical" antimetabolite because of its small substituent) inhibits the enzyme from all sources, while 6-benzylaminouracil and 6-(2,3-dichloroanilino)uracil ("nonclassical" antimetabolites because of their bulky substituents) exert a differentiated action against enzymes from different species. It follows (once again) that bacterial enzymes in experiments on cancer problems have to be used with caution.

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REFERENCES

1. P. LANGEN und G. ETZOLD, *Biochem. Z.* **339**, 190 (1963).
2. G. ETZOLD, B. PREUSSEL, R. HINTSCHE und P. LANGEN, *Acta biol. med. germ.* **20** 437 (1968).
3. P. LANGEN and G. ETZOLD, *Molec. Pharmac.* **2**, 89 (1966).
4. P. LANGEN und G. ETZOLD, *Acta biol. med. germ.* **17**, K1 (1966).
5. P. LANGEN, G. ETZOLD, D. BÄRWOLFF and B. PREUSSEL, *Biochem. Pharmac.* **16**, 1833 (1967).
6. B. R. BAKER, *J. Med. Chem.* **10**, 297 (1967).
7. B. R. BAKER and W. RZESZOTARSKI, *J. Med. Chem.* **10**, 1109 (1967).
8. B. R. BAKER and W. RZESZOTARSKI, *J. Med. Chem.* **11**, 639 (1968).
9. W. BERGMANN and T. B. JOHNSON, *J. Am. chem. Soc.* **55**, 1733 (1933).
10. G. ETZOLD, D. BÄRWOLFF, B. PREUSSEL and P. LANGEN, *Germ. Pat.* (DDR) **62, 840**, July 10, 1968.
11. W. PFLEIDERER and G. NÜBEL, *Liebigs Ann. Chem.* **631**, 168 (1960).
12. A. PAUL and D. SEN, *Indian J. Chem.* **1**, 98 (1963).
13. H. GOLDNER, G. DIETZ and E. CARSTENS, *Liebigs Ann. Chem.* **691**, 142 (1960).
14. Unpublished experiments.

15. M. FRIEDKIN and D. ROBERTS, *J. biol. Chem.* **207**, 245 (1954).
16. W. E. RAZZELL and P. CASSHYAP, *J. biol. Chem.* **239**, 1789 (1964).
17. M. ZIMMERMAN, *J. biol. Chem.* **239**, 2622 (1964).
18. B. R. Baker, *The design of active site directed irreversible enzymic inhibitors. The organ chemistry of enzymic active site.* John Wiley, New York (1967).

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Effect of analeptics on brain pentobarbital levels and sleeping time in mice

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IN THE treatment of barbiturate poisoning the therapeutic emphasis has shifted from the use of analeptics and efforts at arousal to a regimen of physiologic supportive therapy.[1] For some time, though, analeptics were drugs of choice, the results being transferred from experimental evidence[2, 3] despite some reports[4, 5] about the ineffectiveness of these drugs in animals given large doses of barbiturates. Theoretically, stimulant therapy with drugs like nikethamide, pentylenetetrazol and picrotoxin might seem appropriate. Nilsson,[6] and subsequently others,[7, 8] have given up their use because of severe untoward side effects and higher mortality. According to Nilsson,[6] when the nervous system is doubly challenged by the depressant action of one compound and the stimulant action of another, the system tends towards instability and the resultant patterns of nervous activity are abnormal. In a previous experiment[9] we observed that mice given hexobarbital and nikethamide simultaneously showed a tremendous increase in the sleeping time (loss of righting reflex), even though there were convulsive movements and increased respiration during sleep. In the present report we have extended this observation to an examination of the brain–barbiturate levels, using pentobarbital as the depressant and nikethamide, pentylenetetrazol and pictrotoxin as analeptics.

Female white mice (Haffkine Institute strain) weighing between 25–28 g were used throughout the experiments. For barbiturate estimation brains of three mice were pooled after decapitation and dissection. The samples were duplicated after extraction in petroleum ether and estimated spectrophotometrically[10] with a modification[11] where the final extraction was in 0·5 N NaOH. This method is specific for unchanged barbiturate. The specificity of this method of estimating pentobarbital was confirmed by chromatographing the petroleum ether extract of the pooled brains on thin layer (silica gel G + alumina, 1:1) using cyclohexane–isopropanol and ammonia solution as a solvent system.[12] This method gave only one u.v. spot with an R_f value of 0·85. The anleptics used did not show any change in this spot and did not give a u.v. spot themselves when used concurrently as standards. The sleeping time was studied in groups of mice and was considered as the interval between the loss of righting reflex after injection and the regaining of the righting reflex (thrice within a minute), measured by a stop watch. The time has been expressed only in minutes, the seconds being rounded off to the nearest minute. Table 1 shows all the results.

It can be seen that treatment with analeptics increases the brain barbiturate level to many times the control. Even where the sleeping time is decreased, as with picrotoxin, the barbiturate level in brain is high. There does not seem to be any particular waking level of brain barbiturate after treatment with analeptics. The stimulation of the central nervous system with analeptics might alter the cellular permeabilities to favor an excess entry of the barbiturate. This would cause a delay in the excretion of barbiturate and the higher mortality with the use of analeptics observed in other literature.[13–15]

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