Changes with increasing temperature of some kinetic parameters of calf brain membrane bound neuraminidase

Parameter	Temperature (°C)			
	37°	45°	55°	70°
Optimum pH	4.0	4.2	4.4	4.7
V_{max} (units/mg protein)	1.5	3.9	8.1	24
K_m ($\times 10^{-5}$ M)	2.1	3.0	5.8	10.4
Substrate concentration at which the inhibitory effect starts (mM)	0.2	0.25	0.35	0.5
Inhibition at 1 mM substrate (%)	65	50	35	15

Substrate used: disialoganglioside GD1a. The data reported are the average of 5 experiments

well-known inhibition[4,7] by excess substrate. The variations of some kinetic data (optimum pH, V_{max}, K_m, inhibition by excess substrate) with increasing temperature are exposed in the Table. From 37 °C to 70 °C the optimum pH shifted from 4.0 to 4.7, the V_{max} from 1.5 units/mg protein to 24 units, the K_m from 2.1×10^{-5} to 10.4×10^{-5}. The inhibition by excess substrate started at 0.2 mM ganglioside GD1a at 37 °C, at 0.5 mM at 70 °C. The inhibition at 1.0 mM ganglioside GD1a was 65% at 37 °C and only 15% at 70 °C.

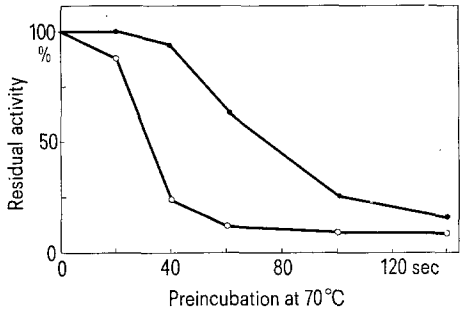

Fig. 3. Effect of the presence of substrate on the stability of calf brain membrane bound neuraminidase at 70 °C. The enzyme preparation was allowed to stand at 70 °C for the indicated time in the presence of 0.15 mM disialoganglioside GD1a (at pH 4.7), then refrigerated in an ice bath and submitted to incubation at 37 °C for 15 min (pH 4.0). The assay mixture contained 880 μg of protein. The data exposed are the average of 4 experiments. -□-□-□-, preincubated in the absence of ganglioside GD1a; -■-■-■-, preininbated in the presence of ganglioside GD1a.

The same behaviour with increasing temperature was shown also with ganglioside GD1b and GT1b as the substrates, and was provided too by the enzyme prepared from the same animal according to LEIBOVITZ and GATT[8]. Also, closely similar findings were observed using the rabbit and human brain enzyme. Thus it can be concluded that the above behaviour is a basic property of brain membrane bound neuraminidase[9].

Riassunto. La velocità di idrolisi del ganglioside GD1a da parte della neuraminidasi di membrana del cervello di vitello è massima a 70 °C. L'aumento della temperatura da 37° a 70 °C provoca: aumento della V_{max} (da 1.5 unità a 24 unità/mg di proteina) e del pH ottimale (da 4.0 a 4.7); aumento del valore di K_m (da 2.1×10^{-5} M a 10.4×10^{-5} M); diminuzione dell'effetto inibitorio da eccesso di substrato.

ADRIANA LOMBARDO, A. PRETI, G. TETTAMANTI and V. ZAMBOTTI

Department of Biological Chemistry, Medical School, University of Milan, Via C. Saldini 50, I-20133 Milano (Italy), 8 April 1974.

[7] R. OHMAN, A. ROSENBERG and L. SVENNERHOLM, Biochemistry *9*, 3774 (1970).

[8] Z. LEIBOVITZ and S. GATT, Biochim. biophys. Acta *152*, 136 (1968).

[9] This work was supported by a grant from the Consiglio Nazionale delle Ricerche (C.N.R.), Italy.

Protection of Vaccinia-Infected HeLa Cells by Lipophilic Benzimidazole Derivatives

Although some activities have been reported[1], the picornaviral-inhibiting derivatives of 2-(α-hydroxybenzyl)benzimidazole (HBB) have tended to be classed as inactive, or of low activity, towards DNA viruses[2]. We now find that some of the more lipid-soluble derivatives of HBB can be very effective at inhibiting the onset of cytopathic effect (CPE) in vaccinia-infected monolayers, but this effect depends on the tissue culture system employed. We have reported a similar phenomenon in relation to herpes simplex virus, D-5-chloro-HBB showing activity in ERK and Hep 2 cultures, but none in HEL cultures[1].

Medium [Eagle's minimum essential medium containing foetal bovine serum (10% v/v), $NaHCO_3$ (0.112% w/v), glutamine 2.0 mM, benzyl-penicillin (100 U/ml) and streptomycin (100 μg/ml)] was removed from HeLa monolayers and replaced by fresh medium [with the serum reduced to 2% and the $NaHCO_3$ increased to 0.224%] containing one of a set of neurovaccinia virus dilutions and containing test- compound at the chosen concentration. The virus was included in this medium immediately before its addition to the monolayers. Infected and uninfected control cultures were simulta-

[1] D. G. O'SULLIVAN, D. PANTIC, D. S. DANE and M. BRIGGS, Lancet *1*, 446 (1969).

[2] H. J. EGGERS and I. TAMM, J. exp. Med. *113*, 657 (1961).

neously prepared. Culture tubes (each containing 2 ml of medium) were rolled continuously at 37 °C and the time elapsing until development of 50% CPE was noted. Viral $TCID_{50}$ doses were calculated from the results obtained from the series of infected controls[3,4].

The variation of time interval t (between infection and 50% CPE) with $\log(TCID_{50})$ is shown in Figure 1 for the stated concentrations of 1-butyl-, 1-phenyl-, 1-benzyl-, 5-bromo-1-propyl-, 5-chloro-1-propyl-, and 5-fluoro-1-propyl-derivatives of HBB and for infected controls. As data from several experiments have been combined to produce Figure 1, experimental points are too numerous to plot in the Figure. Instead, the data from which each curve was constructed was fitted to Equation (1)[4]:

$$\log_{10}(5\text{-}t) = n \log_{10}\log_{10}(TCID_{50}) + \log_{10}B. \quad (1)$$

This is a linear relation between $\log(5\text{-}t)$ and $\log\log(TCID_{50})$. Values of the constants n and $\log B$, the number of culture tubes N, and the correlation coefficient r for each line are given in the Table. The difference between the n value for a compound and for the infected control is related to the effectiveness of the concentration of compound tested[4].

Figure 1 and the Table show that the 1-butyl-, 1-phenyl-, 1-benzyl-, and 5-bromo-1-propyl-derivatives of HBB can exert substantial protective power against CPE in HeLa cells caused by vaccinia virus and that the 5-bromo-derivatives is much more active than its chloro or fluoro analogue. It will be noted that 1-butyl-HBB (30 μM) and 1-phenyl-HBB (70 μM) failed to show any activity, and 1-benzyl-HBB (50 μM) showed only slight activity, when tested against the same virus in ERK cultures[1]. Both HeLa and ERK are continuous cell lines of human origin[5].

5-Bromo-1-propyl-HBB was more effective than IUdR[6] when both were tested at 50 μM by the method described above. Surprisingly, isatin 3-thiosemicarbazone and its 1-ethyl derivative, tested at 10 μM[7] and 5 μM[8] respectively, were toxic in this infected cell system and gave no protection. In case metabolic removal of IUdR in tissue culture reduced the drug's activity, the effect of replacing the compounds periodically during incubation of the infected cells was examined. Data plotted in Figure 2 suggest that metabolic removal of IUdR did reduce its activity to some extent. Lines B, I, and C were obtained with 5-bromo-1-propyl-HBB, IUdR and infected controls respectively, by incubating infected treated and untreated cells in the usual way without further interference with the medium. Lines B′, I′, and C′ were obtained by adding 5-bromo-1-propyl-HBB, IUdR and no test-

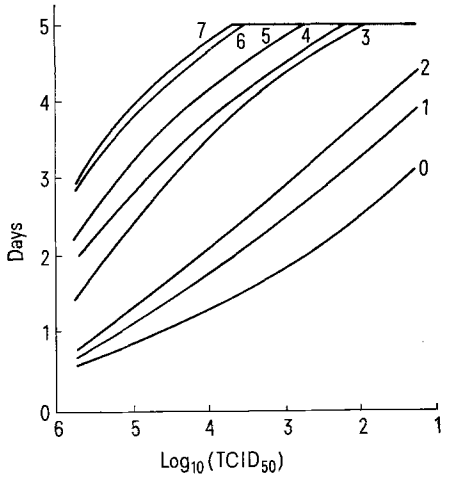

Fig. 1. Dependence of time interval (between infection of HeLa cells and 50% CPE) on vaccinia virus dose for cultures containing inhibitors as follows: curve 0, control cultures with no test-compound; 1, 5-fluoro-1-propyl-HBB (40 μM); 2, 5-chloro-1-propyl-HBB (50 μM); 3, 5-bromo-1-propyl-HBB (50 μM); 4, 1-benzyl-HBB (50 μM); 5, 1-phenyl-HBB (50 μM); 6, 1-butyl-HBB (20 μM); 7, 1-phenyl-HBB (70 μM). 50% cell death occurred at day 5 in uninfected control cultures.

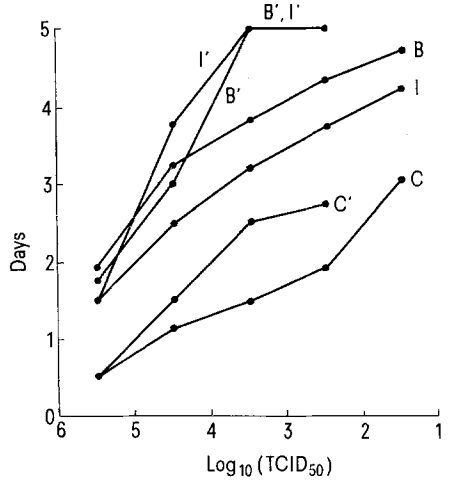

Fig. 2. Comparison of the protection of vaccinia-infected HeLa cells by 5-bromo-1-propyl-HBB (50 μM) (lines B and B′) and by IUdR (50 μM) (lines I and I′) in relation to infected controls (lines C and C′). Lines B, I, and C refer to cultures in which media were not changed after the initial infection of the cells and lines B′, I′, and C′ refer to cultures in which the media were replaced by fresh media (not containing virus, but containing 50 μM of appropriate test-compound) every 24 h following the initial infection of the cells. 50% cell death occurred at day 5 in uninfected control cultures.

Protection of vaccinia-infected HeLa cells by HBB derivatives at quoted micromolarities

Test-compound	μM	n (SD)	$\log_{10}B$ (SD)	N	r
1-Bu-HBB	20	5.06 (0.49)	— 3.50 (0.35)	16	0.995
1-Ph-HBB	70	5.28 (0.63)	— 3.64 (0.45)	12	0.993
1-Ph-HBB	50	3.49 (0.42)	— 2.20 (0.27)	12	0.992
1-Benzyl-HBB	50	2.87 (0.15)	— 1.60 (0.10)	16	0.997
5-Br-PHBB	50	2.65 (0.52)	— 1.44 (0.30)	28	0.915
5-Cl-PHBB	50	1.10 (0.12)	— 0.19 (0.07)	12	0.995
5-F-PHBB	40	0.90 (0.21)	— 0.02 (0.12)	16	0.993
None	—	0.58 (0.04)	0.22 (0.02)	52	0.976

Data in relation to Equation (1). N, number of culture tubes; r, correlation coefficient; PHBB, 1-propyl-HBB.

[3] L. J. Reed and H. Muench, Am. J. Hyg. *27*, 493 (1938).
[4] D. G. O'Sullivan and C. M. Ludlow, Arch. ges. Virusforsch. *41*, 295 (1973).
[5] R. R. A. Coombs, M. R. Daniel, B. W. Gurner and A. Kelus, Nature, Lond. *189*, 503 (1961).
[6] W. H. Prusoff, Pharmac. Rev. *19*, 216 (1967).
[7] F. W. Sheffield, D. J. Bauer and S. M. Stephenson, Brit. J. exp. Path. *41*, 638 (1960).
[8] D. G. O'Sullivan, P. W. Sadler and L. Rice, Proceedings of the Third International Congress of Chemotherapy (Georg Thieme, Stuttgart 1964), p. 879.

compound, respectively, in infected medium to the cells, incubating, and subsequently pouring away the medium and replacing with fresh medium, containing the appropriate compound, every 24 h. The effect of replacing the medium was to delay onset of CPE in all 3 sets of tubes from day 2 onwards. However, this effect was greater for IUdR-containing tubes than for tubes containing the HBB-derivative. With daily replacement of active compounds, there was little overall difference in protective activity between 5-bromo-1-propyl-HBB (50 μM) and IUdR (50 μM). However, the toxicity of IUdR for cells is much less than that of the benzimidazole drivative, although, of course, its long-term effect on cells in general might be suspect.

The observed cell protective effects of the 1-butyl-, 1-phenyl-, 1-benzyl-, and 5-bromo-1-propyl-derivatives of HBB suggest that further investigation of the activities of lipophilic benzimidazole derivatives against DNA viruses is desirable, particularly in relation to the dependence of activity on the specific cell culture employed.

Zusammenfassung. 5-Bromo-1-propyl-, 1-Benzyl-, 1-Phenyl- und 1-Butyl-2-(α-oxy-benzyl-benzimidazol hemmen die cytopathische Wirkung (HeLa Zelle) von Vaccinia-Virus.

D. G. O'SULLIVAN, CAROLYN M. LUDLOW and V. C. DOROMAL

Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W1P 5PR (England), 16 May 1974.

Oxidation and Excretion of D-Lactic Acid by Rats

The role of L(+)lactic acid as the 'physiological isomer' in animal lactate metabolism is since long established. As the classical experiments of CORI and CORI[1] with rats have shown, D(−)lactic acid is poorly utilized and 30 to 40% of the dose ingested is excreted in the urine. These results were fully confirmed by more recent investigations[2] using ^{14}C-D-lactate. However, the wide-spread occurrence of D-2-hydroxyacid dehydrogenase (EC1.1.99.6) in liver, kidney, heart, brain and spleen of the rat and other animals, as well as reversible activation of this enzyme in vitro[3], indicated the need for a re-examination of D-lactate metabolism.

When rats of 250–300 g body weight (BW) were fed a purified diet containing DL-lactate, only 1–2% of the D-lactate ingested was recovered in urine (Table I). Contrary to earlier experiments, a specific enzymatic assay (Boehringer, Mannheim) was used for D-lactate determinations. The excretion of such low levels on the first days of lactate feeding excluded long-term adaptation. In further experiments, oxidation and excretion of D-lactate were measured by $^{14}CO_2$-exhalation radiometry (Exhalameter Berthold & Frieseke, Karlsruhe) in 24 h-fasted rats injected with ^{14}C(u)-D-lactate (Amersham) in appropriate dilution with inactive D-lactate (Serva, Heidelberg). As indicated by the results (Table II), D-lactate was readily oxidized. The somewhat higher excretion in urine after i.p. injection as compared to feeding, can be attributed to faster absorption and to increased diuresis due to sodium surplus. The fraction of metabolites of D-lactate in urine was calculated from radioactivity and enzymatically determined D-lactate. Intermediary isomerization to L-lactate could not be excluded since the metabolites were not isolated. Enzymatic determination of L-lactate in urine indicated, however, that this isomer accounted for only 0.9 and 1.1% of the D-lactate injected into animals on control diet and lactate diet, respectively. Thus, isomerization could not have occurred to a large extent. Surprisingly, lactate feeding did not enhance D-lactate oxidation but tended to increase the excretion of D-lactate and its metabolites in urine. The mechanisms envolved may deserve further investigation.

Table I. Urinary excretion of D-lactate in rats fed a purified diet with 5% DL-Ca-Na-lactate

Measurements	Days on DL-lactate diet	
	1	2
Number of animals	5	5
D-lactate consumed (mg/kg BW)	490 ± 135	629 ± 143
D-lactate in urine (mg/kg BW) [a]	7.4 ± 1.9	7.1 ± 0.4
Dosis (%)	1.5	1.1

[a] 24 h collections.

[1] C. F. CORI and G. T. CORI, J. biol. Chem. *81*, 389 (1929).
[2] F. MEDZIHRADSKY and W. LAMPRECHT, Z. Lebensmitteluntersuch. *130*, 171 (1966).
[3] R. CAMMACK, Biochem. J. *115*, 55 (1969).

Table II. Oxidation and excretion of D-lactate after i.p. injection in rats fed lactate and control diets

Measurements [a]	Lactate-free diet	5% DL-lactate diet
Number of animals	12	12
D-lactate injected (mg/kg BW) [b]	247.0 ± 20.2	247.0 ± 8.4
% of dosis recovered within 6 h as:		
CO_2 in exspiration	84.4 ± 1.3	81.9 ± 2.3
D-lactate in urine	2.9 ± 1.5	4.7 ± 2.7
Metabolites in urine	3.0 ± 2.2	5.5 ± 3.2
Total	90.3 ± 2.2	92.1 ± 5.3

[a] Groups of 3 animals measured simultaneously, $\bar{x} \pm s$ based on $n = 4$. [b] 22 nCi ^{14}C(u)D(−)lactic acid/mg, neutralized with NaOH i.p.