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Neuraminidase Activity in HeLa Cells: Effect of Hydrocortisone

Abstract. *Homogenates of HeLa cells contain neuraminidase activity. This enzyme is particle-bound, and it has a pH optimum of 4.2. Hydrocortisone-regulated cells contain two to three times as much neuraminidase as the corresponding controls. The hydrocortisone treatment also causes an increase in the cell content of β-glucuronidase and acid deoxyribonuclease.*

The regulation of glycoprotein turnover in dividing cells underlies important aspects of cell biology due, in part, to the known involvement of these compounds in the biochemistry of cell membranes and in the determination of the immunological characteristics of the cells. The HeLa cells cultured in the presence of added hydrocortisone contain more sialic acid than do their corresponding untreated controls (*1*). We have now established the presence of a particle-bound neuraminidase (E.C. 3.2.1.18) in $HeLa_{T1}$ cells, and we have studied some properties of this enzyme and the effects of hydrocortisone on the enzyme activity.

The $HeLa_{T1}$ cells (*2*) were grown in monolayer culture with Eagle's minimum essential medium supplemented by calf serum (10 percent), penicillin (50 unit/ml), streptomycin (50 μg/ml), and kanamycin (50 μg/ml). The hydrocortisone-regulated (Hcr) cells were obtained by subculturing the cells in a medium containing 1.0 μg of hydrocortisone per milliliter for a minimum of 1 month. The Hcr state is characterized by a new steady-state level of growth and a new cellular physiological constitution (*3*). The cells, grown in monolayers at 37°C, were harvested from the Blake bottles near the end of exponential growth (about 72 hours) by gentle scraping into 10 ml of saline, buffered with 5 m*M* tris(hydroxymethyl)-aminomethane-hydrochloride (tris-HCl) (*p*H 7.4). The cells were washed twice in the same buffered saline, and a small sample was taken for cell counting in a hemocytometer. The cells were packed by centrifugation, and the pellet was suspended in ice-cold RSB buffer (10 m*M* NaCl, 5 m*M* $MgCl_2$, and 10 m*M* tris-HCl; *p*H 7.4). The suspension (about 1 ml per bottle) was homogenized in a tight-fitting, all-glass Dounce homogenizer. Complete homogenization was obtained after ten strokes, and the thoroughness of the homogenization was checked by phase-contract microscopic examination. The homogenate was centrifuged for 10 minutes at 700*g* to sediment the nuclear fraction, and the supernatant fraction was decanted with a Pasteur pipette. These cytoplasmic extracts, which gave very low blank values during the neuraminidase assay, were utilized for the studies of the properties of this enzyme. The enzyme preparations consisted of nine volumes of cytoplasmic extract and one volume of 1*M* sodium acetate–acetic acid buffer.

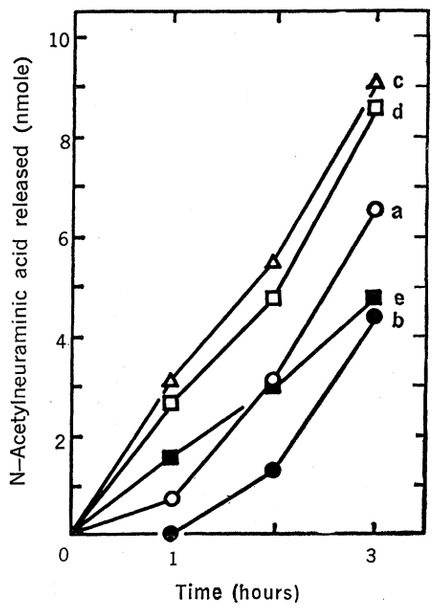

Fig. 1. Progress curves of $HeLa_{T1}$ neuraminidase. (*a*) Control preparation; (*b*) control preparation subjected to ultrasonic treatment. The other curves represent control preparations assayed in the presence of Triton X-100 at the following concentrations (in percent by volume): (*c*) 0.02, (*d*) 0.04, and (*e*) 0.10.

For the neuraminidase assay (*4*), 400 nmole of neuramin-lactose (*5*) dissolved in 0.1 ml of distilled water was added to 0.3 ml of enzyme, and the mixture was incubated for 3 hours at 37°C. The reaction was stopped by adding 0.2 ml of periodate reagent (*6*), and the free *N*-acetylneuraminic acid was determined by the thiobarbituric acid reaction of Warren (*6*). The neuraminidase from preparations of control and Hcr cells showed essentially identical properties. The progress curve (Fig. 1) showed a nonlinear response, that is, a low rate of hydrolysis for the first hour, followed by an apparent activation and linear response afterward. Addition of low concentrations of Triton X-100 resulted in linear response (Fig. 1) and higher activity. The optimum conditions were obtained with a final concentration of detergent between 0.02 and 0.04 percent (by volume). Higher concentrations of Triton X-100 caused increasing inhibition of the enzyme (Fig. 1). A marked inhibition of the neuraminidase activity occurred when the cytoplasmic extract was subjected to a 2-second burst of ultrasound delivered by the microprobe of a Branson Bio-Sonicator at 80 percent efficiency with a 14-kc output (Fig. 1). The *p*H optimum was about 4.2. The activities between *p*H 4.0 and 4.4 were essentially identical and decreased gradually below *p*H 3.8 and above *p*H 4.6. At *p*H 5.8 only 20 percent of the maximum activity was detected. The supernatant fraction of homogenates centrifuged for 1 hour at 105,000*g* showed no neuraminidase activity when assayed either at *p*H 4.2

or at *p*H 5.8. The results indicate that HeLa cells lack the soluble neuraminidase with *p*H optimum 5.8 previously reported in liver, brain, and lactating mammary glands of rats (*4, 7*). The absence of neuraminidase activity at *p*H 4.2 in the high-speed supernatant and the high recoveries (75 to 80 percent) of this activity in the corresponding pellet indicate the particulate nature of this enzyme in HeLa cells. The Michaelis constant (K_m) for neuramin-lactose was 5.84×10^{-4} and $6.95 \times 10^{-4} M$ for preparations of control and Hcr cells, respectively.

Because initial experiments suggested a higher neuraminidase content in Hcr cells than in control cells, a quantitative comparative study was conducted. For those determinations, whole-cell homogenates were utilized in order to eliminate possible error due to loss of variable amounts of neuraminidase activity retained in the nuclear fraction. The incubations were conducted at *p*H 4.2 in presence of 0.03 percent Triton X-100 (by volume) under the conditions described above. Protein determinations were done by the method of Lowry *et al.* (*8*) with crystalline bovine serum albumin as the standard. Although the specific activity of neuraminidase, expressed per milligram of protein, tends to be higher in the Hcr cells, the differences are small and probably not significant (Table 1). However, when the neuraminidase activity is expressed per million cells, a marked increase of enzyme content was observed in the Hcr cells. The average value for the ratio of Hcr to control cells for neuraminidase activity per million cells was 2.62, while the corresponding average ratio for the protein content was 2.42, thus indicating a direct correlation between the increase in cell size (*3*) and the increase in neuraminidase content in Hcr cells. The neuraminidase activity of preparation 3 (Table 1) was also assayed with neuramin-lactose sulfate (*5*) as the substrate. The rates of hydrolysis obtained in this experiment (Table 2) were more than twice those obtained with neuramin-lactose as substrate, and the ratio between the activities obtained with the two types of cells remained almost identical (Table 1, experiment 3, and Table 2).

Since the particulate neuraminidase of rat liver and rat mammary glands belongs to the group of the acid lysosomal hydrolases (*4, 7*), two other lysosomal markers, β-glucuronidase and acid deoxyribonuclease, were assayed by the methods of Talalay *et al.* (*9*) and Schneider and Hogeboom (*10*), respectively. The β-glucuronidase assay was slightly modified for these studies by conducting the incubation in the presence of 0.013 percent Triton X-100 (by volume), which is the optimum concentration for HeLa homogenates. In three different cell preparations, the activities of these acid hydrolases, on a per cell basis, were from two- to threefold higher in the Hcr cells than in the control cells (Table 2). The ratios of Hcr to control cells for these three hydrolases indicate a similar relation between the activities of these enzymes in the control and Hcr cells, respectively. The increased β-glucuronidase activity in Hcr cells is consistent with the findings of Suzuki *et al.* (*11*) who showed that β-glucuronidase is elevated about 80 percent in HeLa cells grown with cortisol for 4 days.

The $HeLa_{71}$ Hcr cells are considerably larger than their corresponding control cells. The extended doubling time of the Hcr cells is due, primarily, to a prolonged G_1 portion of the cell cycle (*3*) which might result from a delayed initiation of DNA synthesis by nuclear membrane–associated DNA polymerase (*12*). Recent studies (*13*) have shown a greatly enhanced sialic acid content of the nuclear membrane in $HeLa_{71}$ Hcr cells, and that most of the glycoprotein synthesis observed in mouse lymphoma cells (*14*) occurs in the S portion of the cell cycle. A study of the neuraminidase activity throughout the cell cycle in synchronized $HeLa_{71}$ control and Hcr cells would probably provide a valuable insight into this problem.

In conclusion, $HeLa_{71}$ cells contain a particle-bound neuraminidase which has properties similar to those of the lysosomal neuraminidase of rat liver and rat mammary gland (*4, 7*). A soluble neuraminidase, similar to that found in the cytosol of rat organs (*4, 7*), was not detected in the high-speed supernatant of $HeLa_{71}$ homogenates. The high neuraminidase content of the Hcr cells is correlated to their larger size. The parallel between the activities of neuraminidase and those of β-glucuronidase and acid deoxyribonuclease, the acid *p*H optimum and the particulate nature of $HeLa_{71}$ neuraminidase, strongly suggests that this enzyme is located in the lysosomes of these cells.

Table 1. Neuraminidase activity and protein content of $HeLa_{71}$ cells grown in the presence and absence of added hydrocortisone. Activity was measured as the amount of *N*-acetylneuraminic acid released per hour with neuramin-lactose used as substrate. Hydrocortisone-regulated cells, Hcr.

Experiment No.	*N*-Acetylneuraminic acid released				Protein	
	Control (nmole/mg protein)	Hcr (nmole/mg protein)	Control (nmole/10^6 cells)	Hcr (nmole/10^6 cells)	Control (mg/10^6 cells)	Hcr (mg/10^6 cells)
1	4.40	4.94	1.15	3.15	0.26	0.64
2	4.03	4.09	1.19	2.62	0.29	0.64
3	4.24	4.89	1.42	4.14	0.33	0.85

Table 2. Effect of hydrocortisone on acid hydrolases of $HeLa_{71}$ cells. Neuramin-lactose sulfate was used as substrate for the neuraminidase assay. Hydrocortisone-regulated cells, Hcr.

Cell preparation	Neuraminidase (nmole/hour per 10^6 cells)	β-Glucuronidase (nmole/min per 10^6 cells)	Deoxyribonuclease (μg/min per 10^6 cells)
Control	3.63	0.05	0.49
Hcr	9.60	0.13	1.05
Hcr/control	2.64	2.60	2.14

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Analysis of Data in a Study of Nest Parasitism, Productivity, and Clutch Size in Purple Martins

Moss and Camin (*1*) used the paired-comparisons *t*-test on mean daily weights of treated and untreated nestling purple martins for 29 days, and concluded that the treated nestlings were heavier ($P < .001$). The test is inappropriate because the 29 "paired comparisons" are not independent, the same birds having been weighed day after day, not to mention that the error variance is probably also changing from day to day.

Similarly, Moss and Camin indicate significant differences in mean maximum weight, resting their tests on standard deviations in each group, without regard to whether there are significant differences between nests. Although perhaps in these data nest means do not differ significantly by the standard of variability of nestlings in the same nest, it cannot be argued that the nest component of variance is zero. Hence, their tests should have used numbers of degrees of freedom derived from numbers of nests, not nestlings, and are mostly nonsignificant. For example, the 1967 comparison of treated to untreated has only two degrees of freedom for nests within treatment. Hence, where Moss and Camin indicate $P < .001$, actually the .05 level is barely reached on the assumption that there is little or no variance between nests. In fact, a comprehensive analysis is needed. Their reference 12, mentioning that a higher mean weight for a brood of five than for (two broods of) four "may be attributable to unusual food-gathering proficiency of a single pair of parents," is on the right track, but apparently they did not perceive how to perform a correct test of significance.

As a final point, in examining a 2×3 table of frequencies of brood sizes according to treatment, Moss and Camin appear to have tested broods of size three against those of size five, omitting broods of four because they were equally frequent for treated and untreated conditions. Presumably their interest lay in progressive change, to which broods of size four are as relevant as other brood sizes. An appropriate test finds that there is progressive change ($P < .01$).

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Norton (*1*) is correct in objecting to the use of the paired-comparisons *t*-test as a test for seasonal difference between martin nestling weights (*2*). However, how crucial is this objection? Statistical tests are often robust, even when an occasional rule of application is unintentionally violated. Would another test give different or comparable results? To examine the validity of his objections, we elected to test our data again following a randomization approach (*3*). With 500 iterations, probability values for equivalence of mean daily weight for each day over the course of the 1966 nesting season were obtained. These were then combined (*4*) to obtain a combined probability value $P_c = .005$ for equivalence of mean weight of treated and untreated nestlings over the course of the season. Thus, our conclusion that parasitized nestlings are lighter than those unparasitized remains unchanged (*2*).

Norton's second criticism relative to our testing of mean maximum nestling weight (MNW) seems somewhat more valid. Reanalysis of our data by using a randomization approach yielded probability values that were in fact higher than those published initially in our table 2 (*2*). In the majority of cases these lay somewhat above the .05 level generally taken as an indicator of statistical significance. However, comparisons within a single year, although still strongly suggestive, are of less interest biologically than conclusions drawn on the basis of several years' data. To test whether unparasitized and parasitized nestlings have the same mean MNW one can combine the seven relevant, recomputed probabilities from modified table 2 to obtain $P_c = .007$, which allows us to reject firmly a hypothesis of equal weight for parasitized and unparasitized young of equal brood size. The case for decrease in mean MNW with increase in brood size is somewhat less strong over the five seasons of data, but is still quite suggestive; a combined probability $P_c = .06$ for equivalence of mean MNW indicates an overall trend toward decrease in weight with increase in brood size. Considering separately brood sizes of three versus four, and four versus five, and combining recomputed probabilities, we obtain for the former case $P_c = .49$, for the latter $P_c = .098$. Thus, a decrease in mean MNW is much more marked as we go from brood sizes of four to five, than from three to four, as suggested earlier in our reference 12 (*2*). In contrast, combining recomputed probabilities in a test of mean MNW reached by unparasitized broods of n versus that reached by parasitized broods of $n-1$ yields a value of $P_c = .96$, which confirms our earlier statement that there is no apparent difference in this case. Finally, if we must include data on broods of four when comparing broods of three and five, then we are glad to know that the probability value determined with this test is somewhat lower than that which we previously reported.

In summary, Norton's conclusions may be of interest from a statistical viewpoint, but their force is considerably blunted by the fact that they do not affect the conclusions drawn in our report.

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