A Method for Detection of Leukocyte Injury Based on Release of a Lysozyme-like Enzyme.* (19800)

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Damage to cells is accompanied by the release of certain cellular contents into the surrounding medium. Various investigators, such as Rossiter(1), have invoked this process as a means of releasing enzymes from rabbit leukocytes for assay and study. Saponin, in particular, has been used to accomplish the liberation of enzymes from cells(1). The effect of hypotonicity was employed by Walker(2) in his studies on the β-glucuronidase content of mouse liver. In connection with studies in progress in this laboratory on the metabolism of human leukocytes, a need was found for a screening test for substances having an adverse effect on cellular metabolism. It seemed that the phenomenon of release of cellular contents into the surrounding medium occurring with injury of the cell might be utilized for this purpose. An intracellular enzyme with the properties of lysozyme was selected as the material to be measured. The method is described here, together with the effects observed on exposure of human leukocytes to varying concentrations of saline and to leukotoxin.†

Procedure and results. Leukocyte suspensions. Whole blood was obtained from human subjects by venipuncture and added to heparin (Liquaemin-Roche) in final heparin concentration of 4 mg %. Sterile siliconed (GE 9987) syringes and glassware were used throughout the preparatory procedures. Sedimentation of red blood cells was accomplished by a dextran method(3) modified in this laboratory by Dr. S. P. Martin and to be reported by him. The supernatant leukocyte-platelet suspension in plasma was centrifuged at 65 g for 7 minutes, a procedure which leaves the platelets and some leukocytes in suspension. The supernatant was taken off, and the sedimented leukocytes were then resuspended in the desired test medium. The final count (or in hypotonic solutions, the equivalent count) of leukocytes is noted in the tabulated data, in each instance. In the sample experiments summarized in the present report, the test media consisted of decreasing concentrations of saline (Table I), and leukotoxin 1:50 in saline (Table II). *Leukocyte lysate.* Sedimented leukocytes as prepared above were resuspended in distilled water. *Lysozyme substrate.* Bacto lysozyme substrate‡ stock suspension was prepared by subjecting a 4% suspension in citrate-phosphate pH 6.2 buffer (as used by Pringle(4)) to 8000 cycles/sec vibration in a Raytheon Oscillator 9 kc for 10 min. For final use, the stock was diluted 5.5 ml per 100 ml of buffer, to 30% light transmission.

Method. In each instance, 1.0 ml of the leukocyte suspension was added to 1.5 ml of lysozyme substrate, and an immediate reading was obtained at 540 mμ in a Coleman Junior Spectrophotometer (75 x 12 mm cuvettes), 100% transmission being set by use of a blank tube containing 1.0 ml of the cell suspension and 1.5 ml of buffer. All tests were run in duplicate. After exactly 20 minutes in a 37°C water-bath without agitation, a second reading was obtained.§ Percent lysis

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† An antiserum to human leukocytes prepared by and supplied through the courtesy of Dr. James Tullis of the Department of Physical Chemistry, Harvard Univ. Med. School.

‡ A dried preparation of *M. lysodeikticus*, supplied through the courtesy of Mr. H. W. Schoenlein of Difco Laboratories.

§ Initially all determinations were done at intervals of 5 to 10 minutes over a period of 1 hour. Subsequently, it was found that a single reading at 20 min. sufficed for the present purpose, although the method of recording repeated observations has yielded interesting information as regards the enzyme *per se*. Studies concerning the absolute lysozyme activity of the cells in various conditions and under various circumstances form a separate investigation which is in progress.

TABLE I. Effect of Decreasing Concentrations of Saline on Human Leukocytes, Leukocyte Lysate and Crystalline Egg-White Lysozyme.

Test material (enzyme source)	Suspending or diluting medium, % conc. NaCl .850	.668	.486	.304	.121	.061
Human leukocytes (A), 6500/mm³	9.5*	15.8	46.3	54.1	77.6	
Human leukocytes (B), 6700/mm³	20.1	32	37	60.9	81.9	
Human leukocytes (C), 800/mm³	7.2	7.5	11.5	18.4	49.4	
Leukocyte lysate (C), 800/mm³ (⁓)	41.7	48	52.4	54.6	48.1	54.3
Crystalline egg-white lysozyme, 3.6 μg/ml	54.6	56.7	59.6	56.1	61.9	55.8

* Recorded as % lysis of lysozyme substrate.

TABLE II. Effect of an Antiserum to Human Leukocytes on Human Leukocytes, as Indicated by Enzyme Release from the Cell.

Leukocytes from subject	% lysis of lysozyme substrate with added: Saline	Leukotoxin	Index of injury*
(A) 5000/mm³	21	50.4	138
(B) 6300	16.6	49.6	194
(C) 4300	10.7	25.7	136

* See text (Method) for derivation of index. An index greater than 24 indicates significant injury.

of the substrate was calculated by the formula: 100 minus (the optical density at 20 minutes, divided by the optical density at 0, times 100). When it is desired to obtain data on the *release of enzyme* from experimentally injured leukocytes as compared to control leukocytes, it is necessary to correct this value for the rate of the enzymatic reaction in the control tubes, since differences noted in a fast-moving system cannot be compared directly with those observed in a slow-moving system. The following index is computed in such circumstances to correct for speed of the reaction: (% lysis in the presence of leukocytes exposed to test substance) minus (% lysis in the presence of control leukocytes), divided by (% lysis in the presence of control leukocytes), times 100. Thus, the larger the index, the greater the lysis of substrate in the presence of test leukocytes, as compared to control leukocytes. Control experiments on the method, using 2 S.D. in the calculations, indicate that an index greater than ±24 is very significantly altered. If a cell-free source of enzyme is used (as in plasma titrations), the S.D. is less, and an index of ±12 is significantly altered. The use of this index of injury is illustrated in Table II.

The rate of the enzymatic reaction in control leukocyte suspensions has been found to be determined to a considerable degree by the injurious effect of the lysozyme substrate itself on the leukocytes during the 20 minute period of observation. It is affected, also, by the care with which the leukocytes are handled prior to the titration and by changes in electrolytes, pH, temperature and time. There is also variability in the reaction rate from day to day in the same individual and from individual to individual, although leukocyte suspensions of closely-approximated count and lysozyme substrate concentrations of identical amount are used in each determination. In all comparative situations, the aforementioned factors operate equally in test and control tubes, so that differences observed have been attributed to the addition of the test substance. When a substance is to be tested for its injurious effect on leukocytes, it has been found desirable to use a suspension of at least 5000 leukocytes per cmm to detect differences consistently. In most of the studies to be reported in later communications, suspensions of 5500-6500 leukocytes per cmm have been used. The present report includes lower counts, as in Table I to compare leukocyte lysate activity with that of a leukocyte suspension of equivalent count.

The method as an indicator of cell damage. The method outlined above has been used as a test for cell injury following the exposure of leukocytes to a variety of physical, chemical and biologic agents. Most of these observations will be the subject of later reports.

Table I is included here as an indication of the changes that are observed following exposure of normal human leukocytes (subjects A and B), leukocytes and leukocyte ly-

sate (subject C) and crystalline egg-white lysozyme‖ to decreasing concentrations of saline. It will be noted that while no consistent change occurs with lysate or crystalline lysozyme, there is a steady increase in lysis of substrate in the presence of leukocytes as the leukocytes are exposed to decreasing concentrations of saline.

Table II compares the percent lysis of substrate in the presence of normal human leukocytes as compared to that observed in the presence of leukocytes from the same suspension after exposure to leukotoxin for one hour. The marked increase in lysis of substrate after exposure of the leukocytes to leukotoxin is evident.

Summary. A method is described for the detection of cell injury by the measurement of intracellular substances released upon injury. The cells studied have been human leukocytes, and the intracellular substance selected for measurement has been an enzyme with the properties of lysozyme. Application of the method is illustrated by data concerning the adverse effect of hypotonicity and of an antiserum to human leukocytes on the cells. In further studies beyond the scope of the present report, the method has been found to be useful in screening a variety of physical, chemical and biologic agents for adverse effect on the metabolism of human leukocytes, as well as for detecting reversal of such injurious effects by protective agents.

‖ Obtained through the courtesy of Armour and Co.

1. Rossiter, R. J., *J. Physiol.*, 1949, v110, 136.
2. Walker, P. G., *Biochem. J.*, 1952, v51, 223.
3. *Separation of Formed Elements, The Protein, Carbohydrate, Steroid, Peptide and Other Components of Plasma.* University Laboratory of Physical Chemistry Related to Medicine and Public Health, Harvard University, July, 1950.
4. Pringle, B. H., DePaulis, D. C., and Pemrick, T. D., *Am. J. Clin. Path.*, 1951, v21, 1039.

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Uracil in Growth and Pyrimidine Nucleotide Synthesis of *Lactobacillus bulgaricus* 09. (19801)

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Orotic acid or a potential source of the compound is required for rapid growth of *Lactobacillus bulgaricus* 09(1-4). Ureidosuccinic acid has small but definite growth-promoting activity(2). Ureides of a number of other mono- and di-carboxylic acids which could not be visualized to yield orotic acid were synthesized and found to be essentially inactive (5). In experiments with *Lactobacillus bulgaricus* orotic acid or ureidosuccinic acid labelled with C^{14} in the ureido carbon gives rise to C^{14}-labelled uridylic and cytidylic acids of the bacterial cell(6). A considerable amount of non-radioactive dilution was observed in the nucleotides isolated despite the essential nature of a utilizable pyrimidine source for growth. Somewhat analogous findings were reported simultaneously by Abrams (7) who observed that a purine-requiring mutant of *Saccharomyces cerevisiae* synthesizes adenine and guanine from 1-C^{14}-labelled glycine as readily as does the wild type parent strain with no purine requirement. It has been reported(1,2) that uracil, uridine, uridylic acid, cytosine, cytidine or cytidylic acid are inactive in satisfying the requirement of *Lactobacillus bulgaricus* 09 for a pyrimidine. These findings led to the hypothesis that nucleic acid synthesis in *Lactobacillus bulgaricus* 09, and possibly in other species as well, normally does not involve the pyrimidine nucleosides and nucleotides as intermediates but instead involves the participation of an orotic acid nucleoside and an orotic acid nucleotide. According to this concept decarboxylation of orotic acid would not occur prior to incorpora-