To ascertain rates of protein degradation it is necessary to minimize reutilization of the isotope[6]. The use of serial extraction eliminates radioactive precursor, especially that appearing in the medium early in the chase period. Moreover, since all experiments were conducted using a large volume of fresh medium relative to cell volume and containing 2 mM L-leucine, reutilization should have been minimal.

Figure 2 shows the kinetics of protein degradation for a representative experiment. At zero time TCA-soluble radioactivity was only 9.6% of total radioactivity. TCA-soluble radioactivity in the cells remained at a fairly constant low level (about 5% of total radioactivity) throughout the experiments, while TCA-soluble radioactivity in the medium accumulated with the chase time. Degradation was approximately exponential with indication of an early phase of fast degradation. The half-lives of the labeled proteins were comparable in resting, PHA-transformed and CL T-lymphocytes: 31.1±6.7, 39.4±7.3 and 27.8±5.8 h respectively. These values are approximately twice the half-life calculated by others for rat lymphocytes using radioautography and grain counting[6]. This discrepancy could possibly be due to species differences in turnover rate, but is more likely to be due to the fact that labeling of rat lymphocytes was carried out 'in vivo', whereas in our experiments labeling was carried out 'in vitro', which, surprisingly, may be more accurate. This is because after i.v. injection of an isotope, there is a preferential labeling of the lymphocytes from peripheral blood, a relatively small pool which is subsequently diluted by the less labeled lymphocytes from spleen, thymus, thoracic duct and other lymphoid systems, thus causing an erroneaous overeste of the degradation rate. Since the life-span of lymphocytes is very long[7], it can be concluded that virtually all protein turnover in human T-lymphocytes is intracellular rather than involving death and renewal of entire cells.

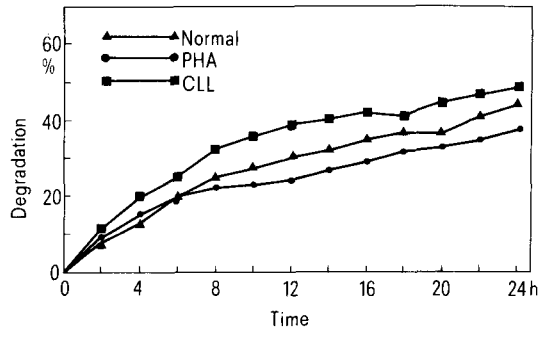

Fig. 2. Kinetics of protein degradation in normal, chronic leukaemic and PHA-transformed T-lymphocytes. Viability of the cells at the end of the experiment was 84.0±9.1, 89.3±7.5 and 86.4±9.1 respectively.

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Correction of the anomalous electrophoretic behavior of ribonuclease A[1]

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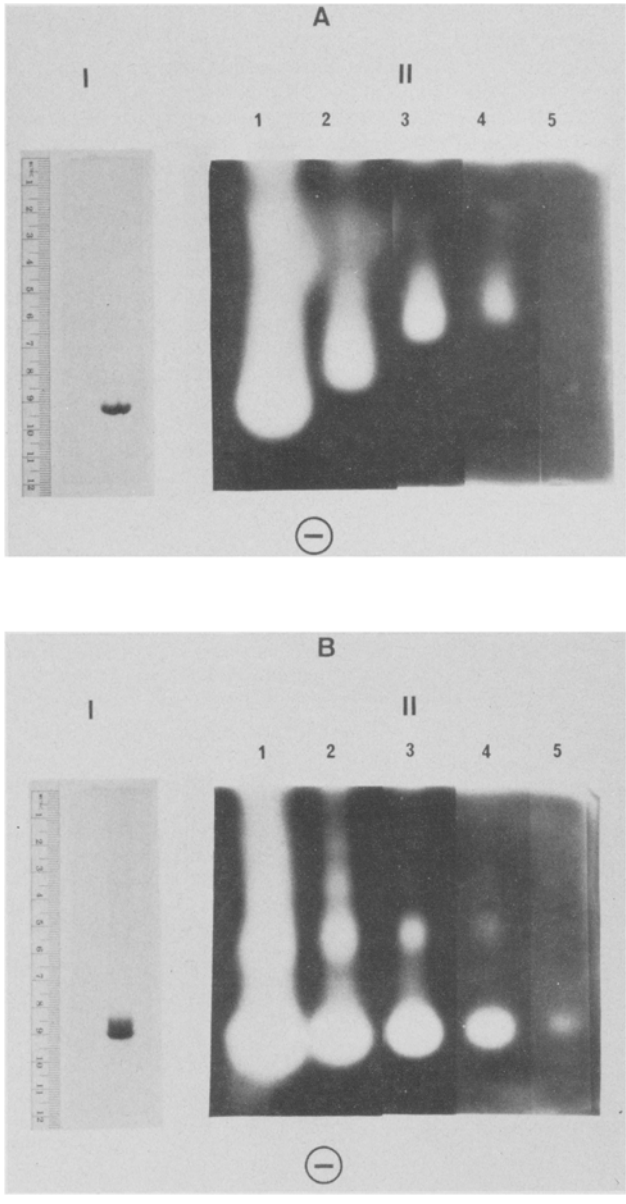
Summary. Ribonuclease A behaves anomalously on polyacrylamide gel electrophoresis at acid pH. The distance traveled by the protein is a function of the amount of enzyme added at the lower range of detectable activity (10 pg to 100 ng). Addition of myoglobin (1 mg/ml) abolishes the anomaly. The observations are consistent with the known affinity of RNase for anions. Caution is warranted in the interpretation of apparent electrophoretic variants of RNase observed at low concentrations of enzyme.

Implicit in the use of electrophoretic techniques for comparing different proteins is the assumption that for a given set of experimental conditions (e.g., supporting medium, buffer, ionic strength, pH, applied voltage and time), a given protein will always migrate the same distance relative to a reference. Relative migration distance (and mobility) is also assumed to be independent of the amount of a given protein subjected to electrophoresis. Deviations from the latter assumption appear to have been rarely observed for any protein and never reported for ribonuclease A (RNase A) from bovine pancreas. We report here that the mobility of RNase A in a common acidic buffer system during polyacrylamide gel electrophoresis (PAGE) is a function of the amount of protein analyzed and that the anomalous electrophoretic behavior at low levels of protein can be rectified by the addition of another polycationic protein, myoglobin. These observations are consistent with the known affinity of RNase A for anions.

Materials and methods. RNase A was purchased as ribonuclease I 'A' from Miles Laboratories, Inc., Elkhart, IN. Myoglobin and bovine serum albumin from sperm whale skeletal muscle were from Sigma Chemical Company, St. Louis, MO.

RNase zymogram. After electrophoresis, the polyacrylamide gel (see legend for fig.) was equilibrated in 0.05 M sodium phosphate buffer, pH 8.0. A 1% agarose gel 0.79 mm thick containing 2 mg/ml RNA in the phosphate buffer was placed over the polyacrylamide gel. After 20 h at



Variation in the migration distance of RNase A in polyacrylamide gel electrophoresis (PAGE) when different total amounts of enzyme are applied to the gel, and the use of myoglobin treatment to overcome this variation. PAGE was performed in thin layer polyacrylamide gels. The PAGE system was a modification of the cathodic disc electrophoresis system at acid pH developed by Reisfeld et al.[16]: Potassium acetate buffer in the gel (pH 6.5 in the stacking gel and pH 4.5 in the separating gel) and β-alanine/acetic acid (pH 4.5) in the electrode buffer. The following amounts (µg) of RNase A were run in the PAGE system for detection by the zymogram (II): 1) 0.1, 2) 0.01, 3) 0.001, 4) 0.0001, and 5) 0.00001. For this purpose, a stock solution of RNase A was serially diluted in 1.0 mg/ml bovine serum albumin, and the forementioned quantities of RNase A were applied in the same total volume (45 µl) of sample. To compare the migration distance of a relatively large quantity of RNase A, a 20-µg load was also run and stained for protein (I). Also, each sample was either: [A] not treated or [B] treated with myoglobin at a final concentration of 1.0 mg/ml. The PAGE run lasted for 7 h. The white areas in II were cleared of RNA by the enzyme whereas RNA in intermediate and surrounding areas was not digested and stained blue (black in the photo). Note that the bulk of the RNase A appears near the cathode, but that at high concentration up to 4 additional less cathodally running bands are visible.

37°, the agarose gel was removed, fixed in cold 0.5 M HCl, washed in 0.5% acetic acid, and stained with toluidine blue: acetic acid and destained with 0.5% acetic acid. RNase activity appears as clear areas against a blue background.
Protein stain. Proteins were stained with Coomassie brilliant blue R-250[2].

Results. The variation of distance of migration with concentration of RNase is clearly shown in the figure A. Application of larger amounts of protein results in greater migration toward the cathode.
To demonstrate that the addition of certain cations has a pronounced effect on the migration of RNase A, the same samples were treated with myoglobin. The results of treatment are shown in the figure B. It is noteworthy that even the 'maximum' migration distance of RNase A (I in fig.) is reduced markedly from that expected on the basis of its isoelectric point (pI) of 9.45 (Miles catalog): For example, myoglobin (pI 8.2) ran slightly ahead of the RNase A toward the cathode. This reduced mobility of RNase A is not due to a sieving effect in the polyacrylamide gel, since the molecular weight of RNase A is less than that of myoglobin. Other data, not shown, indicate that the variation in mobility of RNase A occurs only at total amounts less than 0.1 µg.

Discussion. There are 2 observations of interest here. The first is that the electrophoretic mobility of a protein may vary with the quantity of protein applied and the second, that this variable mobility may be overcome and corrected by the addition of myoglobin. The obvious questions raised are why has this observation not been made before and what is the cause of this phenomenon? There appears to be only one other documented case of protein exhibiting mobility differences at different load levels in electrophoresis. Vessell[3] showed that during starch gel electrophoresis at pH 7, the cathodal mobility of isozyme 5 (the most basic of the 5 isozymes) of lactate dehydrogenase (LDH) depends on the amount applied. It is rather strange that this behavior of RNase A has been overlooked. The enzyme has been analyzed by zone electrophoretic techniques by many investigators. The probable reason for this oversight is the use of the enzyme at relatively high protein loads (greater than 1 µg). In our PAGE system, the variation in mobility of RNase A occurred only at total amounts less than 0.1 µg. The observation was made possible by the use of an extremely sensitive RNase zymogram method capable of detecting approximately 10 pg of RNase A. Similar variations in the migration and mobility of RNase isozymes from a single urine specimen was evident when the urine was sufficiently dilute.
There are several possible explanations for the anomalous electrophoretic behavior. In the case of LDH, Ressler et al.[4] suggested that the behavior was due to interaction with anionic components in the gel matrix. There is no evidence that interaction with the gel is responsible for the behavior of RNase on PAGE.
The observation that anomalous migration occurs only at low concentrations of RNase A or urine RNase (data not given) suggests that the variation occurs when the ratio of protein to buffer falls below some critical level. The finding of a decreased cathodal mobility of one protein relative to another may be generally interpreted in 1 of 2 ways: 1. The protein having lower cathodal mobility also has a lower isoelectric pH or 2. this protein has a relatively greater tendency to bind anions and this interaction causes a decrease in the net positive charge of the protein. The fact that RNase A with the higher isoelectric point is less mobile than myoglobin with lower isoelectric point may be due to the binding of anions from the buffer by the RNase. The cationic agent myoglobin will then compete with the RNase

A and negate the effect of the buffer anions on decreasing amounts of RNase A.

The phenomenon of anion binding to proteins is common[5-8]. RNase A contains 4 arginine residues and 11 lysine residues[9] and the positively charged side groups on these residues may interact with the phosphate groups in substrates. In addition, the binding of phosphate from buffers has been demonstrated by dialysis and gel filtration experiments[10]. The paper electrophoresis experiments of Crestfield and Allen[11] demonstrated a marked decrease in the isoelectric pH (from 7.8 to 5.9) as the ionic strength of phosphate buffer was increased. When the ionic strength was held constant, the isoelectric pH value in phosphate buffer was 6.4 whereas in ammonia-ammonium acetate buffer it was 9.1 These data demonstrated the differential effect of buffer ions on the mobility of RNase A. Electrophoretic experiments by a number of other authors[12-15] demonstrate the variation of isolelectric pH with changing ionic strength of various buffers. Comparison of the isoelectric pH values determined from RNase A mobility vs pH curves with the pI values determined by isoelectric focusing also suggest that the unusually low isoelectric pH values determined for RNase A are artifacts owing to interaction with buffer anions.

Our present results with RNase A provide a caveat in the use of electrophoretic mobility to discriminate proteins. We suggest that the phenomenon portrayed here may be particularly likely to occur with other RNase enzymes, and perhaps other cationic proteins, present at low concentrations in comparison to the buffer ions in electrophoresis. The use of equally sensitive zymogram techniques for other enzymes or improved methods of protein detection should allow a determination of whether this phenomenon is relatively common or is as rare as presently appreciated.

1 Acknowledgment. This is publication No. 80-33 from the Department of Medical Genetics and was supported in part by the Indiana University Human Genetics Center, PHS GM 21054.

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Properties of γ-glutamyltranspeptidase, and glutathione levels in rat mammary gland

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Summary. γ-Glutamyltranspeptidase activity and glutathione levels were studied in rat mammary gland during the lactogenic cycle; both increased during mid-lactation. The enzyme's specific activity with several amino acids showed that glutamine and methionine were the best substrates. Maleate decreased the transpeptidation reaction and increased the hydrolytic activity. These results suggest that γ-glutamyltranspeptidase from the mammary gland is similar to the enzyme described in other tissues in relation to these properties and the physiological role proposed in amino acids transport.

γ-Glutamyltranspeptidase is a widely distributed enzyme which has been specially studied in kidney[1]. As it is a membrane-bound enzyme, an important role in the transport of amino acids into the cells has been atributed to it; this is achieved in a cycle of six enzymatic reactions known as the γ-glutamyl cycle[2]. The natural substrate postulated for the enzyme is reduced glutathione (GSH), a metabolite which is found in high concentrations in tissues like liver, kidneys, the intestinal mucous villi, erythrocytes, etc., which contain some or all of the cycle enzymes.

The metabolism of this tripeptide, the functions of which are the protection of −SH groups, detoxification, the provision of a reservoir of cysteine, etc.[3,4], has not been studied in the mammary gland. In a previous paper we described the hormonal dependence of γ-glutamyltranspeptidase of rat mammary gland[5]; now we present a study of the activity of the partially purified enzyme with some L-amino acids as substrates; the effect of maleate on enzyme activity, and the GSH levels in the lactogenic cycle.

Materials and methods. L-γ-Glutamyl-p-nitroanilide, GSH and glycylglycine were obtained from Sigma Chemical Co.; L-U-^{14}C-glutamic acid from the Radiochemical Centre, Amersham.

Primiparous Sprague-Dawley rats (150–200 g b.wt) were taken at different stages of the lactogenic cycle. During lactation the rats were maintained with 8–10 pups.

The enzyme assay was carried out using L-γ-glutamyl-p-nitroanilide as donor and glycylglycine as acceptor[6]. The enzyme activity was also assayed with GSH as the γ-glutamyl moiety donor, and glycylglycine and several amino acids as acceptor substrates; when L-U-^{14}C-glutamic acid was used as the acceptor, the transpeptidation product was isolated by paper electrophoresis[6] and counted in a Searle Delta 300 scientillation counter. GSH was assayed as described by Ball[7] and proteins were determined by the Lowry method[8]. Enzymatic activity for all methods is expressed as μmoles of product formed (or γ-glutamyl donor utilized) per min at 37 °C (units); specific activity is expressed as units per mg of protein.

The enzyme was purified from lactating mammary gland using the method already described for the kidney enzyme[9]. For the kinetic studies the double reciprocal plot of