Active and Passive Cation Transport and L Antigen Heterogeneity in Low Potassium Sheep Red Cells

Evidence against the Concept of Leak-Pump Interconversion

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ABSTRACT Several lines of experimental evidence are presented suggesting that the L antigens in low potassium (LK) sheep red cells are associated with separate Na⁺K⁺ pump and K⁺ leak pathways. The stimulating effect of anti-Lp on K⁺ pump flux is distinct from the action of anti-Lt on K⁺ leak flux, implying that K⁺ leak transport sites may not be converted into active pumps by the L antiserum. Treatment of LK red cells with trypsin completely abolished both the stimulation of K⁺ pump flux and the enhancement of the rate of ouabain binding brought about by anti-L. That this effect is due to a total destruction of the Lp determinant associated with the LK pump was evident from the complete failure of anti-Lp to bind to trypsinized LK red cells. The Lp antigen can be effectively protected against the trypsin attack by prior incubation with anti-L, indicating that the sites for antibody binding and trypsin action may be closely adjacent at the structural level. Trypsin treatment, however, did not interfere with anti-Lq reducing ouabain insensitive K⁺ leak influx, nor did it prevent binding of anti-Lq, the hemolytically active L antibody which is probably identical with anti-Lp. The functional independence of the Lp and Lq sites was documented by the observation that anti-Lq still reduced K⁺ leak influx in LK cells with experimentally induced high potassium concentrations, at which K⁺ pump flux is fully suppressed, whether or not anti-Lp was binding to the Lp antigen associated with the LK pump.

INTRODUCTION

It is now well established that active Na⁺K⁺ transport (pump flux) in low-potassium (LK) sheep red cells (Kerr, 1937; Evans, 1954; Tosteson and Hoffman, 1960) is stimulated several-fold when these cells are exposed to the L antiserum, which recognizes specific membrane surface antigens (Ellory and Tucker, 1969; Rasmusen, 1969; Lauf et al., 1970). The activation of Na⁺K⁺ pump flux involves kinetic changes rather than an increase in the number of pumps per cell (Joiner and Lauf, 1975, 1977). The kinetic changes consist mainly of an increase in the affinity of the cytoplasmic aspect of the Na⁺K⁺ pump for
Na\(^+\) ions relative to K\(^+\) ions, i.e., a reduction of the potent inhibitory action of intracellular K\(^+\) ions at the Na\(^+\) loading site of the pump (Lauf et al., 1970; Glynn and Ellory, 1972). The kinetic alteration of the pump by anti-L is also manifested by an increased rate of ouabain binding, signifying an enhanced turnover of cations per pump (Joiner and Lauf, 1975, 1977). A similar qualitative alteration of Na\(^+\)K\(^+\) transport has been reported for LK goat red cells (Sachs et al., 1974a, b). The cation transport and immunological properties of these ungulate red cells have been recently reviewed (Lauf, 1975).

The number of L antigens per cell is at least an order of magnitude greater than that of the Na\(^+\)K\(^+\) pumps (Lauf and Sun, 1976; Tucker et al., 1976; Joiner and Lauf, 1975). Treatment of LK cells with trypsin permitted the distinction of two different L antigenic determinants: the trypsin-labile L\(_p\) antigen through which anti-L stimulates the pump, and the trypsin-resistant L\(_v\) antigens which are involved in the L\(_y\) antibody-mediated complement hemolysis of these cells (Lauf et al., 1971). The L\(_p\) antibodies can be separated from anti-L\(_y\) by adsorption to and elution from LK goat red cells (Ellory and Tucker, 1970; Kropp and Sachs, 1974) or by their failure to bind to newborn red cells of phenotypically LK lambs (Tucker et al., 1976). A recent report proposed that anti-L\(_y\) is identical with a third antibody activity (anti-L\(_v\)) found in L antisera which reduces K\(^+\) leak transport by about one-half (Dunham, 1976a). On kinetic grounds, it was proposed that part of the activation of K\(^+\) pump flux in LK cells is due to a conversion by anti-L of K\(^+\) leak transport sites into pumps (Dunham, 1976a, b). This would be consistent with the hypothesis that both pumps and leak sites may be controlled by a single gene locus, which is an attractive explanation for the genetic cation transport polymorphism in sheep, goat, and cattle red cells (Dunham and Hoffman, 1971).

The present study confirms the finding of Dunham (1976a, b) that anti-L\(_y\) reduces K\(^+\) leak transport in LK sheep red cells and lends support to the concept that anti-L\(_y\) and anti-L\(_v\) may be indeed identical antibodies. However, experiments involving treatment of LK cells with trypsin resulted in a clear distinction of two independent effects of the L antiserum on K\(^+\) pump and leak fluxes in LK red cells. Trypsin destroyed the L\(_p\) antigen associated with the LK pump, but not the L\(_v\) antigen related to the K\(^+\) leak pathway. When K\(^+\) pump flux and the effect of anti-L\(_y\) were completely suppressed by preparing high-potassium LK cells, anti-L\(_y\) still affected fully K\(^+\) leak transport. These and other data make it unlikely that anti-L converts inactive pumps (K\(^+\) leak sites) into active ones but rather suggest that pump and leak transport are carried out by different membrane proteins, controlled in a genetically complex fashion. A preliminary report of these data was presented elsewhere (Lauf et al., 1977).

**MATERIALS AND METHODS**

**Sheep and Goat Red Cells**

Blood was drawn from healthy Dorset sheep and Nubian goats by jugular venipuncture, with heparin as anticoagulant, and was used on the day obtained. The cation types are based on measurements of intracellular sodium [Na\(^+\)]\(_c\) and potassium [K\(^+\)]\(_c\) concentrations using an atomic absorption spectrophotometer (model 460, Perkin Elmer Corp.,
Norwalk, Conn.). The presence of the M and L antigens in heterozygous and homozygous LK sheep red cells was determined by immune hemolytic assays with anti-M and anti-L sera or their purified antibodies and guinea pig serum as complement source, as previously described (Lauf and Tosteson, 1969; Lauf and Dessen, 1973b). The red cells were washed by centrifugation in an isotonic solution containing 150 mM NaCl and 10 mM Tris-Cl, pH 7.6, and subsequently suspended in the various incubation media indicated below.

**Incubation Media, Reagents, and Antibodies**

The general composition of our incubation media was (mM): 150 NaCl; 10 sucrose; 10 glucose; 10 Tris-Cl, pH 7.5. In the isotopic flux studies KCl was added in quantities to yield a final extracellular K⁺ concentration [K⁺]₀ of 5-7 mM when ¹⁴KCl was introduced at the beginning of the flux experiments. The proportion of cold to labeled KCl varied according to the specific activity of ²⁴K available as the experiment was started. ²⁴K (sp act ~14.5 Ci/mol K₂CO₃) was obtained from the Burlington Nuclear Research Facilities, North Carolina State University, Raleigh, N.C., and converted into the chloride form by titration with 0.07 N HCl. In the experiments involving trypsinization of LK sheep red cells, the standard medium contained in addition 5 mM KCl and 2 mM CaCl₂.

[³H]Ouabain, in benzene-ethanol (1:9), was obtained from New England Nuclear, Boston, Mass.). The specific activity given by the manufacturer (about 13 Ci/mmol) was carefully checked by thin-layer chromatography and a biological assay, employing dilution with nonradioactive ouabain and saturation binding to red cells as described elsewhere (Lauf and Joiner, 1976; Joiner, 1977; Joiner and Lauf, 1977). The specific activity of the [³H]ouabain used in this study was estimated to be 17 Ci/mmol.

Pilot experiments using trypsin preparations from various commercial sources led us to the conclusion that only the Worthington enzyme (lot TRL 36C876, 194 U/mg, Worthington Biochemical Corp., Freehold, N.J.) released maximum amounts of sialic acid containing peptides from the surface of LK sheep red cells. We therefore decided to use only this enzyme in all the experiments reported in the present study. Ovomucoid (Worthington, lot 01-36E690) served as trypsin inhibitor. All other reagents used were of analytical grade.

Six different L antisera produced by injection of LK sheep red cells into adult HK sheep were obtained from Dr. B. A. Rasmusen, Laboratory of Animal Genetics, University of Illinois, Urbana, Ill. All sera were heated for 30 min at 56°C to inactivate complement. One of the antisera (denoted S37) was made L antigen specific by previous absorption with a panel of L antigen-negative sheep red cells. The six antisera were tested for their effect on cation transport in LK sheep red cells, and then subjected to precipitation by 33% ammonium sulfate to remove most of the nonimmunoglobulin proteins (Snyder et al., 1971). The purified immunoglobulins of each serum, containing the active L antibodies, were dialyzed against standard medium before use and their protein concentrations adjusted to approximately those found in the native sera as based on protein determination by the method of Lowry et al. (1951). After the dose response curve for each antibody preparation was established by means of K⁺ influx experiments, further experiments were carried out with a dilution of anti-L immunoglobulin (IgG) still capable of yielding maximum effects on K⁺ influxes. Among the six antibodies tested, the S44 reagent proved to be one of the most powerful reagents and was therefore used for all experiments reported here. The S37 L antibody was used as hemolytic reagent because it mainly contained the hemolytically active L antibody and only little anti-L affecting K⁺ pump influx. Guinea pig serum absorbed with HK and LK sheep red cells (to prevent nonspecific lysis by anti-T, Lauf, 1975) served as complement source.
Pretreatment of Sheep Red Cells

Trypsin treatment of HK and LK sheep red cells was carried out in a manner similar to that of our earlier report (Lauf et al., 1971) with some minor modifications. For example, the concentration of CaCl₂ was reduced to 2 mM, and it was found useful to incorporate 10 mM sucrose in the incubation medium to minimize hemolysis. Furthermore, 10 mM glucose was always present during the proteolytic digestion period to maintain intracellular adenosine triphosphate levels close to the control values of about 1 μM/ml packed cells as measured by the method of Lowry et al. (1964).

In the experiments designed to study the time dependency of the protection of the L antigen by anti-L against the action of trypsin, 5 ml of a 20% (vol/vol) suspension of LK cells was exposed to 5 ml S44 IgG anti-L (ca. 5 mg/ml final suspension) and incubated for 30 min at 37°C. Then 0.5 ml trypsin solution (1.5 mg/ml final suspension) was added followed by additional incubation at 37°C. 1-ml samples were removed at given time intervals and transferred into test tubes containing 3 mg ovomucoid/0.1 ml in order to inhibit the hydrolytic activity of trypsin. All samples were kept on ice until the start of the K⁺ influx experiment. Controls included cells treated with anti-L, and with no addition of antibody or enzyme.

The cation composition of LK sheep red cells was altered with Nystatin (Mycostatin 30 μg/ml cell suspension, E. R. Squibb & Sons, New York) by the method of Cass and Dalmark (1973). Minor modifications included the use of 50 mM MgSO₄ instead of sucrose in the cation-variable media to minimize cell volume changes (Joiner, 1977).

Separation of Anti-Lp and Anti-Lt with LK Goat Red Cells

10 ml of packed LK red cells ([K⁺],e = 22 mM) from a Nubian goat were mixed with 10 ml S44 anti-L IgG (10 mg/ml) and incubated for 30 min at room temperature. The suspension was centrifuged at 15,000 rpm (Sorvall RC5 Superspeed centrifuge, DuPont Instruments, Sorvall Operations, Newtown, Conn.) for 5 min. The supernate was carefully removed, a 2-ml sample kept aside, and the remainder absorbed again with an equal volume of packed LK red cells. This procedure was repeated twice more. The three separate portions and the final supernate (4 ml) as well as an unabsorbed anti-L IgG solution (control) were dialyzed against 4 mM K⁺ medium and kept frozen at −90°C until used in the flux experiment. The packed LK goat red cells left in the centrifuge tubes after each of the four absorptions were pooled (total volume 28 ml packed cells equivalent to about 1.68 × 10⁹ goat red cells) and washed in Tris-buffered saline pH 7.5 (TBS) to remove unbound L antibody. The washed cells were then hemolyzed in 10 mM Tris-Cl buffer pH 7.6 and the ghosts obtained were washed in the same solution until the membrane suspension exhibited a white appearance. This suspension was adjusted to about 1 mg membrane protein/ml and the bound L antibodies were eluted by using a butanol extraction technique described earlier (Snyder et al., 1971). In brief, this technique partitions membrane phospholipid and cholesterol into the organic phase while the bulk of the membrane protein as well as the L antibody remain solubilized in the aqueous layer. Since n-butanol destroys the L antigen activity (Lauf, 1974), the L antibody dissociates from the antigenic determinant and will again bind to intact LK red cells. The aqueous phase was lyophilized, resuspended in a small volume of 4 mM K⁺ medium, and dialyzed vs. the same medium overnight. The dialyzed proteinaceous suspension was centrifuged at 20,000 rpm, and the clear supernate carefully removed. The remaining protein pellet was washed again in the same medium and the final supernate pooled with the first one. On the basis of the known volume of eluted L antibody and on the assumption that most of the bound L antibody was recovered, the concentration of the eluted Lp antibody could be approximately related to that present in the unabsorbed L
antibody preparation. In addition, the protein concentration of the final extract was determined by the Lowry method. The total protein recovered amounted to 53 mg which includes the L antibody protein as well as some solubilized membrane protein. It should be pointed out that solubilization of lyophilized membrane protein obtained by n-butanol-water extraction (Maddy, 1964) yields about 1-2% of the total membrane protein in soluble form which constitutes mostly the hydrophilic membrane glycoproteins (Poulik and Lauf, 1965). See also Results.

Isotopic Flux Measurements, [3H]Ouabain Binding, and Hemolytic Assay

Unidirectional K⁺ influx was measured by ⁴²K uptake as previously reported by this laboratory (Lauf et al., 1970, 1971; Lauf and Sun, 1976; Lauf and Joiner, 1976) using the dibutyl-phthalate technique (Kepner and Tosteson, 1972; Joiner and Lauf, 1975; Lauf and Joiner, 1976). The total uninhibited K⁺-influx (M̄K) was calculated from Eq. (1) without correction for the small back flux:

\[ iM_K = \frac{c_{42K}}{c_{42K_0}} \times \frac{\left[K^+\right]_o}{t} \]

where \( c_{42K} \) and \( c_{42K_0} \) denote the counts per minute per volume cells or medium, \([K^+]_o\) the concentration of K⁺ in the medium, and \( t \) the 60-min time interval over which \( 42K \) uptake was measured. The active or K⁺ pump influx (\( IM_K \)) is given by Eq. (2):

\[ IM_K = iM_K - iM_K^{\text{leak}} \]

where \( iM_K \) defines the ouabain-insensitive K⁺ leak influx. In order to compare the effect of anti-L on the K⁺ leak flux it was found to be useful to convert \( iM_K \) into the leak rate coefficient (\( k_k \)) defined by Eq. (3):

\[ k_k = \frac{iM_K}{[K^+]_o} \]

This conversion corrects for the small changes (~1-4%) in \([K^+]_o\), observed between the samples at the end of the flux experiment, which is due to the usual variability occurring when \( 42K \) is introduced into the test tubes.

Binding of [³H]ouabain was performed essentially as described by Joiner and Lauf (1975). In brief, prewarmed [³H]ouabain was added at time zero to a 20% (vol/vol) cell suspension pre-equilibrated in K⁺-free medium at 37°C, from which at desired time intervals 0.5-ml samples were withdrawn and transferred into ice-cold 103 mM MgCl₂ (pH 7-8) layered over 2 ml dibutyl-phthalate. Cells were partitioned from the suspending medium by centrifugation at 15,000 rpm and 0°C through the organic layer. The pelleted cells were hemolyzed in 8 ml ice-cold 10 mM Tris-Cl, pH 7.5, the ghosts pelleted and repeatedly washed by centrifugation until essentially hemoglobin free. The supernate obtained after the very first centrifugation was analyzed spectrophotometrically at 527 nm for total hemoglobin content from which the number and volume of cells counted could be computed on the basis of the mean corpuscular hemoglobin content. It was reported earlier that this procedure did not result in a loss of labeled ouabain because of the extraordinary low dissociation rate of ouabain from its receptor (Joiner and Lauf, 1975; Joiner, 1977). The membranes were solubilized in 0.1 N NaOH, then neutralized with HCl and counted in a Beckman Liquid Scintillation Counter (model L 250, Beckman Instruments, Fullerton, Calif.). The counting efficiency (\( E \)) was about 27%. The number of ouabain molecules bound per cell (\( B \)) was computed according to Eq. (4) (Joiner and Lauf, 1975):

\[ B = \frac{cpm \times (100/E) \times N}{C \times V \times S \times Ci} \]
where cpm represents counts per min corrected for background; \( N \), Avogadro's number; \( C \), the number of cells/ml (3.3 \( \times 10^{10} \)); \( V \) the volume of cells/ml per sample; \( S \), the specific activity of \([3H]\)ouabain in Ci/mol and Ci the Curie number (2.22 \( \times 10^{12} \) dpm/Ci).

To test for the presence of L\(_p\) antigenic determinants in trypsinized and control LK cells, the specifically prepared anti-L IgG of serum S37 was absorbed with these cells at 37°C for 60 min and at 0°C for 10 min. After centrifugation at 15,000 rpm and 4°C for 5 min, the supernates were carefully removed and tested for residual hemolytic L\(_p\) antibody activity in the presence of complement, by use of ATP-depleted LL (LK) cells as described by Lauf and Dessent (1973b). The purpose of ATP depletion is to augment the susceptibility of the sluggish anti-L-mediated immune hemolysis by complement and thus increase the resolution of the technique.

**RESULTS**

**Effect of Anti-L on \( K^+ \) Pump and Leak Fluxes**

Table I shows the effect of six different L antisera on \( K^+ \) pump and leak fluxes in LL (LK) sheep red cells (sheep no. 44N). Five of the six antisera stimulated \( \mu M_k \) more than sixfold which is consistent with previous reports (Ellory and Tucker, 1969; Lauf et al., 1970; Lauf, 1974). One antiserum (S37) increased \( \mu M_k \) by only twofold, which may be due in part to dilution, since this antiserum was previously absorbed with a panel of HK cells to remove all L-nonspecific antibodies. After this absorption step, the S37 antiserum turned out to be a better reagent for detection of the L antigen by immune hemolysis than by \( K^+ \) pump stimulation. It has been reported that during prolonged immunization of HK sheep with LK cells the hemolytically active antibody (anti-L\(_p\)) gradually increased while anti-L\(_p\), the antibody affecting \( \mu M_k \), decreased in potency (Ellory and Tucker, 1970).

All antisera also reduced the ouabain-insensitive \( K^+ \) leak influx as evident in 30–50% lower \( k_k \) values. This result contrasts with our earlier finding (Lauf et al., 1971; Lauf, 1974) and supports the observation of Dunham (1976a, b) that L antisera exert two effects on the cation transport system in LK sheep red cells: one activating the \( Na^+K^+ \) pump (anti-L\(_p\)), and the other reducing \( K^+ \) leak influx.

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**Table I**

| Serum   | \( \mu M_k \) | Ratio anti-L: control | \( k_k \) | Ratio anti-L: control |
|---------|---------------|------------------------|----------|------------------------|
| None    | 0.19          | --                     | 0.027    | --                     |
| Anti-L S33 | 1.21        | 6.4                    | 0.019    | 0.70                   |
| Anti-L S37 | 0.42        | 2.2                    | 0.017    | 0.63                   |
| Anti-L S41 | 1.17        | 6.2                    | 0.014    | 0.52                   |
| Anti-L S42 | 1.45        | 7.5                    | 0.015    | 0.56                   |
| Anti-L S44 | 1.15        | 6.1                    | 0.015    | 0.56                   |
| Anti-L S45 | 1.20        | 6.3                    | 0.015    | 0.56                   |

Exp. 451. Test cells: LL (LK) 44 N. \([K^+]_o = 7.0 \text{ mM}\).
(anti-L4). The relationship of anti-L4y to anti-Lp and anti-L4 will be discussed further below.

For subsequent flux experiments we used mainly the S44 L antiseraum, which showed the best dose response with respect to \( \frac{M_r}{U} \) and \( \frac{K}{K_k} \), and of which we had ample supply. The anti-L-containing IgG fraction was obtained by ammonium sulfate precipitation (Snyder et al., 1971) and the concentration of IgG reconstituted to 30 mg/ml equivalent to about two to three times the immunoglobulin concentration of the native serum. Table II shows the effect of S44 anti-L IgG on \( \frac{M_r}{U} \) and \( \frac{K}{K_k} \) in 5 LL and 3 LM (LK) sheep red cells. It can be seen that the L antibody stimulated \( \frac{M_r}{U} \) four- to fivefold in four out of five LL red cells, while a sixfold stimulation was obtained in red cells of sheep 44N. It should be pointed out that the red cells of this particular sheep had 90 ouabain binding sites (\( Na^+K^+ \) pumps) as opposed to 46–60/cell in all the other animals, which in part explains the higher \( \frac{M_r}{U} \) value in the presence of anti-L. In general, stimulation of \( \frac{M_r}{U} \) was lower in the LM cells tested consistent with earlier reports (Lauf and Dessent, 1973a; Tucker et al., 1976). The effect of anti-L on \( \frac{K}{K_k} \) varied between 15% and 46% depending on the red cells tested but independently of their LK genotypes.

Fig. 1 illustrates the response of \( \frac{M_r}{U} \) and \( \frac{K}{K_k} \) to increasing concentrations of anti-L IgG. There was a steep rise in the activation of \( \frac{M_r}{U} \) as the IgG concentration was increased from 1 to 2.5 mg/ml suspension. Maximum stimulation of \( \frac{M_r}{U} \) was 6.1-fold at concentrations above 5 mg IgG/ml, similar in magnitude to the results of Tables I and II for the same sheep (44N). The value for \( \frac{K}{K_k} \) was 0.017 × h\(^{-1}\) in the absence of anti-L and decreased by 35% at 5 mg anti-L IgG/ml. In general, all \( \frac{K}{K_k} \) values of this experiment are somewhat lower than those of Table I and II for the same cell but such small variations between experiments are typical.

### Table II

| Sheep | Antigens | \( [K^+]_o \) | \( \frac{M_r}{U} \) | \( \frac{K}{K_k} \) | \( \frac{M_r}{U} \) | \( \frac{K}{K_k} \) |
|-------|---------|---------------|----------------|----------------|----------------|----------------|
| 44N   | LL      | 21.8          | 0.23           | 0.020          | 1.43           | 0.017          | 6.2           | 0.85 |
| 67N   | LL      | 14.7          | 0.24           | 0.029          | 1.03           | 0.020          | 4.3           | 0.69 |
| 71N   | LL      | 13.0          | 0.25           | 0.041          | 1.30           | 0.024          | 5.2           | 0.59 |
| 98N   | LL      | 15.0          | 0.28           | 0.037          | 1.17           | 0.020          | 4.2           | 0.54 |
| 62N   | LL      | 17.5          | 0.14           | 0.041          | 0.61           | 0.024          | 4.4           | 0.59 |
| 58N   | LM      | 13.1          | 0.14           | 0.053          | 0.61           | 0.029          | 4.4           | 0.55 |
| 65N   | LM      | 15.2          | 0.16           | 0.099          | 0.39           | 0.059          | 2.4           | 0.63 |
| 66N   | LM      | 16.4          | 0.15           | 0.047          | 0.43           | 0.026          | 2.8           | 0.55 |

Exp. 463 and 483. Anti-L IgG: 5 mg/ml 10% suspension of cells \( [K^+]_o = 5-6 \text{ mM} \).
Effect of Trypsin on the L Antisera Activities Modifying Cation Transport

Fig. 2 shows an experiment in which LL (LK) cells of sheep 44N were incubated for 60 min at 37°C, suspended in solutions of increasing trypsin concentrations. After this treatment the cells were exposed to anti-L IgG. As the trypsin concentration was increased to 5 mg/ml cell suspension, the effect of anti-L on $i_M^+$ was virtually abolished, a finding at variance with Dunham's observation (1976a) that after trypsin treatment there remains a residual twofold stimulation of $i_M^+$ by anti-L. However, our findings that trypsin did not alter the effect of

\[ i_M^+ \]

anti-L on $i_k^+$ are consistent with Dunham's data. At all trypsin concentrations employed there was a 40% reduction of $i_k^+$ by anti-L. There is no obvious explanation for the shape of the curve relating $i_k^+$ to the trypsin concentrations.

With LL (LK) sheep red cells of animal 62N, the influence of preincubation time in trypsin (2.5 mg/ml) on the two activities of the L antiseraum was studied. Samples of LK cells exposed to trypsin were taken at given time intervals and the action of the enzyme was inhibited by addition of the trypsin inhibitor ovomucoid (twofold molar excess). The cells were washed in standard medium and then incubated with anti-L (5 mg/ml suspension) and $K^+$ influx measured as

\[ i_k^+ \]

The effect of anti-L IgG on the 44 N cells is only 4.2-fold, see Table I. In this experiment the $K^+$ leak flux was also found to be lower.

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**Table I**

| Anti-L IgG (mg/ml Cell Suspension) | $i_M^+$ (mmol K+/L Cells h) | $i_k^+$ (mmol K+/L Cells h) |
|-----------------------------------|-----------------------------|-----------------------------|
| 0                                 | 1.5                         | 0.025                       |
| 5                                 | 0.5                         | 0.015                       |
| 10                                | 0.01                        |                             |

**Figure 1.** The effect of various L antibody concentrations on K$^+$ pump and leak fluxes in LL (LK) sheep red cells. Left-hand ordinate, ouabain-sensitive K$^+$ pump influx ($i_M^+$) which was measured at 6-7 mM [K$^+$]. Right-hand ordinate, the ouabain-insensitive K$^+$ leak influx was divided by [K$^+$] measured at the end of the flux experiment to yield the leak rate coefficients ($i_k^+$). S44 denotes the anti-L batch used throughout these experiments.
described. Fig. 3 shows that by 30 min most of the effect of anti-L on \( ^{14}M_\text{K} \) was abolished and that by 60 min virtually no difference was seen between the \( ^{14}M_\text{K} \) values of cells treated with trypsin and anti-L and those of control cells (Fig. 3 inset). Again these findings are at variance with those of Dunham (1976a) and clearly demonstrate the complete inactivation by trypsin of the anti-L effect on the pump. However, independent of the time of exposure to trypsin, the effect of anti-L on \( ^{14}k \) was unchanged (about 20%, Fig. 3 inset), confirming Dunham’s finding (1976a, b) that the interaction of anti-L with the leak transport sites is not sensitive to trypsin.

![Graph](image)

**Figure 2.** Alteration by increasing trypsin concentrations of the anti-L effect on \( K^+ \) pump influx in LL (LK) sheep red cells. After pretreatment with the indicated trypsin concentrations for 60 min at 37°C, the cells were washed and exposed to anti-L (5 mg IgG anti-L/ml 10% cell suspension) for another 30 min at 37°C before addition of \( ^{42}K \). *Left-hand ordinate,* ouabain-sensitive \( K^+ \) pump influx \( (^{14}M_\text{K}) \) measured at 4 mM [\( K^+ \)]. *Right-hand ordinate,* the ouabain-insensitive \( K^+ \) leak influx was divided by \( [K^+]_o \) measured at the end of the flux experiment to yield the leak rate coefficients \( (^{14}k) \). Solid lines, preincubation with anti-L. Broken lines, controls in 1% bovine serum albumin.

From the data in Figs. 2 and 3 it is apparent that the \( L_p \) antigen was the principal target for the action of trypsin since L antibodies stimulating \( ^{14}M_\text{K} \) (anti-\( L_p \)) did not show an effect in trypsinized LK red cells. This conclusion is further supported by the \(^{3}H\)ouabain binding experiment presented in Fig. 4. Trypsinized and untreated LL (LK) red cells were preincubated with 5 mg anti-L IgG/ml 20% cell suspension for 30 min at 37°C. Then \(^{3}H\)ouabain \((10^{-7} \text{ M final concentration})\) was added and samples were taken at various time intervals up to 4 h to insure saturation binding. Fig. 4A shows the typical effect of anti-L on the kinetics of ouabain binding to untreated LK cells: as reported earlier (Joiner and Lauf, 1975) the rate of ouabain uptake during the 1st h of the experiment was accelerated in the presence of anti-L, but the maximum number of ouabain molecules bound per cell is indistinguishable from that of control cells. This
finding is consistent with our hypothesis that the apparent affinity of the \( \text{Na}^+\text{K}^+ \) pump complex for ouabain is altered, which is related to an increase in the turnover number caused by anti-L (Joiner and Lauf, 1977). Trypsin treatment completely prevented the effect of anti-L on the rate of ouabain binding (Fig. 4B) which was found to be identical with that of control cells. Thus, since trypsin did not affect the rate of ouabain binding typical for LK red cells in the absence of anti-L, the enzyme probably did not alter the apparent affinity of the unstimulated pump for the cardiac glycoside.

**Figure 3.** Time course of trypsin effect on anti-L-induced \( \text{K}^+ \) pump flux activation in LL (LK) sheep red cells. Cells were pre-exposed to trypsin (2.5 mg/ml 42% cell suspension at 37°C). At the time intervals indicated, 0.25-ml samples of the suspension were removed, squirted into tubes containing 0.25 ml of a 0.5% ovomucoid solution in \( \text{K}^+ \) medium, washed, and further incubated with 5 mg anti-L IgG/ml 10% cell suspension for another 30 min at 37°C before addition of \( ^{42} \text{K} \). *Left-hand ordinate*, ouabain-sensitive \( \text{K}^+ \) pump influx (\( i_{\text{MP}} \)) measured at 4.3 mM [\( \text{K}^+ \)]\(_o\). *Right-hand ordinate*, the ouabain-insensitive \( \text{K}^+ \) leak influx was divided by [\( \text{K}^+ \)]\(_o\) measured at the end of the flux experiment to yield the leak rate coefficients (\( i_{\text{MP}} \)). Inset, \( i_{\text{MP}} \) and \( i_{\text{K}} \) for untreated cells exposed only to anti-L or 1% bovine serum albumin (control).

**Specificity of the Trypsin Effect**

An important question concerns the specificity of the trypsin action on the membrane level. It was already reported earlier that trypsinization of anti-L itself did not affect its action on \( i_{\text{MP}} \) (Lauf et al., 1971). However, these early experiments did not rule out that trypsin, once absorbed to a membrane area in the vicinity of the L antigen, might be capable of specifically hydrolyzing the antibody-combining site as the L antibody combines with the L antigen. To exclude this possibility, LK and HK red cells were pretreated with trypsin (LK\(_T\) and HK\(_T\) cells), washed, and then exposed to anti-L for 90 min at 37°C (time required for pre-exposure to anti-L and flux experiment). Subsequently, the cells were removed by centrifugation and the supernates tested at various
dilutions for the residual effect of anti-L on $M_{K}^{M}$ and $M_{K}^{s}$ in fresh, untreated LL (LK) cells of animal 44N. Fig. 5 reveals that, as compared to the effects of the unabsorbed control anti-L, there was no difference in the response of $M_{K}^{M}$ and $M_{K}^{s}$ to the two effects of the L antiserum, previously exposed to LKT and HKT cells. This experiment involved exposure of anti-L to only $2-3 \times 10^{9}$ cells. No significant removal of the antibody by this number of cells should be expected (provided the L antigen remained intact after trypsinization of LK cells), since the number of $L_{b}$ antigens per LK cell is very small (Lauf and Sun, 1976; Tucker et al., 1976). Therefore, it is unlikely that proteolytic degradation of the L antibody-combining sites occurred.

Further evidence for the specificity of the action of trypsin is presented in Fig. 6. It is known that the proteolytic activity of the enzyme can be prevented by about equimolar concentrations of the trypsin inhibitor ovomucoid (Kassell, 1970). Thus, we incubated LL (LK) red cells with constant amounts of trypsin (2.5 mg/ml final suspension) but increasing amounts of ovomucoid (lower abscissa) for 60 min at 37°C, after which the cells were washed and exposed to anti-L (5 mg/ml) before addition of $^{42}$K and ouabain. As long as the molar ratio of

![Figure 4](image-url)
ovomucoid:trypsin was kept above unity (upper abscissa), full protection against the action of trypsin was maintained. Upon reducing the molar ratio of inhibitor:enzyme to below unity, the action of the enzyme on the $L_p$ antigen became apparent: at a molar ratio of 0.25 virtually no protection by the inhibitor was observed. Note that also in this experiment anti-L reduced $i_{MP}$ by 50%, independent of the pretreatment conditions.

Finally, additional strong evidence for a direct proteolytic action of trypsin on the $L_p$ antigen associated with the Na$^+$K$^+$ pump mechanism stems from our earlier observation that anti-L, once bound to the LK membrane surface,

\[
\begin{array}{c}
1.4 \\
1.2 \\
1.0 \\
0.8 \\
0.6 \\
0.4 \\
0.2 \\
0.0 \\
\end{array}
\]

**Figure 5.** Recovery of anti-L activity after exposure to trypsin-treated LK$_T$ and HK$_T$ sheep red cells. Washed, trypsinized LK$_T$ or HK$_T$ sheep red cells were suspended in K$^+$ medium containing anti-L IgG to give a final concentration of 8 mg antibody protein/ml 10% cell suspension. After 90 min of incubation at 37°C the cells were spun down and samples of the supernates (abscissa) were added to fresh LK red cells to test the effect of residual antibody activity on K$^+$ transport (see Fig. 1). *Left-hand ordinate,* ouabain-sensitive K$^+$ pump influx ($i_{MP}$) measured at about 5.0 mM [K$^+$]$_o$. *Right-hand ordinate,* K$^+$ leak rate coefficient ($i_{KL}$), obtained by dividing K$^+$ leak flux by [K$^+$]$_o$.

prevents the attack of the enzyme (Lauf et al., 1971). However, the flux activation by the protecting anti-L was only small in these earlier experiments. It is possible that anti-L slowly dissociated during the second exposure to the enzyme, or that other transient changes in the membrane permitted a gradual time-dependent attack by the enzyme (i.e. that the $L_p$ antigen is not fully protected by the L antibody). Fig. 7 shows that anti-L fully protects the $L_p$ antigen against the proteolytic action of trypsin in a time-independent manner. A fivefold stimulation of $i_{MP}$ (compare with *inset* of Fig. 7) was found which was stable up to 45 min after addition of trypsin to anti-L pre-exposed cells. The 22% higher $i_{MP}$ value found in anti-L-treated control LK cells (*inset*) not treated with
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**Figure 6.** Inactivation by ovomucoid of the trypsin effect on the anti-L-mediated K⁺ pump stimulation. LK (LL) sheep red cells were treated for 1 h at 37°C with 2.5 mg trypsin/ml 20% cell suspension in the presence of increasing concentrations of ovomucoid (lower abscissa). After washing in K⁺ medium, the cells were incubated in K⁺ medium with 5 mg anti-L IgG/ml (final concentration) for 30 min at 37°C before addition of ⁴²K. *Left-hand ordinate,* ouabain-sensitive K⁺ pump influx (\( i_{\text{MP}} \)) measured at 6 mM [K⁺]₀. *Right-hand ordinate,* K⁺ leak rate coefficients (\( k_{\text{L}} \)) obtained by dividing K⁺ leak influx by [K⁺]₀. *Upper abscissa,* molar ratio of ovomucoid/trypsin during pretreatment. *Inset,* \( i_{\text{MP}} \) and \( k_{\text{L}} \) for untreated cells exposed only to anti-L or 1% bovine serum albumin (control).

**Figure 7.** Protection of the L⁺ antigen by anti-L against trypsin attack as a function of exposure time to trypsin. The LK (LL) sheep red cells were pretreated with 5 mg anti-L IgG/ml 10% cell suspension for 30 min at 37°C. Trypsin (15 mg/1.0 ml) was added at time 0 and samples were taken at various time intervals and transferred into test tubes containing 3.0 mg ovomucoid in 0.1 ml medium to halt the hydrolytic activity of trypsin. *Left-hand ordinate,* ouabain-sensitive K⁺ pump influx (\( i_{\text{MP}} \)) measured at 6-7 mM [K⁺]₀. *Right-hand ordinate,* K⁺ leak rate coefficients (\( k_{\text{L}} \)) obtained by dividing K⁺ leak influx by [K⁺]₀. *Inset,* transport parameters for untreated control cells and unprotected control cells (trypsinized cells) in the absence and presence of anti-L.
trypsin can be explained in terms of a longer incubation time of these cells with anti-L (experimental data not shown).

Evidence for the Independence of \( L_p \) and \( L_L \) Sites and the Relationship to the \( L_{ty} \) Antigen

The experiments shown in the previous section suggested that there might be two separate antibodies present in the L antiserum which alter both K\(^+\) pump (anti-\( L_p \)) and leak fluxes (anti-\( L_L \)) in LK cells, supporting similar observations made by Dunham (1976a, b). On the membrane level, these observations would indicate antigenic site heterogeneity, for which we now present further evidence. In addition, the interrelationship with the \( L_{ty} \) antigen will be further illuminated.

Previous work from this laboratory suggested that trypsinized cells still removed anti-L from the serum though to a lesser extent than control cells (Lauf et al., 1971). We have re-examined the capacity of trypsinized LK red cells to bind anti-\( L_p \) by employing an \( L_p \) antibody preparation essentially devoid of the anti-\( L_L \) component. The separation of anti-\( L_p \) from anti-\( L_L \) was achieved by absorbing anti-\( L_p \) to and eluting from LK goat red cells (Ellory and Tucker, 1970; Dunham, 1976a) which apparently do not bind anti-\( L_L \). Figure 8B shows that the \( L \) antibody eluted from four adsorptions to LK goat red cells (and then pooled) activated K\(^+\) pump flux in LK sheep red cells almost identically to the unabsorbed control anti-L, while there was virtually no stimulation of \( \frac{1}{k_k} \) by the anti-L IgG remaining in the supernates. It should be noted that only one absorption with LK goat red cells sufficed to remove all anti-\( L_p \) from the anti-L preparation. When the eluted anti-\( L_p \) was tested for its effect on \( \frac{1}{k_k} \), a 25% reduction of this parameter was seen only when very high concentrations of the eluted anti-\( L_p \) component were employed (Fig. 8A). However, the anti-L IgG fraction not binding to LK goat cells and present in the supernates of the four absorption steps clearly reduced \( \frac{1}{k_k} \) at lower IgG concentrations, comparable to the reduction seen with the unabsorbed control. There was no significant decrease of the anti-L activity even after the fourth consecutive exposure to LK goat red cells.\(^2\)

Fig. 9 shows an experiment in which anti-\( L_p \), purified by elution from LK goat red cells, was absorbed by increasing numbers of trypsinized and control LK sheep red cells (abscissa) and then tested for residual K\(^+\) pump stimulatory activity. There was a progressive fall of the anti-\( L_p \) activity as the number of control cells used per absorption was increased to \( 2.1 \times 10^{10} \). The slope of the curve was similar to that reported earlier for whole anti-L serum (Lauf, 1974).

\(^2\) In this absorption experiment 53 mg protein was eluted from \( 0.6 \times 10^{12} \) LK goat red cells present during the first fully effective absorption step (see Materials and Methods). If the eluted protein constitutes only anti-\( L_p \), \( 3.1 \times 10^9 \) anti-\( L_p \) molecules must have bound per LK goat red cell. However, this number must be corrected for contamination of the eluate with 1-2% solubilized LK membrane glycoprotein derived from all absorptions since \( 1.7 \times 10^{12} \) cells were extracted. This contamination amounts to about 3-6 mg membrane protein if one assumes \( 2 \times 10^{-19} \) g membrane protein/LK goat red cell. Correcting for said contamination, LK goat red cells would still bind \( 3.0 \times 10^9 \) anti-\( L_p \) molecules/cell. With 10% contamination by membrane proteins this number would be about 10\(^6\)/cell. Although this type of analysis has its limitations, it is evident that the number of \( L_p \) sites per LK goat red cell may be higher than that reported by Kropp and Sachs (1979).
However, full anti-I₄ activity was recovered in the supernate after exposure to trypsinized LK cells, indicating that these cells did not bind the antibody. Note that absorption of anti-I₄ with either control or trypsinized LK cells did not affect ⁴ᴷ which was not different from ⁴ᴷ in control cells without anti-L (zero point on abscissa). This follows because at the concentration of anti-I₄ used (equivalent to 0.25 ml on Fig. 8 A, B) there was virtually no measurable anti-L activity present. We conclude from this experiment that the L₄ antigen was effectively destroyed or cleaved off by the trypsin treatment, which is consistent with our other experiments in this report that anti-I₄ is unable to stimulate K⁺ pump flux and the rate of ouabain binding in trypsinized LK sheep red cells.

The preceding experiments suggested that the L₄ and L₄ antigens may be considered as separate entities, possibly associated with structurally different membrane molecules. This immunological and functional dissociation strongly suggests that proteins involved in pump transport do not mediate K⁺ leak transport. One possible test for this hypothesis is to determine whether anti-I₄ still would affect ⁴ᴷ in LK cells with functionally inactive pumps which cannot be

![Image of Figure 8](https://example.com/image.png)
stimulated by anti-Lα. Table III reveals an experiment in which [K+]c was raised to 165 mM and [Na+]c kept at 10 mM by the nystatin method (Cass and Dalmark, 1973), conditions under which \( iM_{K}^{p} \) in the presence and absence of anti-L is completely inhibited (Lauf et al., 1970; Hoffman and Tosteson, 1971). It can be seen that the leak flux component was reduced by anti-L to a degree similar to that in cells with 25 or 1.0 mM [K+]c, implying a functional independence of the K^+ leak pathway affected by anti-Lα and the K^+ pump affected by anti-Lp.

Earlier we reported that trypsin treatment did not prevent the interaction of the hemolytically active anti-Lα antibody with the Lα antigens on LK sheep red cells (Lauf et al., 1971). With the clear distinction of Lp and Lα antigens reported here, a question arose as to the relationship of the Lα, antigen with the two other L determinants. In light of our recent findings that enzymatic treatment of sheep red cells may uncover L-nonrelated T antigens (Lauf, 1975), against which T antibodies may be present even in purified anti-L sera or in the guinea pig serum used as complement source and thus obscure an L specific hemolysis, we felt it would be important to establish the integrity of the Lα antigen after trypsin treatment by means other than the direct hemolysis test. We chose to test the absorption of anti-L S37, which predominantly contained anti-Lα, and only weak anti-Lp activities (see Table I). Fig. 10 shows Lα, antibody-mediated immune hemolysis of LK sheep red cells after anti-Lα was absorbed with control or
trypsinized LK red cells from two donors. In these experiments, complement alone produced some 20% "nonspecific" lysis, while in the case of absorption of anti-L$_{4y}$ by untreated cells less than 20% hemolysis was observed. This phenomenon is due to some removal of complement by nonbound, aggregated immunoglobulins. Nevertheless, the data reveal that absorption with trypsinized LK cells removes most of the anti-L$_{4y}$ antibody present in unabsorbed anti-L S37 IgG. These findings then confirm our earlier report that anti-L$_{4y}$ is still capable of binding to trypsinized LK red cells, though to a slightly diminished extent.

Serum S37 also decreased $V_k$ and therefore it is impossible at present to separate, either functionally or immunologically, the anti-L$_{4y}$ and anti-L$_4$ activi-

### Table III

**EFFECT OF ANTI-L IMMUNOGLOBULIN FROM SERUM S 44 ON K$^+$ PUMP AND LEAK FLUXES IN LL (LK) SHEEP RED BLOOD CELLS WITH ALTERED CELLULAR CATION COMPOSITION**

| Serum   | $[\text{K}^+]_{\text{cell water}}$ | $[\text{Na}^+]_{\text{cell water}}$ | $\%_\text{M}$ | $\%_\text{A}$ |
|---------|-----------------------------------|------------------------------------|----------------|---------------|
| Control | 25.0                              | 145                                | 0.11           | 0.041         |
| Anti-L  | 25.0                              | 145                                | 0.94           | 0.025         |
| Control | 1.0                               | 165                                | 0.44           | 0.071         |
| Anti-L  | 1.5                               | 165                                | 1.10           | 0.080         |
| Control | 165.0                             | 10                                 | 0.01           | 0.054         |
| Anti-L  | 165.0                             | 10                                 | 0.08           | 0.024         |

Exp. D 122. LL (LK) 98N sheep red cells. Anti-L IgG = 5 mg/ml final cell suspension.

**Figure 10.** Test for binding of anti-L$_{4y}$ to trypsinized and control LL (LK) sheep red cells. Anti-L IgG (S37, free of L-nonspecific antibodies) was absorbed with trypsinized or control LL (LK) 62 N (same as 116, solid line) and 71 N (interrupted line) sheep red cells. Dilutions of the cell-free supernates (abscissa) were then tested for the hemolytic L antibody activity (ordinate) in the presence of complement with LL 67 N cells. Negative values on the ordinate are due to inhibition of L nonspecific hemolysis produced by complement alone (see text).
ties of this serum since both are insensitive to trypsin treatment of the target cells. Several other attempts were unsuccessful to demonstrate directly anti-Lγ activity in the purified anti-L or anti-Lp preparation from S44 serum, since contaminating L-nonspecific antibodies were present in both fractions. Furthermore, it was impossible to separate Lγ, Lp, and Luv antibodies on the basis of charge heterogeneity (Snyder et al., 1971) since we now have found that all three antibodies were present within the same, electrophoretically fast IgG1 fraction (data not shown, see also Lauf and Sun, 1976).

**Discussion**

The results of this study speak to the genetic association of the cation transport system with the L-antigenic determinants in LK sheep red cells and on the mechanism by which the L antiserum stimulates the Na\(^+\)K\(^+\) pump flux and reduces K\(^+\) leak flux in these cells.

The following findings warrant emphasis: (a) all L antisera tested affected cation transport in both LL and LM (LK) sheep red cells by stimulating K\(^+\) pump flux and by reducing K\(^+\) leak influx; (b) trypsin pretreatment of LL red cells completely abolished the response of iM\(_K\) to the stimulating action of anti-L but did not alter the effect of anti-L reducing ik\(_K\); (c) the increase in the rate of ouabain binding, characteristic of the effect of anti-L on the Na\(^+\)K\(^+\) pump of LK red cells, was not observed when these cells were trypsinized before antibody exposure; (d) the effect of trypsin was specifically on the membrane level—anti-L was not inactivated by trypsin-treated LK or HK red cells, ovomucoid inhibited the action of trypsin on the L antigen at molar ratios of unity, and the protection of the L antigen by anti-L against the enzymatic attack was complete and stable for at least 45 min; (e) anti-Lp, eluted from LK goat red cells and acting mainly on the pump, failed to bind to trypsin-treated LK red cells while anti-Lγ, the antibody not binding to LK goat cells, reduced ik\(_K\) in control and trypsin-treated LK red cells. Anti-Lγ also reduced iM\(_K\) in LK red cells, made into high potassium cells by the nystatin method where normal as well as anti-L stimulated K\(^+\) pump influx was virtually inhibited; (f) anti-Lγ, the antibody hemolyzing LK cells in the presence of complement, may be identical with anti-Lγ since it still could be adsorbed to trypsinized LK red cells.

Our observation that all L antisera tested and their purified immunoglobulins contained two antibodies, anti-Lp and anti-Lγ, acting on the pump and leak systems, respectively, corroborates two recent reports by Dunham (1976a, b) who showed that anti-Lγ affected only passive K\(^+\) flux and not passive Na\(^+\) flux. These findings, therefore, supercede our earlier reports that anti-L affects only the pump component of cation transport (Lauf et al., 1970; Lauf, 1974). In these earlier studies the effect of anti-L on ik\(_K\) was invariable, present in some studies (Lauf, 1972) but not in others (Lauf, 1974). The possible reasons for this variability are multiple: differences between the early antisera (made specific for the L antigen by prior absorption) and the batch of L antisera used in this report (Tables I, II) or genetic differences between sheep (Western breed against pure Dorset of this study) may account for this discrepancy. It should be noted that the basic K\(^+\) leak influx before addition of anti-L was much higher in the LK red cells of Dunham's report (1976b) than in the LK cells of our studies (Lauf et al.,
1970, 1971; Lauf, 1974; Joiner and Lauf, 1975; see Results). Thus, in light of the
dramatic stimulation of $\frac{\mu M}{kg}$ by anti-L, a further small reduction by the antibody
of an already small $K^+$ leak flux was initially considered to be insignificant.
However, with the availability of more potent L antisera, up to 50% reduction of
$\frac{\mu M}{kg}$ was observed, an effect of the magnitude seen by Dunham (1976a, b).

This report confirms and extends our previous finding that trypsin inactivates
the response of the LK pump to anti-L (Lauf et al., 1971). The enzymatic attack
was on the membrane level. The possibility that trypsin, either free in the fluid
phase or membrane bound, hydrolyzes the L antibody combining site was
excluded by several experiments. Pretreatment of anti-L with trypsinized LK or
HK cells did not inactivate the L antibody (Fig. 5), the enzyme effect was blocked
by equimolar concentration of ovomucoid (Fig. 6), and the protection of the L
antigen by anti-L against the enzyme action was complete and stable for at least
45 min (Fig. 7). We also have shown previously that trypsin did not alter the
effect of anti-L when the L antibody was exposed to trypsin before addition to
LK cells (Snyder et al., 1971). Hence in terms of the topography of the LK red
cell membrane, a site for attack by the enzyme (involving peptide bonds adjacent
to lysyl or arginyl residues) and the binding site for the L$^-$ antibody affecting $K^+$
pump flux must be identical.

Dunham reported (1976b) that trypsin treatment did not entirely abolish the
response of the LK pump to anti-L: at 10 mg trypsin/ml he still observed a 2.4-
fold stimulation of active $K^+$ influx and attributed this effect to the remaining
action of anti-L on the pump (Dunham, 1976a), i.e. interconversion of leak sites
to pumps. Our experiments, however, clearly demonstrate that trypsin treat-
ment completely prevented the action of anti-L on the pump (while the reduc-
tion of $\frac{\mu M}{kg}$ by anti-L was unaffected) (Figs. 2, 3). Moreover, the change in the rate
of ouabain binding, characteristic of LK red cells treated with anti-L (Joiner and
Lauf, 1975), was not seen in LK red cells exposed to trypsin before anti-L (Fig.
4). In addition, anti-L$_p$, separated from anti-L$_4$ by absorption to and elution from
LK goat red cells (Ellory and Tucker, 1970; Kropp and Sachs, 1974; Dunham,
1976a; Fig. 8 of this report), did not bind to trypsinized LK red cells (Fig. 9),
implying that the L$_p$ antigen associated with the pump is either lost or inactivated
on the membrane level, while anti-L$_4$ still affected $\frac{\mu M}{kg}$ in trypsinized cells as well as
being bound to the L$_4$ antigen associated with the $K^+$ leak pathway. In one
experiment, not shown here, we were able to demonstrate binding of anti-L$_4$ to
trypsinized LK red cells as evident from the failure of the absorbed antibody to
reduce $\frac{\mu M}{kg}$ in control cells. These data are strong evidence that there are at least
two L-antigenic determinants, the L$_p$ antigen related to the pump and the L$_4$
antigen associated with the $K^+$ leak system. This conclusion can be supported in
quantitative terms since there are between 900 and 1,500 L antigens per LK
sheep red cell of which about one-third appear to be associated with the effect of
anti-L$_p$ on the pump (Lauf and Sun, 1976; Tucker et al., 1976). The remainder
of the L antigens have been identified as L$_4$ antigens and are not hydrolyzed by
trypsin (Lauf et al., 1971; Tucker et al., 1976). Since both anti-L$_4$ (reduction of
$\frac{\mu M}{kg}$ as well as anti-L$_p$ (immune hemolysis in the presence of complement) still
affected trypsinized LK cells, and since anti-L$_p$, certainly was absorbed by
trypsinized LK cells (Fig. 10), it is highly likely that the L$_4$ antigen is identical with
the $L_p$ antigen. In addition, Dunham (1976a) reported that $L_p$ antibodies failed to hemolyze LK red cells while the $L_l$ antibody contained lytic activity.

In his recent studies Dunham (1976a, b) reiterated an earlier concept (Dunham and Hoffman, 1971) that anti-$L$ may interconvert $K^+$ leak sites into active pumps. This concept assumes that a single locus controls the membrane expression of pumps and leaks in HK and LK sheep red cells and was further based on a theoretical computation showing that the total number of pumps and leaks in LK and HK cells is the same (Dunham and Hoffman, 1971). In his subsequent study Dunham (1976b) showed kinetic similarities between $K^+$ pump and leak fluxes in LK red cells and, on the basis of the residual $L$ antibody stimulation of the pump in trypsinized LK red cells, concluded that anti-$L_l$ may convert inactive pumps (passive $K^+$ transport sites) into active Na$^+$K$^+$ (coupled) pumps. This conclusion was supported by experiments on immature LK red cells showing that during maturation pumps may be converted from an active into an inactive mode (Dunham and Blostein, 1976).

Our data are not compatible with this concept. First, we have previously shown that anti-$L$ does not increase the number of ouabain binding, and therefore pump, sites (Joiner and Lauf, 1975, 1977). Second, the results of this report distinguish two antigens of which the $L_p$ antigen can be destroyed by trypsin, and the effect of anti-$L_p$ on the $K$ pump and on the ouabain binding rate abolished, without affecting the $L_l$ antigen. Third, when the $K^+$ pump flux as well as the effect of anti-$L_p$ are completely inhibited by high intracellular potassium (Lauf et al., 1970; Hoffman and Tosteson, 1971), anti-$L_p$ still reduces $\frac{1}{2} k$ to the same extent as in high sodium LK cells (Table III). Since anti-$L_p$ still binds to high potassium LK cells (data not shown; see also Sachs et al., 1974a), we believe that the experimental evidence of our present study does not support the hypothesis of a simple genetic association between both leaks and pumps being interconvertible by anti-$L$ or during maturation of the cell. Rather, a more complex system must be envisioned whereby separate pathways for active and passive cation transport may be regulated by the "single locus" phenotypic inheritance characteristic of the HK/LK system.

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