There does not seem to be any work done on the variations in transaminase activity at different times of the year. A definite change exists in the transaminase activity of *R. tigrina*. The average values of SGOT and SGPT recorded for the whole year can be divided into 3 phases. In the first phase, from November to March, the values are relatively low (SGOT 50.0–80.0 and SGPT 15.0–19.0). The 2nd phase is close to average values, and in the 3rd phase the values are higher with the peak in August. The high values of transaminase activity (3rd phase) seem to be in direct correlation with spawning period. The high level of this enzyme may be indicative of active phase and higher metabolic activities in frogs in this period. This may also be related to rise in serum glucose[14] and protein[15] reported in this animal, during the same period.

Low values of SGOT and SGPT observed in December and January appear to be related with fall in temperature (40 °C in summer to 15 °C in winter). This is also corroborated by the results of the experiments on cold acclimation of the frog. Low values of SGOT and SGPT are observed when the frogs are acclimatized at low temperatures. Rat tissues[16] exposed to cold for 72 h also show a rapid fall in transaminase activity at early stages, but they regain the normal level after some time, but no such change was observed in frog serum upto 168 h. This may be due to the poikilothermic nature of these animals.

1 Acknowledgments. Thanks are due to Dr M.M. Goil and Dr R.K. Sharma for their invaluable help in various ways. Dr K. Singh would also like to thank S.C.S.T., U.P., for the financial assistance.
2 J. Awapara and B. Seale, J. biol. Chem. *194*, 497 (1952).
3 B. Bulos and H. Philip, J. biol. Chem. *240*, 3283 (1965).
4 W.T. Jenkins, D.A. Yphantis and I.W. Sizer, J. biol. Chem. *234*, 51 (1959).
5 B.A. Kilby and E. Neville, J. exp. Biol. *34*, 276 (1957).
6 J.W. Daugherty, Exp. Parasit. *1*, 331 (1952).
7 D.V. Aldrich, A.C. Chandler and J.W. Daugherty, Exp. Parasit. *3*, 173 (1954).
8 W.B. Foster and J.W. Daugherty, Exp. Parasit. *8*, 413 (1959).
9 R.K. Sharma and K. Singh, Z. ParasitKde *54*, 251 (1977).
10 S. Reitman and S. Frankel, Am. J. clin. Path. *28*, 56 (1957).
11 P.P. Cohen, The enzyme, vol. I, part 2. Academic Press, N.Y. 1951.
12 D. Marjanovic, L. Kozic, M. Petrovic, T. Palic and V. Litricin, Vet. Glasn. *28*, 793 (1974).
13 E. Shimizu and Y. Naito, J. Fac. Agric. Iwate Univ. *11*, 37 (1952).
14 S. Kumar and S.S. Khanna, Zool. Jb. Physiol. *76*, 80 (1971).
15 K. Singh, Zool. Anz. *197*, 39 (1976).
16 J.R. Puchol and A. Carballido, Med. exp. *6*, 158 (1962).

On the modification of sulfhydryl groups by 4-cyclopentene-1,3-dione[1]

E. Toro-Goyco, C. Zuazaga de Ortiz and J. del Castillo

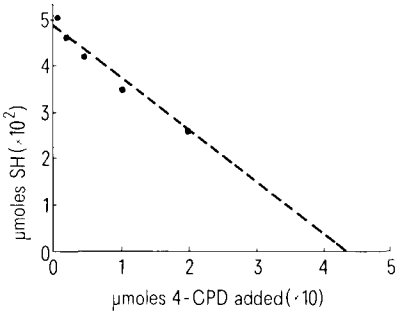
Department of Biochemistry and Nutrition and Laboratory of Neurobiology, University of Puerto Rico, Medical Sciences Campus, San Juan (Puerto Rico 00936, USA), 13 February 1978

Summary. 4-Cyclopentene-1,3-dione reacts quantitatively with sulfhydryl groups of both cysteine and the sulfhydryl containing protein pinguinain. A 10fold excess of the reagent totally modifies cysteine after 15 min at room temperature and the single sulfhydryl group in native pinguinain is totally modified by a 48–86fold excess of reagent.

Recent work from this institution[2,3] has shown that the sulfhydryl reagent NEM induces electrical excitability in the ventroabdominal flexor muscles of the crustacean *Atyas occidentalis*, a naturally nonexcitable tissue. As a result of this work it has been proposed that the effects of NEM in these tissues depend on the induction of new properties in the membrane proteins, likely to arise from the conversion by NEM of $-CH_2-SH$ side chains to polar thioethers. Neither the nitrogen nor the chains attached to it appear to be essential for this effect, as 4-CPD[4], a compound similar to NEM but containing a methylene group instead of a tertiary nitrogen also induces excitability[5]. Although this compound has not been regarded as an SH reagent we decided to study whether 4-CPD does in fact react with sulfhydryl groups. The modification of a protein with 4-CPD may have interesting effects on its tertiary structure because of the presence of 2 symmetrical carbonyl groups in the reagent molecule which will enable it to form hydrogen bonds with neighboring NH_2 groups in the protein.

Materials and methods. Chemicals. 4-CPD was from Aldrich; cysteine from Sigma; DTNB from Calbiochem; BAPNA from Merck; precoated silica gel plates from Kontes/Quantum; Sephadex gels from Pharmacia.

Reaction of 4-CPD with cysteine: TLC. 1-mM solutions of cysteine and 10-mM solutions of 4-CPD were prepared in 5 mM phosphate buffer at pH 6.0. 0.5 ml of each solution was mixed for 15 min at room temperature and 30 µl of the reaction mixture was spotted in 20×4 cm precoated silica gel plates and chromatographed using butanol: acetic acid: H_2O (4:1:1) as solvent. Equal volumes of the cysteine and 4-CPD solutions were spotted as references. Cysteine and its thioether were detected by staining with ninhydrin spray and 4-CPD with iodine vapors. An identical experiment was performed with the same solutions at pH 7.6.



Modification of SH groups in the protease pinguinain by 4-CPD. The ordinate shows the DTNB titratable groups (µmoles) left after addition of varying amounts of 4-CPD. Modification performed in 5 mM phosphate at pH 7.6. The reaction was allowed to proceed for 1 h.

Table 1. Disappearance of DTNB titratable SH groups in cysteine after exposure to 4-CPD

µl 4-CPD added*	OD_{412}
0	0.894
5	0.769
10	0.692
15	0.561
20	0.435
30	0.229
50	0.172

*Total cysteine in solution was 0.2 µmoles. Each µl of the CPD contained 0.01 µmoles. Titration performed, at pH 7.6 and room temperature using a Gilford Model 2400 spectrophotometer.

Table 2. Enzymatic activity of pinguinain after addition of varying amounts of 4-CPD

4-CPD added	ΔOD 412 nm	Residual ΔOD 412 nm (%)	Enzymatic activity units	Residual activity (%)
None	1.23		23.3	
100 µl	0.675	55	12.8	55
200 µl	0.364	30	6.8	29

* 1 unit = µmoles BAPNA hydrolyzed per h. The absorbancy of the nitroaniline produced in hydrolysis was measured at 405 nm, using an $\varepsilon^{405nm}_{1mM}$ of 8.8 The total enzyme used for the assay was 150 µg.

Reaction of 4-CPD with cysteine: spectrophotometric titration of the unreacted SH groups. Aliquots of 0.2 ml of the cysteine solution prepared above were allowed to react with increasing amounts of 4-CPD in solution at pH 7.6 and the sulfhydryl contents of the aliquots titrated photometrically with DTNB following the procedure described by Jocelyn[6]. The $\varepsilon^{412nm}_{1mM}$ assumed was 13.6.

Reaction of 4-CPD with the sulfhydryl groups of pinguinain, a sulfhydryl plant protease. 10 mg of chromatographically pure pinguinain, a compound described and routinely purified in our laboratory[7,8] were dissolved in 2 ml of 10 mM phosphate buffer at pH 6.0. 50 µl of β-mercaptoethanol were added to the mixture and allowed to react for 1 h at room temperature. This step was accomplished to fully reduce the reactive sulfhydryl group of the enzyme. Excess reducing agent was removed by filtration through a column packed with Sephadex G-25, in the same buffer used to dissolve the enzyme. Aliquots of the eluate, (1.2 ml each; 0.96 mg protein) were collected and varying amounts of a 10-mM solution of 4-CPD in 5 mM phosphate buffer pH 6.0 were added. All aliquots were brought to the same volume by addition of buffer. The samples were allowed to react for 1 h and 0.2 ml aliquots removed from each tube for enzymatic assays. Spectrophotometric titration of the SH groups in the unreacted and the reacted protein were performed as described for cysteine. Enzymatic activity was assayed at 25 °C using 0.2 ml aliquots of unreacted and reacted enzyme using BAPNA as substrate in 0.1 M phosphate buffer at pH 6.0 as suggested by Erlanger et al.[9].

Results and discussion. TLC indicated that using 4-CPD and cysteine in a 10:1 ratio, the later is quantitatively modified after 15 min at room temperature. Under the experimental conditions used, 4-CPD remains at the origin, cysteine shows an R_f of 0.26 and its thioether, product of the reaction with 4-CPD, barely moves from the origin.

The titration experiments showed that using the same 4-CPD: cysteine ratio, the ability of cysteine to react with DTNB is almost totally abolished (table 1). Although similar results were obtained after reaction of the reagent with pinguinain, higher 4-CPD: pinguinain ratios had to be used to accomplish the almost complete modification of pinguinain (figure). Assuming the presence of 1 SH group/19,000 daltons in pinguinain[7], addition of 430 µl of a 10-mM solution of 4-CPD (4.3 µmoles) were required at pH 7.6 to modify almost totally the SH group content (0.05 µmoles) of 960 µg of pinguinain. This is a 86:1 ratio. At pH 6.0, however, the ratio needed was 48:1, suggesting that conformational changes in the enzyme caused by pH changes facilitate the accessibility of the SH groups to the reagent. Additional evidence that 4-CPD reacts with the SH in pinguinain was obtained by performing activity assays after the enzyme was exposed to various amounts of 4-CPD, using as reference values the enzymatic activity in the absence of the reagent. The results are summarized in table 2. Enzyme activity (expressed as nmoles of BAPNA hydrolyzed per h) was significantly reduced to 29% of the control after addition of a 40fold excess of the reagent at pH 7.6 and room temperature. A 20fold excess of 4-CPD caused a reduction to 55% of the control values, indicating a close agreement between the titration data which measures disappearance of SH groups and the enzymatic activity data which is also indirect evidence of the modification of the SH groups.

Dicarbonyl compounds have been used successfully for specific arginine modification, but at a more alkaline pH than that used by us[10]. It can be argued that likewise 4-CPD will react with arginine. However, in dicarbonyl compounds used for protein modification (hexanedione and diketobutane) the carbonyl groups are in the ortho position to each other while in 4-CPD they are meta. The point stressed here is that the excitatory effects induced in the otherwise nonelectrically excitable muscle are due to the reaction of 4-CPD with the sulfhydryl group. The saturated analog of 4-CPD, 1,3-cyclopentanedione, does not induce electrical excitability in crustacean muscle[5]. These results confirm that 4-CPD is an SH reagent and that it probably exerts its activity in biological membranes by modifying the cysteinyl residues to carbonyl containing thioethers, which in turn can easily form hydrogen bonds with nonprotonated amino groups in proteins.

1 This research was supported in part by USPHS grants NS-07464, NS-14938 and GM-05784. We thank Drs A.F. Habeeb and H. Jering for critically reviewing the manuscript. This is contribution No. 79 of the Laboratory of Neurobiology.

2 J. del Castillo and C. Zuazaga de Ortiz, Satellite Symposium of the XXVII International Congress of Physiology, Monaco 1977.
3 C. Zuazaga de Ortiz and J. del Castillo, in: Symposium on Abnormal Neuronal Discharges, Raven Press, in press.
4 Abbreviations used: 4-CPD, 4-cyclopentene-1,3-dione; DTNB, 5, 5'-dithiobis-(2-nitrobenzoic acid); NEM, N-ethylmaleimide; BAPNA, N,α-benzoyl-L-arginine-p-nitroanilide.
5 C. Zuazaga de Ortiz and J. del Castillo, Experientia *34*, (1978).
6 P.C. Jocelyn, Biochem. J. *85*, 480 (1962).
7 E. Toro-Goyco, A. Maretzki and M.L. Matos, Archs Biochem. Biophys. *126*, 91 (1968).
8 E. Toro-Goyco and I. Rodríguez-Costas, Archs Biochem. Biophys. *175*, 359 (1976).
9 B.F. Erlanger, N. Kokowsky and W. Cohen, Archs Biochem. Biophys. *95*, 271 (1961).
10 M.Z. Atassi, in: Immunochemistry of Proteins, vol. 1, p. 33. Ed. M.Z. Atassi, Plenum, New York 1976.