

## Studies on Lysozyme from Human Leucemic Urine by Isoelectric Focusing

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Lysozyme from normal human serum purified by bentonite absorption, followed by pyridine elution<sup>1</sup> could be separated by chromatography on Sephadex gel into two enzymatically active components.<sup>2</sup> The assay was made viscosimetrically with glycol chitin as substrate.<sup>3</sup> The existence of two different lysozymes in human serum may be correlated to the demonstration of two lysozyme peaks after gel chromatography of extracts of human leucocytes.<sup>4</sup> In order to study the different molecular forms further, the method of isoelectric focusing was used.<sup>5,6</sup> By using serum and urine from a patient with a special form of monocytic leukemia a much higher amount of lysozyme was available for this study than from normal serum and urine.

Earlier isoelectric focusing attempts to fractionate lysozymes with carrier ampholytes, Ampholine, with isoelectric points up to pH 10 were not successful because of the high pI of the proteins. However, new ampholytes for the alkaline pH range have recently been synthesized by one of us (O.V.), making it possible to study these proteins by isoelectric focusing.

**Material and methods.** Normal human serum was obtained from healthy blood donors. Serum and urine from a patient suffering from monocytic leukemia was kindly supplied by Dr. J. Palmblad, Södersjukhuset, Stockholm, which is gratefully acknowledged. Dried *Micrococcus lysodeikticus* cells were purchased from Koch-Light, England, and glycol chitin from Seikagaku Kogyo Co., Tokyo, lot 4401. Enzymatic assay was performed with the *Micrococcus* lyso-plate method<sup>7,8</sup> or the

viscosimetric glycol chitin method.<sup>3</sup> The activity is expressed as  $\mu\text{g/ml}$  of lysozyme and Hultin units (H.U.)/ml, respectively, calculated as described in Refs. 7 and 3.

Twenty ml of fresh human leucemic urine was applied on a column of Sephadex G-25 ( $2.5 \times 28$  cm) equilibrated with 0.5 % glycine, pH 6.3. Glycine was used to obtain a saltfree protein solution for subsequent isoelectric focusing. Dialysis could not be used because of great losses of lysozyme. The result of Sephadex separation can be seen in Fig. 1. The fractions of each peak were analysed in separate isoelectric focusing columns.

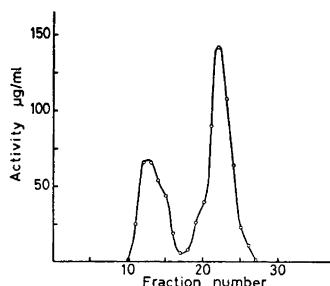


Fig. 1. Gel chromatography of fresh urine from a patient suffering from monocytic leukemia. A 20 ml sample (activity 225  $\mu\text{g}$  lysozyme/ml) was applied to a Sephadex G-25 column ( $2.5 \times 28$  cm) equilibrated with 0.5 % glycine pH 6.3, eluted in the same medium and collected in 5.0 ml fractions. Flow rate 60 ml/h. The lysozyme activity was assayed according to the *Micrococcus* lyso-plate method.

A column of 110 ml capacity (Typ 8101 LKB-produkter AB, S-161 25 Bromma, Sweden) was used for isoelectric focusing. The carrier ampholytes were aliphatic amino-carboxylic acids with pI in the pH interval 9.4 to 11.2; the method of synthesis will be described later.\* Of a suitable mixture thereof 0.5 g was used for each experiment. In order to obtain a sufficient conductivity in the neutral pH range 0.1 ml of a 40 % w/v solution of Ampholine (LKB) pH 6–8 and 1/4 of the alkaline ampholytes were added to the less dense solution. A sample of the protein solution was used instead of water when preparing the less dense solution. The dense solution contained 25 g of sorbitol (Hopkin and Williams) and the alkaline ampholytes. The density

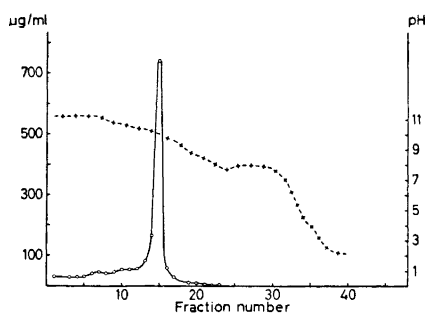
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\* The substances will be available from LKB within the nearest future.

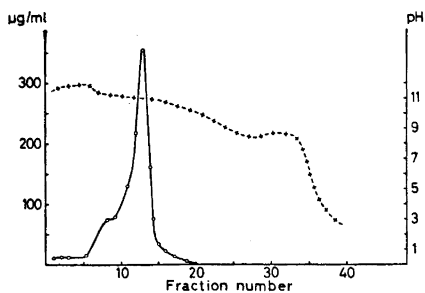
gradient was made with the aid of a gradient mixer (LKB). The central tube and the bottom part were filled with a water solution of sorbitol, 55 % w/v, to which 2 ml of 1 M NaOH was added. The central tube was used as cathode. After filling the column 10 ml of 0.05 M acetic acid was added. In the cooling jacket water at +4°C was circulated. The potential was 280 V at the start and was increased to 400 V after 6 h. After 65 h 2.5 ml fractions were taken.

The specimens were assayed for enzymatic activity without preceding dialysis.

**Results and discussion.** Figs. 2 and 3 show that purified human lysozyme in an alkaline pH gradient could be focused into



**Fig. 2.** Isoelectric focusing of monocytic leucemic urine purified by gel chromatography as described in Fig. 1. A 30 ml sample from fractions 11–16 (Fig. 1) was applied to the 110 ml column. O: lysozyme activity assayed with *Micrococcus* lyso-plates at pH 6.5; +: pH at +4°C.



**Fig. 3.** Isoelectric focusing of monocytic leucemic urine purified as described in Fig. 1. A 20 ml sample from fractions 20–23 (Fig. 1) was applied to the column. For other details see legend to Fig. 2.

two main components with pI 10.0 and 11.0. The high pI found for the human lysozymes is consistent with the general observation that lysozymes have a high isoelectric point; e.g., egg white lysozyme has been reported to have a pI of about 11.<sup>9</sup>

The pI values reported here for human lysozyme are not absolute values because they were obtained in density gradients of sorbitol. It has been found from studies on cytochrome *c*<sup>10</sup> that the pI can be influenced by the sucrose concentration especially at alkaline pH. Other investigators have reported that lysozyme from human leucocytes can be separated by ion exchange chromatography into two enzymatically active components.<sup>11,12</sup> This supports our findings that lysozyme is heterogeneous.

The two major components found are consistent with the two components separated by Sephadex chromatography. The fraction series were also assayed viscosimetrically. A comparison of the muramidase activity (*Micrococcus* lyso-plates) and of the glycol chitin splitting activity (viscosimetric method) of the two components shown in Fig. 1 demonstrated different substrate affinity.<sup>2</sup>

The separation on Sephadex can be due to ionic or possibly nonionic adsorption. When the same column was equilibrated with 0.5 M NaCl and 0.5 % glycine only one component of lysozyme was obtained. At present this observed heterogeneity cannot be explained. The amino acid composition of these components is presently under investigation. Two components of lysozyme can also be obtained after isoelectric focusing on lysozyme from serum and urine purified by the bentonite procedure. These results will be described in a later publication.<sup>2</sup> The fact that two components can be detected in samples of unpurified leucemic urine means that they are not artifacts caused by the pretreatment of the protein. No losses of enzymic activity were observed after isoelectric focusing.

Recently the method of isoelectric focusing has been used for purification in studies on lysozyme from turnip.<sup>13</sup>

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## Specificity of RNA-DNA Hybrids in Differentiating Erythroid Cells

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In a previous paper we described molecular hybridization between newly synthesized RNA from differentiating foetal erythroid cells and DNA from adult livers.<sup>1</sup> It was shown that as differentiation proceeded erythroid cell RNA hybridized less efficiently with DNA. We interpreted this to indicate increasing repression of the genome. This view was supported by experiments in which RNAs from cells at different stages of differentiation simultaneously competed for sites on DNA.

The interpretation of RNA-DNA hybridization experiments in mammals is

hampered by several difficulties.<sup>1</sup> Firstly, reactions may not display locus specificity.<sup>2</sup> Secondly, the extent of cross reaction among related base sequences is dependent upon reaction conditions such as temperature and salt concentration.<sup>3</sup> Thirdly, repetitious nucleic acid sequences which make up about one third of the mouse genome<sup>4</sup> hybridize at a much faster rate than unique sequences.<sup>5</sup> Therefore, the reaction of such repetitious nucleic acids may completely obscure the contribution from unique or less repetitious sequences.<sup>6</sup> Significant nucleotide mispairing occurs under ordinary reaction conditions when related nucleic acid sequences of repetitious type are used.<sup>6</sup>

The work described here was an attempt to minimize those difficulties of interpretation that stem from the lack of locus specificity. Some apparently fundamental facts relating to the use of RNA-DNA hybridization in our mammalian system were discovered.

Our previously reported reactions were carried out at 67°C. At this temperature chain scission and depurination pose a problem which is only partially overcome by the use of appropriate salt concentrations (in our experiments 4×SSC; the abbreviation SSC denotes 0.15 M NaCl–0.015 M sodium citrate). A significant improvement was achieved by using formamide in the reaction mixtures<sup>7,8</sup> because it greatly reduces the thermal stability of nucleic acids so that the reactions can be carried out at low temperatures.<sup>9,10</sup> It has been shown that in formamide at low temperatures rates of reaction between

*Table 1.* Hybridization of foetal mouse yolk-sac erythroid cell RNA with adult mouse liver DNA. Numbers given under "Hybridization" were calculated as cpm hybridized at saturation level divided by the specific activity (cpm/μg) of the labelled RNA used. Results are means of 5 experiments (without formamide), and 2 experiments (with formamide) ± S.E.

Day of gestation	Hybridization	
	Without formamide	With formamide
11	0.078 ± 0.010	0.010 ± 0.004
12	0.053 ± 0.011	0.006 ± 0.003