presence of cumene hydroxiperoxide, only peaks I, II and III are active, while in the case of peroxide dicumene, only peak III is.

The results obtained with chromatographically separated isoenzymes were confirmed with electrophoretic data. As shown in Figure 2, the development of isoenzymes with benzidine and hydrogene peroxide leads to the appearance of 7 electrophoretic bands: 5 cathodic and 2 anodic ones, while in the presence of benzidine, as donor of hydrogen, and of cumene hydroxiperoxide or of peroxide dicumene, 3 and respectively 1 band are evidenced.

From these experimental results it ensues that the real peroxidase oxidating substratum is generally the peroxidic grouping and not only H_2O_2. The 7 chromatographically and electrophoretically separated isoenzymes have different specificities for the 3 oxidating substrata tested. Isoenzyme III decomposes all used peroxidic compounds, evincing maximum activity in the presence of peroxide dicumene (ROOR).

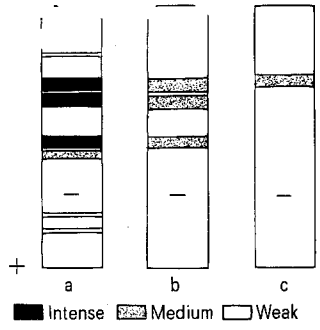

Fig. 2. Variation of the spectrum of horse radish isoperoxidases depending on the oxidating substratum used: a) H_2O_2; b) cumene hydroxiperoxide (ROOH); c) dicumene peroxide (ROOR).

Zusammenfassung. An sieben aus Meerrettichwurzeln isolierten Isoenzymen der Peroxydase konnte substratspezifisches Verhalten nachgewiesen werden.

DANA IORDĂCHESCU, I. F. DUMITRU and S. NICULESCU

Faculty of Biology, Bucharest University, Splaiul Independentei 91–95, Bucuresti (Rumania), 5 June 1972.

Inhibition of Cyclic 3′, 5′-Nucleotide Phosphodiesterase Activity by Diuretics and Other Agents

Adequate intracellular levels of adenosine 3′,5′-monophosphate (c-AMP) are essential for the maintenance of cardiac function[1] but drugs which cause inordinately elevated cardiac c-AMP levels[2] produce necrotic lesions in cardiac tissue[3] and can cause tachyarrhythmias or fibrillation[4,5]. Because cardiac arrhythmias have been reported to result from diuretic therapy and the i.v. administration of mercurial diuretics have been shown to cause ventricular fibrillation[6] the effect of various diuretic agents on cardiac c-AMP phosphodiesterase was investigated.

Methods. Ventricles of female Syrian guinea-pigs were homogenized at 0–4° in 0.01 *M Tris*-HCl buffer pH 7.4 containing 0.25 *M* sucrose. The homogenate was centrifuged at 10,000 × *g* for 10 min at 4 °C, the supernatant decanted, lyophilized and used as the source of c-AMP phosphodiesterase. The preparation was stored at −10 °C and did not lose activity over a period of 6 months. c-AMP phosphodiesterase of rat heart was similarly prepared but not lyophylized. Purified beef heart c-AMP phosphodiesterase was obtained from Sigma Chemical Co.

Platelet phosphodiesterase was prepared according to WOLFE and SHULMAN[7] and the rat brain enzyme according to BROOKER et al.[8].

Enzyme activity was assayed as described in Table I legend and contained sufficient enzyme to degrade 25 to 30% of the substrate and a [H³]c-AMP concentration equal to the Michaelis constant of the enzyme being tested.

Following incubation at 37 °C for 13 min the enzyme was inactivated by heat treatment and the denatured protein was separated by centrifugation. A 10 µl aliquot from each incubation mixture was then applied to cellulose thin layer chromatography sheets and developed in ethanol (95%)-ammonium acetate (1 *M* pH 5.0 containing 0.01 *M* Na_2 EDTA) (70/30). This solvent system separated c-AMP from AMP, adenosine, inosine and hypoxanthine. These purines were produced from AMP due to contaminating enzymes present in some of the enzyme preparations. The c-AMP, AMP and nucleoside areas were cut into small pieces and placed into counting vials containing 15 ml of toluene phosphor solution[9].

Results. The effects of several diuretics, organomercurial compounds and sulfhydryl reagents on guinea pig phosphodiesterase activity were investigated and the results are shown in Table I. Of the compounds tested, mersalyl, meralluride, phenylmercuric acetate, methyl mercuric chloride and PCMB were the most potent inhibitors of the enzyme. Ethacrynic acid, a non-mercurial diuretic was found to be the least effective inhibitor.

Preincubation of the enzyme preparation in the presence of some of the test drugs resulted in greater enzyme inhibition than with no preincubation. Combinations of several concentrations of the 2 mercurial diuretic drugs with theophyl-line resulted in greater inhibition of the enzyme activity than with the diuretics alone. This observation may be of clinical importance because commercial preparations of both mersalyl and meralluride are formulated with large amounts of theophylline to prevent breakdown of the organo-mercurial complex.

[1] W. R. KUKOVETZ and G. POCH, Naunyn-Schmiedebergs Arch. Pharmak. *266*, 236 (1970).
[2] B. M. BRECKENRIDGE, A. Rev. Pharmac. *10*, 19 (1970).
[3] P. A. MARTORANA, J. Pharm. Pharmac. *23*, 200 (1971).
[4] J. GY. PAPP and L. SZEKERES, Eur. J. Pharmac. *3*, 4 (1968).
[5] A. S. LEON and W. B. ABRAMS, Am. J. med. Sci. *262*, 9 (1971).
[6] M. S. PATHY, Geront. clin. *13*, 261 (1971).
[7] S. M. WOLFE and N. R. SHULMAN, Biochem. biophys. Res. Commun. *35*, 265 (1969).
[8] G. BROOKER, L. J. THOMAS and M. M. APPLEMAN, Biochemistry *7*, 4177 (1968).
[9] E. RAPKIN, Int. J. appl. Radiat. Isotopes *15*, 69 (1964).

Table I. The effect of theophylline and preincubation on inhibition of guinea-pig heart phosphodiesterase by various drugs and sulfhydryl reagents

Compound	Conc. (mM)	Theophylline (2.0 mM)	Enzyme activity (% control)	
			No. preincubation	10 min preincubation
None		—	100	100
		+	75	78
Mersalyl	0.2	—	41	23
	0.2	+	25	15
Meralluride	0.2	—	56	32
	0.2	+	29	11
Ethacrynic acid	2.0	—	65	58
	2.0	+	19	26
Phenylmercuric acetate	0.2	—	10	9
	0.2	+	9	5
Methylmercuric chloride	0.2	—	46	6
	0.2	+	30	11
p-Chloromercuribenzoic acid	0.2	—	48	12
	0.2	+	26	11
Mercuric chloride	0.2	—	32	18
	0.2	+	13	11

The reaction mixtures contained 35 nmoles [^{3}H] c-AMP, 5 µmoles *Tris*-HCl (pH 7.4), 0.2 µmoles $MgCl_2$, 27.5 µg enzyme preparation and the indicated test compound (s) in a final volume of 0.125 ml. Preincubations were carried out in the presence of enzyme, buffer, $mgCl_2$ and test compound (s) and the enzymatic reaction initiated by the addition of [^{3}H] c-AMP. The K_m value for the enzyme was 2.8×10^{-4} M and the V_{max} was 1.3 nmoles c-AMP per min per reaction mixture (47.5 nmoles/min/mg protein).

Because mersalyl, meralluride, ethacrynic acid and PCMB have all been shown to react with protein sulfhydryl groups[10–13], the possibility that these compounds inhibit c-AMP phosphodiesterase of guinea-pig heart by reacting with a sulfhydryl group essential for enzyme activity was investigated. Table II shows that dithiothreitol only partially reverses the inhibition of enzyme activity by PCMB and that reversal by dithiothreital was less effective at higher PCMB concentrations. These results therefore do not convincingly demonstrate that the enzyme possesses a sulfhydryl group for catalytic activity.

Table II. Reversal of enzyme inactivation by *p*-chloromercuribenzoate

p-Chloromercuribenzoate (M)	Dithiothreitol (M)	Enzyme activity (% control)
—	—	100
—	10^{-2}	100
1.6×10^{-5}	—	53
	10^{-2}	70
2.0×10^{-5}	—	37
	10^{-2}	44
4.0×10^{-5}	—	25
	10^{-2}	31

27.5 µg of enzyme preparation per reaction mixture was incubated with various concentrations of PCMB, 5 µmoles *Tris*-HCl pH 7.4 and 0.2 µmoles $MgCl_2$ for 10 min at 37°C. Dithiothreitol (10^{-2} M) was added and the incubation was continued for 10 min at 37 °C. The reaction mixtures were completed by the addition of 35 nmoles [^{3}H] c-AMP and enzyme activity was assayed as described previously.

Table III shows the results of a comparative study of the effect of sulfhydryl reagents and diuretics on enzymes from various sources. All mercury containing compounds and ethacrynic acid inhibited all of the enzymes tested (with the exception of PCMB with rat brain), although the magnitude of the inhibitions differed. The test compounds had a similar effect on both the high and low K_m enzymes of human platelets.

Discussion. The importance of c-AMP phosphodiesterase in the regulation of cardiac integrity is suggested by the observations that inhibition of this enzyme coincident with activation of adenyl cyclase is associated with an increase in the incidence of cardiac necrosis and of arrhythmias and the susceptibility towards fibrillation[3,14]. The possibility must therefore be considered that cardiac arrhythmias arising from diuretic therapy may be associated with elevated levels of c-AMP resulting from inhibition of the c-AMP phosphodiesterase as demonstrated in this study.

The basal activity of guinea-pig cardiac adenyl cyclase has been shown by WEINRYB et al.[15] to be inhibited by several sulfhydryl reagents. Because the heart in vivo is under catecholamine control[16], the effect of drugs on the catecholamine stimulated but not the non-stimulated

[10] A. ROTHSTEIN, Expl. Eye Res. *11*, 329 (1971).
[11] A. FARAH, C. H. BENDER, R. KRUSE and E. CAFRUNY, J. Pharmac. exp. Ther. *126*, 309 (1959).
[12] E. J. CAFRUNY and A. FARAH, J. Pharmac. exp. Ther. *117*, 101 (1956).
[13] R. M. KOMORN and E. J. CAFRUNY, Science *143*, 133 (1963).
[14] I. UEDA and F. OKUMURA, Biochem. Pharmac. *20*, 1967 (1971).
[15] I. WEINRYB, I. M. MICHEL, J. F. ALICINO and S. M. HESS, Archs Biochem. Biophys., *146*, 591 (1971).
[16] S. F. EPSTEIN, C. L. SKELETON, G. S. LEVEY and M. ENTMAN, Ann. intern. Med. *72*, 561 (1970).

Table III. Effect of various drugs and sulfhydryl reagents on cyclic 3', 5'-nucleotide phosphodiesterase of various tissues

Compound	Concentration (mM)	Enzyme activity (% control)						
		Guinea-pig heart	Beef heart	Rat heart low K_m	Human platelet low K_m	Human platelet high K_m	Rat platelet	Rat brain
Mersalyl	0.2	41	24	38	54	30	—	84
	2.0	28	11	—	—	5	31	—
Meralluride	0.2	56	42	54	64	46	—	81
	2.0	33	9	—	—	3	67	—
Ethacrynic acid	2.0	65	14	33	17	22	45	12
Phenylmercuric acetate	0.2	10	30	47	47	35	—	56
	2.0	4	22	—	—	—	—	—
Methylmercuric chloride	0.2	46	54	66	76	62	—	94
	2.0	24	33	—	—	7	28	—
Mercuric chloride	0.2	32	37	37	26	23	—	87
	2.0	8	8	—	—	2	15	—
p-chloromercuribenzoic acid	0.2	48	56	56	69	41	—	100
	2.0	35	21	—	—	—	—	—
N-ethylmaleimide	2.0	95	74	—	—	—	—	—
Iodoacetamide	2.0	100	—	—	—	—	—	—
Iodoacetic acid	2.0	95	—	—	—	—	—	—
Theophylline	2.0	75	53	—	—	36	92	—

Incubation conditions were as previously described. The amount of the substrate [H³] c-AMP used was as follows: a) 35 nmoles for the guinea-pig heart, rat brain and rat platelet preparations. b) 4.38 nomles for the beef heart preparation (equal to the reported K_m value[27]). c) 0.38 nmoles for the rat heart preparation (equal to the K_m value of 3.0×10^{-6} M as determined in this laboratory). d) 60 nmoles and 4.13 nmoles for the human platelet enzyme (equal to K_m values of 4×10^{-6} M and 3.3×10^{-5} M respectively as determined in this laboratory).

enzyme is of physiological importance. Since sulfhydryl reagents have been shown to inhibit the basal but not the ACTH or NaF stimulated activity of adrenocortical adenyl cyclase[17], drugs used in this study may be expected to elevate in vivo c-AMP levels in the normal catecholamine stimulated hearts by inhibiting c-AMP phosphodiesterase.

Platelet aggregation can be prevented by elevated intracellular levels of c-AMP[18]; therefore the present observation that the organomercurial diuretics inhibit platelet c-AMP phosphodiesterase may account for the inhibition of platelet aggregation in vitro[19].

Résumé. Différent composés organo-mercuriels ainsi que l'acide éthacrinique inhibent les phosphodiestérases de l'AMP cyclique, du cœur de cobaye, de bœuf et de rat, des plaquettes de rat et de l'homme ainsi que du cerveau de rat.

R. E. A. Gadd, S. Clayman and D. Hébert

Research Laboratories, Health Protection Branch, Department of National Health and Welfare, Tunney's Pasture, Ottawa (Ontario K1A OL2, Canada), 14 May 1973.

[17] L. A. Kelly and S. B. Koritz, Biochem. biophys. Acta *237*, 141 (1971).
[18] E. W. Salzmann and L. L. Neri, Nature Lond., *224*, 609 (1969).
[19] R. E. A. Gadd, S. Clayman and D. Hebert, Experientia *27*, 1339 (1971).

Effect of Age on Distribution of Acidic Glycosaminoglycans in Normal Human Urine: Special Reference to Proportion of Chondroitin Sulfate A to the C-Isomer

The qualitative and quantitative analyses of urinary acidic glycosaminoglycans (AGAG) in relation to connective tissue disorders are the focus of interest and study. There is no general agreement, however, regarding the proportional constituents of AGAG in normal human urine, though previous studies have indicated that the major components of urinary AGAG are chondroitin sulfates A and/or C[1-3]. Age-dependent changes of the excreted urinary AGAG in normal human have been reported with respect to the comparative changes of the AGAG resistant to testicular hyaluronidase[4]: dermatan sulfate (chondroitin sulfate B) or heparan sulfates[5]. In these circumstances, we have studied the effects of age on a possible change of urinary AGAG in normal subjects, chondroitin sulfates in particular, at the disaccharide subunits by an enzymatic assay with chondroitinases[6,7].

[1] N. di Ferrante, J. lab. clin. Med. *61*, 633 (1963).
[2] D. P. Varadi, J. A. Cifonelli and A. Dorfman, Biochim. biophys. Acta *141*, 103 (1967).
[3] E. Wessler, Biochem. J. *122*, 373 (1971).
[4] D. Allalouf and A. Ber, Biochim. biophys. Acta *201*, 61 (1970).
[5] N. Taniguchi, Clin. chim. Acta *37*, 225 (1972).
[6] K. Murata, T. Ishikawa and Y. Oshima, Clin. chim. Acta *28*, 213 (1970).
[7] K. Murata, T. Harada, T. Fujiwara and T. Furuhashi, Biochim. biophys. Acta *230*, 583 (1971).