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Liberation of Bactericidal Histone from Inactive Deoxyribonucleohistone Complex by DNase.* (29775)

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In 1887, Ribbert(1) observed the extracellular destruction of microorganisms surrounded but not ingested by leucocytes. This extracellular antibacterial activity has been substantiated more recently by Dubos and MacLeod(2) and Cromartie *et al*(3). Skarnes and Watson in 1956(4) isolated Leukin, a bactericidal factor from peritoneal leucocytes, under conditions simulating the acidic environment existing in an inflammatory area. They suggested that this cationic protein was derived from the nucleus upon cell destruction and was then able to exert its extracellular bactericidal activity.

The present studies demonstrate a plausible means by which the cationic histones might be liberated from the deoxyribonucleohistone complex (DNP) within the nucleus by means of deoxyribonuclease (DNase).

Materials and methods. DNase I was obtained from Worthington Biochemical Corp., Freehold, N. J. The fast green FCF and safranin counterstain (Gram) were obtained from Hartman-Leddon Co., Philadelphia, Pa.

Group A streptococci, T-18, were isolated at NMRU-4, Great Lakes, Ill. The organisms were grown out of a lyophilized state, transferred at 12 hours and the subsequent 12-hour culture was used in the bactericidal assays.

The modified Stock's dialyzable medium (5) was employed because it was capable of supporting the growth of the streptococci and yet it did not combine nonspecifically with the cationic histones.

Calf thymus obtained from local meat packing plants was the source of nuclei. The method employed was that of Allfrey(6) in which the calf thymus is mildly homogenized in 0.25 M sucrose solution with a Waring blendor followed by centrifugation to obtain the nuclei. The nuclei were then resuspended in the dialyzable medium at a concentration of 50% T at a wave length of 600 mμ.

The nucleohistones were extracted from calf thymus with solutions of low ionic strength according to the method of Crampton *et al*(7). This procedure also provides a method for preparation of histone and DNA based upon the observations that high salt concentrations (2.6 M) tend to dissociate the nucleohistone complex. Addition of alcohol to this high salt solution precipitates out the DNA. All 3 substances, nucleohistone, DNA and histone were lyophilized after their isolation.

The antibacterial effects were determined in two ways: The first method was performed by adding the substances to be tested for bactericidal activity to the solvent (water or medium) and incubating them for 1 hour at 37°C. These solutions were then inoculated with a "standard loop" of a 12-hour culture and reincubated for 30 minutes at 37°C. The assay was designed so that a subsequent loop inoculation from the above solutions into tubes of trypticase soy agar at 45°C contained approximately 50-100 organisms. Bactericidal activity was expressed as a decrease in the colony counts on the poured plates after 24 hours incubation at

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TABLE I. Liberation of Cationic Bactericidal Factor from Isolated Calf Thymus Nuclei with DNase.

	Staining characteristic	Plate counts	% Reduction
Control nuclei*	Anionic	76 64 73	†
Nuclei*+ DNase (100 μg/ml)	Cationic	32 40 35	50

* 50% T at 600 mμ.
† % Reduction is calculated as a decrease in plate counts relative to control nuclei.

37°C relative to the control plates. In the second method the substances were added to the medium and incubated for 1 hour at 37°C. These tubes were then inoculated with a "standard loop" containing about 10^6 organisms. Turbidity measurements were then made in a Coleman Junior spectrophotometer at a wave length of 600 mμ at hourly intervals. All assays were performed in triplicate.

The air-dried smears were stained by a modification of the Alfert method for staining histones(8). The slides were stained for 30 minutes at room temperature in a 0.1% solution of fast green dye adjusted to pH 8.1, rinsed in distilled water and then stained for 30 seconds in safranin solution, washed one minute and dried. This is essentially the modification of Spitznagel(9) except that safranin was substituted for azure to give better contrast with the acidic fast green dye.

Results. Calf thymus nuclei isolated by the method of Allfrey contain an intact DNP complex as revealed by incorporation studies. These thymus nuclei stained with safranin thereby showing the relative absence of the cationic reactive groups; however, upon addition of 500 μg DNase I to 5 ml of the 50% T nuclei suspension the nuclei were stained by the fast green after a one-hour incubation at 37°C. This demonstrated the release or uncovering of the cationic groups within the nucleus. Table I summarizes the results of the bactericidal assay using the pour plate method; there was a 50% reduction in the colony count in the DNase treated in relation to the control nuclei.

These results suggest the release of a cationic bactericidal factor from the nucleus upon DNase treatment, but it was felt that more direct proof might be obtained by getting the same results with the DNP which consists of DNA plus histone. DNP (200 μg/ml) or DNP plus DNase were dissolved in water and another set in the dialyzable medium. This was done to determine whether the medium could be used for subsequent studies. Preliminary experiments indicated that various other media complexed non-specifically with the cationic proteins such as histones. This has also been noted by other investigators(10,11,12). Although the cationic substances caused no observable precipitation within the medium, we were also concerned that the ionic strength of the medium might be sufficiently high to reverse the bactericidal effects of histone noted by Hirsch(10). Table II shows that there was no bactericidal activity in tubes containing only DNP or DNase whereas conversely no growth occurred in tubes containing DNP and DNase. The results for water and medium suspensions were identical.

Since it was shown that the medium did not in any way block the bactericidal activity of histone, a turbidimetric assay for the bactericidal activity of the soluble substances was used. The experiment was set up with tubes containing medium plus DNA (100 μg/ml) or histone (100 μg/ml) or DNP (200 μg/ml) or DNase (50 μg/ml) or finally DNP plus DNase. The streptococci attained identical turbidity in the DNA and DNase tubes (Fig. 1). A slight inhibition occurred in the DNP control which can be explained in one of two ways; first, there is some inherent bactericidal activity in the DNP preparation, or the T-18 group A streptococci which produce DNase as shown by the method of Jeffries

TABLE II. Liberation of Bactericidal Histone from Intact DNP Complexes with DNase.

	Water		Medium	
	Plate counts	% Reduction	Plate counts	% Reduction
DNP (200 μg/ml)	37	22	32	22
	18		29	
	34		31	
DNase (20 μg/ml)	40	*	38	*
	36		41	
	42		46	
DNP + DNase	0	97	1	94
	0		0	
	1		1	

* % Reduction is calculated as a decrease in plate counts relative to DNase control.

et al(13) might be inhibiting its own growth through the enzymatic liberation of the histone. And finally, an almost complete inhibition of growth occurred in tubes containing the histone and the DNP plus DNase.

Discussion. On the basis of these results we envisage DNases, produced by pathogenic bacteria or those contained within the inflammatory leucocytes, acting upon the extracellular nucleohistone or nuclei of nonviable leucocytes within the inflammatory area liberating cationic histones. Maver and Greco (14) showed that the enzymatic dissociation of the nucleohistone complex occurred more readily than the enzymatic degradation of histones with tissue cathepsins containing nuclease activity. The release of histones does not assure the host of bacterial destruction because histones can react non-specifically with the nearest acidic substance. This electrostatic interaction with substances such as the hyaluronic acid capsule of the streptococci or the connective tissue mucopolysaccharides results in the blockage of the bactericidal activity of the histones. Furthermore, histones in addition to having antibacterial activity are known to be toxic to tumor cells, liver cells and leucocytes(15). This toxic action might be attributed to any one of the number of *in vitro* inhibitory actions of histones(16). The leucocyte-histone interaction would therefore result in cell death and consequently cell degeneration. The bacteria, somewhat paradoxically, might bring about their own death following the production of DNase which results in liberation of histone from the leucocyte nuclei.

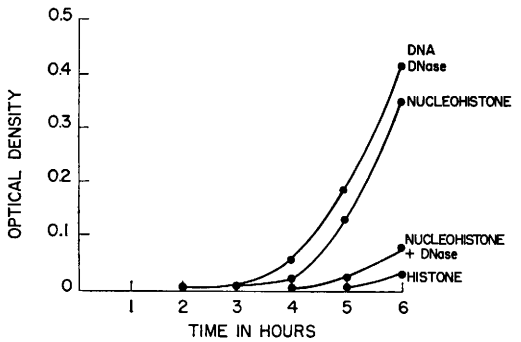

FIG. 1. Liberation of bactericidal activity from inactive deoxyribonucleohistone complex (200 μg/ml) by DNase (50 μg/ml). DNA and histone are at concentrations of 100 μg/ml. Bacterial growth is measured by optical density.

Summary. DNase effectively liberates cationic bactericidal histones from intact nuclei or isolated DNP as shown by bactericidal assays and staining techniques. The possible importance of this mechanism in innate resistance is discussed.

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Effects of Acid Catabolites on Activity *in vitro* of Phenylalanine Hydroxylase from Rat Liver.* (29776)

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Wang and Waisman(1) demonstrated a significant decrease of phenylalanine hydroxylase in male rats which received high levels of phenylalanine for 3 weeks. Woods and McCormick(2) confirmed these findings, quantitated more exactly the amount of phenylalanine necessary to produce a given lowering in hydroxylase activity, and indicated that the primary decrease in measured activity may not be due to accumulation of catabolites directly inhibitory to the enzyme, since loss in activities upon dialysis of preparations from both experimental and control rats is approximately the same. However, such indirect evidence does not preclude some secondary inhibition of the hydroxylase caused by certain of the phenylalanine catabolites, *e.g.*, phenylpyruvate. The potential influence of accumulated levels of catabolites and similar compounds has not been sufficiently assessed, although Udenfriend and Cooper(3) showed that even pyruvate added in a concentration equimolar to substrate phenylalanine markedly inhibits the hydroxylase, and recently Ross and Haljasmaa(4) found that esculetin and some dihydroxyphenylacetamide derivatives are quite active inhibitors of the enzyme *in vitro*. In addition, the relative ability of the pteridine cofactor of phenylalanine hydroxylase, characterized by Kaufman(5), in effecting the degree of any such inhibition has not been defined, though Gal *et al*(6) showed a differential inhibition of the hydroxylase is relieved by a synthetic analogue of the cofactor.

The present investigation was undertaken to assess the influence of common acids and catabolites of phenylalanine on phenylalanine hydroxylase, to quantitate any inhibition of crude and purified enzyme in the absence and presence of additional cofactor, and to characterize the nature of any such inhibition elicited.

Materials and methods. The organic acids were obtained from Eastman Organic Chemicals. 6,7-Dimethyl-5,6,7,8-tetrahydropteridine, a synthetic cofactor for phenylalanine hydroxylation, was purchased from Calbiochem. Young, male Sprague-Dawley rats were fed *ad libitum* on commercial diet. For measuring activities of crude phenylalanine hydroxylase in supernatant solutions from liver homogenates, preparations were made according to Kenney *et al*(7); partially purified enzyme was prepared by carrying the fractionation through an ammonium sulfate precipitation as described by Kaufman(8). Incubation mixtures were made generally according to Freedland *et al*(9), and contained 10^{-3} M L-phenylalanine, 10^{-4} M reduced diphosphopyridine nucleotide, 2.5×10^{-3} M nicotinamide, 3.3×10^{-4} M pteridine cofactor when added, and enzyme in 0.1 M potassium phosphate buffer, pH 6.7. Tyrosine formation was determined by the colorimetric method of Udenfriend and Cooper (10).

Results. The effects of phenylalanine ca-

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