tional activity as a whole can be detected not only in thrombocytoasthenias but also in other conditions, as for instance during menses[1]. (See previous researches on the positivity of the prothrombin consumption test during menses[2].) In such cases, as well as in other conditions, significant modifications can be detected, regardless of the actual platelet count, which might influence the results in other methods.

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Department of Internal Medicine, University of Pavia, October 16, 1953.

Zusammenfassung

Eine Methode zur Auswertung der funktionellen Plättchenaktivität wird beschrieben. Isolierte Plättchen in konstanter Zahl (300000/cu.mm) werden einem alle übrigen Gerinnungsfaktoren in konstanter Menge enthaltenden Substrat zugesetzt. Prozente können von den Gerinnungszeiten durch eine Eichkurve ermittelt werden.

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The Influence of the Nucleus and of Heat Regulation on the Dipeptidase Activity of the Nucleated and Anucleated Erythrocytes of Vertebrates

Researches in Comparative Cytochemistry

The study of the peptidases—hydrolytic enzymes which split the CO-NH bond of the amino acids combined to form the peptides—has been greatly advanced by the work of the CARLSBERG Group (LINDERSTRÖM-LANG, HOLTER an others[1]). These workers have put forward the hypothesis that these enzymes, known to be ubiquitous, may play a part in the process of protein synthesis which occurs in the cells; such a suggestion has arisen from the fact that the peptidases are found in very considerable quantities in cells with notable power of synthesis (DUSPIVA[1], SMITH[2]). It has moreover been possible to locate the peptidases in the cytoplasm of the cells (DUSPIVA, HOLTER and LÖVTRUP[3]), and not in the nucleus. URBANI[4] was able to notice that in amoebas experimentally enucleated, the dipeptidases are partially affected by the removal of the nucleus, from which one must conclude that the nucleus, although it does not itself contain the enzyme, partly controls its presence in the cytoplasm.

We may recall briefly that researches on dipeptidase activity have even been carried out on the cells of human haematopoietic tissues, in normal conditions and in connection with the relation between *d-peptides* and malignant processes (MERTEN, ADAMS, SALVIDIO, and PARREIRA[1]). Recently ADAMS *et al.*[2] have assayed Mammalian and Avian red blood corpuscles for four separate peptidases; they found that each peptidase varied only a 3-fold range from species to species, and that nucleated avian erythrocytes showed no distinctive differences in activity from mammalian corpuscles.

To explain more clearly the relationship between nucleus and cytoplasm, G. COTRONEI suggested to us that a piece of comparative research, extended to all the Vertebrates, might be of interest in the evaluation of the influence of the nucleus on the enzymatic content of haematic cells.

We have therefore undertaken an analysis of the dipeptidases in the erythrocytes of the Vertebrates, from Fish to Man; we have, however, not been able for the moment to carry out research on the Cyclostomata, owing to lack of material.

Material and Method. Erythrocytes of the following species: *Carassius auratus, Triton cristatus, Bufo vulgaris, Rana esculenta, Lacerta viridis, Anser anser, Gallus domesticus, Mus musculus,* and *Homo sapiens.* Determination of the dipeptidase activity (substrate *dl*-alanylglycine 0·2 m) was carried out according to the titrimetric micromethod of LINDERSTRÖM-LANG and HOLTER[3]. After taking the sample the red corpuscles were washed, counted and hemolysed with distilled water. We calculated the standard cleavage of alanylglycine 100,000 cells and for one hour's incubation at 38°C, and the cleavage per cellular volume unit, obtaining this latter by means of a microhematocrit.

Results. Having ascertained that in the erythrocytes of the Urodela also, the enzymatic activity occurs almost entirely in the cytoplasm, we established the pH opimum of the peptidases in the various classes of Vertebrates, and by comparing them, determined the enzyme content of the erythrocytes of the Vertebrates examined.

As one can see from the table, the optimum pH in the various species examined is around 7·70, though it has an optimum of 7·40 in Fish, and 7·90 (with a secondary maximum at 7·10) in Man. Quantitatively, on a superficial examination of the data, it would seem that there exists a *direct proportionality* between cellular volume and dipeptidase activity. On a more careful examination, however, one notes that while the relationship between the volume of the erythrocytes of Triton and Mouse is 50 to 1 (2500 micron^3 to 51 micron^3), the relationship between the respective standard cleavages is 103 to 1, and the cleavage per volume is 1·46 for the Triton, 0·60 for the Mouse, and 0·41 for Man. These facts show that the enzymatic activity of the erythrocytes in Mouse and Man is *less* that one would have expected from the relationship to cellular volume. An examination of the connection between cellular volume and enzymatic activity per volume unit of the erythrocytes of the cold blooded animals shows, on the other hand, that the dipeptidase activity is proportional to the cellular size and practically equal in the various cold blooded species, in relation to the unit of cellular volume (seeTable). From the Figure it can be seen that the cleavage per volume unit (in grey) is almost the same in the cold blooded Vertebrates, while there is a clear difference between cold and hot blooded species. Between

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[2] E. ADAMS, M. MCFADDEN, and E. L. SMITH, J. Biol. Chem. *198*, 664 (1952).

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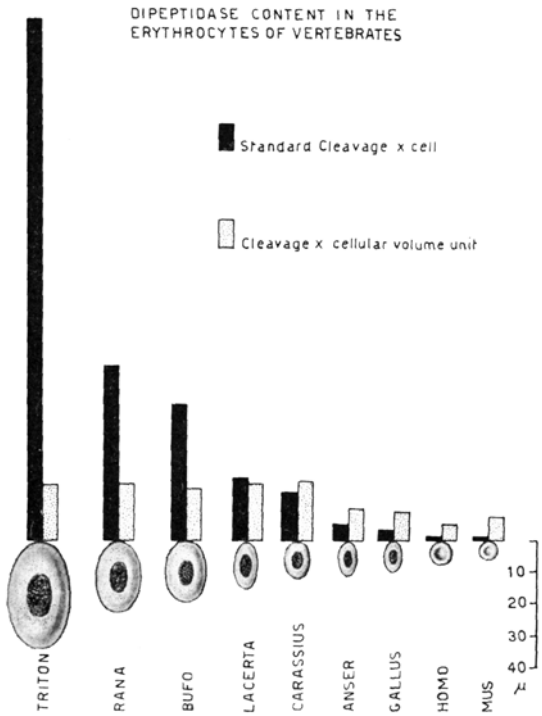
No. of samples	Species	pH optimum	Cellular volume	Standard/Cleavage	Volume/Cleavage
6	Triton	7·70	2,500	270	1·46
1	Rana	7·70	930	93	1·45
5	Bufo	7·70	815	70	1·33
1	Lacerta	7·70	290	33	1·40
3	Carassius.	7·40	225	27	1·50
1	Anser	7·70	130	8	0·80
2	Gallus	7·70	100	6	0·75
1	Mus	7·70	51	2,6	0·60
3	Homo	7·90	80	2,50	0·41

The cellular volume is in cubic micron; the standard cleavage and the cleavage per cellular volume unit (Volume/Cleavage) are in microliter HCl 0·06 N.

Birds and Mammals one sees that the cleavage/volume is greater for the former, and for Mammals it is the lowest found in the zoological scale. From an examination of these data we can establish the following points:

(1) The dipeptidases are present in the cytoplasm of erythrocytes, and absent from the nucleus.
(2) The quantity of peptidases in an erythrocyte is proportional to the volume of the erythrocyte itself.
(3) The quantity of dipeptidases per volume unit of cytoplasm is constant in cold blooded Vertebrates. In warm blooded animals, the quantity of enzyme per volume unit of protoplasm is less than in the cold blooded animals. It is also possible to differentiate the hot and cold blooded Vertebrates from the point of view of enzyme content, by comparing the enzymatic activity.
(4) In hot blooded Vertebrates with nucleated erythrocytes (Avians), and anucleated erythrocytes (Mammals) the enzyme content is less in Mammals, whose red blood corpuscles have lost the nucleus; moreover, the enzymatic content of the erythrocytes of Mammals is less than that of all other Vertebrates.



For a full discussion of the matter, reference should be made to another work in process of publication (SALVIDIO and URBANI[1]).

Conclusions. The comparative criterion on which this piece of research is based has permitted us to obtain objective results on the dipeptidase content of the erythrocytes in the range of Vertebrates, from Fish to Man, and it is the first time that this particular field of research has been studied. We have been able to distinguish the influence of the nucleus on the enzymatic content of the red blood corpuscles, and we have also been able to establish that there is a correlation between the enzymatic activity and the phenomena of heat regulation. Examination of the erythrocytes of five classes of Vertebrates has shown that from the enzymological point of view also, one can distinguish between Vertebrates with nucleated erythrocytes and Vertebrates with anucleated erythrocytes. Finally Avians, in which the erythrocytes present the morphological appearance (nucleus) of cold-blooded Vertebrates in a warm-blooded organism, have an enzymatic content quantitatively intermediate between that of Fish, Amphibians, and Reptiles from one side, and Mammals on the other.

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Riassunto

Applicando il metodo comparativo gli autori hanno studiato l'influenza del nucleo sul tenore in dipeptidasi (substrato *d–l* alanilglicina) degli eritrociti di Vertebrati eterotermi ed omeotermi dai Pesci all'uomo. La mancanza di materiale non ha permesso di comprendere per il momento, nelle osservazioni gli eritrociti dei Ciclostomi.

Con il micrometodo di LINDERSTRÖM-LANG e HOLTER si è potuto localizzare l'enzima prevalentemente nel citoplasma degli eritrociti, inoltre si è potuto accertare:

I. La quantità di dipeptidasi di un eritrocita è proporzionale al volume dell'eritrocita stesso.
II. La quantità di dipeptidasi per unità di volume protoplasmatico è una costante negli eterotermi.
III. Negli omeotermi la quantità di enzima, per unità di volume protoplasmatico, è minore che negli

[1] E. SALVIDIO and E. URBANI, to be published in: Haematologica *37* (1953).

eterotermi. Il tenore enzimatico degli eritrociti dei Mammiferi è inferiore (a parità di volume protoplasmatico) a quello di tutti gli altri Vertebrati studiati.

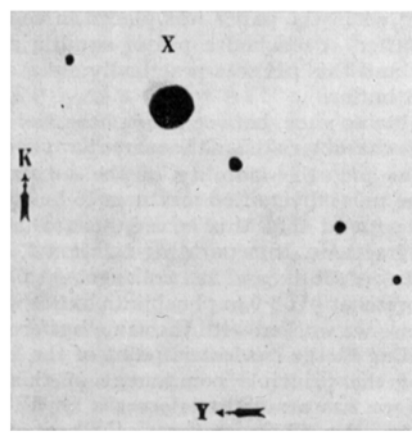
Dall'insieme delle ricerche è risultata l'influenza del nucleo e della termoregolazione (dedotta dalle condizione eteroterme e omeoterme degli organismi studiati) sul tenore in dipeptidasi degli eritrociti nucleati e anucleati dei Vertebrati.

Some Remarks on Two-Dimensional Paper Electrophoresis[1]

Paper electrophoresis has been making rapid development during recent times both on account of the simplicity of carrying out the method and the low cost of the equipment required. But to be sure that it is really paper electrophoresis and not just chromatography with applied voltage, the process should be carried out under conditions which ensure having a movement of the substance which depends essentially on the electric field applied, and exclude other factors which might interfer with the final result. From this point of view, flat electrophoresis presents undoubted advantages over that carried out on an inclined surface, even with continuous evaporation of the solvent according to MACHEBOEUF and his associates[2] (electrorheophoresis).

In any case, paper electrophoresis effectively opens up a large field of inquiry on account of the various opportunities for successful combination with chromatography[3], also for effecting a continuous separation[4] of the different substances and for carrying out two-dimensional electrophoresis[5], that is, twice successively in directions at right angles to each other and under different electrical conditions.

Our working conditions[6] for the study of two-dimensional paper electrophoresis have been the same as those described by KUNKEL and TISELIUS[7], with the exception of one particular: the two opposite sides of the square sheet of paper did not dip into buffer, but were electrically connected there with small siphons of the same paper (four at each side) not larger than 15 mm. This arrangement, even though it forced us to work with a lower current intensity, operated in such a way that the movement of the substances was independent of their position on the paper and due exclusively to the forces of the electric field. In fact, also in electrophoresis in a single direction, we have noticed many times that when the paper strip was dipping at the two extremities for its entire width into buffer contained in the vessels, there was—even without the current—a movement of the substances due to the strong influx of electrolyte. This movement was negligible in the central part of the strip and increased little by little gradually as it approached the buffer in the vessels. These movements had a really noticeable influence on the results of the electrophoresis, particularly for amino acids and the more diffusible ions. When, in order to have a larger path available, a starting point was selected near one of the ends of the strip, it happened sometimes that, at first, the substances migrated towards the nearest vessel (pole with the same charge) before starting their normal route towards the opposite pole; other conditions being equal, the path taken by the substances differed according to the initial point. Even when the starting point was chosen in the central part of the strip, during the electrophoresis with the movement due to the electric field, the substances fell under the influence of the attraction exerted by the buffer. Although it was possible to determine this influence exactly at each point, both without the application of the current and when the strip was inserted in the circuit (and in this case such influence adds up algebraically to the value of the electroendosmotic flow), we preferred to try to eliminate this interference. The use of narrow siphons (one each seven centimetres of the width of the strip) eliminates, as we already said, this difficulty.



Two-dimensional paper electrophoresis of human plasma fraction I (according to COHN *et al.*). *X* = origin. *K* = direction in the first phase. Potential, 300 V. Duration, 360 min. Phosphate buffer, pH 8·0, ionic strength 0·01. *Y* = direction in the second phase. Potential, 450 V. Duration, 600 min. Phosphate buffer, pH 5·7, ionic strength 0·1. – From the left descending to the right: gamma-globulins, fibrinogen, beta- and alpha-globulins, albumin.

In our work we have always carried out two-dimensional electrophoresis on the horizontal plane and we have checked that the path taken is due almost exclusively to the migration of the ions under the difference of potential and to the electroendosmotic flow (the effects of this latter are proportionately reduced if MUNKTELL paper 20/150 is used). Working in the two phases of the process, with the same buffer (same pH and same ionic strength) the ratio between the speed of the individual components remains constant with variations of the difference of the potential applied and, at the end of the second phase, the various substances are found along a straight line, the position of which on the plane depends solely on the characteristics and duration of the passage of the current. Each individual component is found at the extremity of the hypotenuse of a right angled triangle, the other sides of which represent the path taken by the substance in the two different phases of the process.

[1] A section of this paper was read at the XIII International Congress of Pure and Applied Chemistry, Stockholm, 1953.

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