did not get the second thymocyte inoculation, were immunized with SRBC and the other half of both groups were immunized with horse red blood cells (HRBC). Figure 1 gives the mean titers of antibody to SRBC in the four groups of mice. The only group that made a significant antibody response to SRBC was the one in which the mice were given normal thymocytes after tolerance induction and immunized with HRBC. Mice that received thymocytes and SRBC responded no better than the mice that were not given thymocytes; these mice failed to respond when immunized with either SRBC or HRBC. Thus, tolerance to SRBC was produced and could be broken only if mice were given an inoculation of normal thymocytes, and then only if immunized with another kind of heterologous red cell (in this case HRBC).

The foregoing observation has been substantiated in three additional experiments, which have also shown the following: (i) The antibodies to SRBC made after immunization with HRBC are not cross-reacting; they were completely removed by one absorption with an equal volume of packed SRBC while their titer was unaffected by four absorptions with HRBC. The absorption with HRBC, however, removed all the agglutinins for HRBC. (ii) The addition of an equal amount of SRBC to the HRBC inoculum not only does not prevent the formation of antibodies to SRBC, but in one experiment boosted the antibody response to SRBC from a peak mean titer of 2.4 ± 0.7 to 4.4 ± 0.4 ($\log_2 2 \pm$ S.E.). (iii) The addition of thymocytes without HRBC is insufficient to break tolerance. (iv) When the tolerant spleen cells are adoptively transferred along with normal thymocytes to syngeneic thymus-deprived mice these secondary recipients make no antibody to SRBC when immunized with HRBC (three separate experiments) although they make a vigorous antibody response to HRBC. The details of the experiments with adoptively transferred cells have been described (*15*).

We offer the following explanation for these results. The multiple SRBC injections rendered the mice tolerant. The added normal thymocytes could not break the tolerance as they were themselves rendered tolerant. [There is direct evidence that normal cells can be rendered tolerant by tolerant cells (*15*).] However, HRBC could stimulate these cells. In a manner which is not entirely clear, the SRBC that remained from the tolerance-inducing injections were able to capture the T cell stimulatory effect produced by the HRBC. Hartmann has described special in vitro conditions where SRBC B cell precursors can capture the effect produced when HRBC stimulate T cells, but only if SRBC are present (*16*). The importance of the residual SRBC antigen in our studies is emphasized by the fact that the HRBC could not break the tolerance of the cells after transfer to a second host and also by the boosting effect produced by the inclusion of SRBC with the HRBC.

Our method of breaking of tolerance differs from previous reports of breaking tolerance with cross-reacting antigens in that the antibodies made did not cross-react with the antigen used for tolerance abrogation (*11, 17*). This shows that specific, as well as cross-reacting B cells, may exist in a nontolerant state in ostensibly tolerant hosts. It also emphasizes the need for T cell activation, as do the above-mentioned experiments with allogeneic thymocytes (*13*), for breakage to occur. Our results differ, however, from those with allogeneic cells in that no immunologic attack, either graft-versus-host or host-versus-graft, is being mounted on B cells which might result in some fundamental changes in that population. Both results, as well as others demonstrating the allogeneic effect, emphasize that at least some parts of the T-B cell interaction may be nonspecific.

The main point we would like to make, however, is that situations may exist where mice are incapable of responding to SRBC, even after the addition of normal T cells and yet still possess significant numbers of nontolerant B cell precursors that can make antibody to SRBC.

RICHARD K. GERSHON
KAZUNARI KONDO
Department of Pathology,
Yale University School of Medicine,
New Haven, Connecticut 06510

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18. Ellen Searle rendered technical assistance. Work supported by grant CA-08593 from the NCI, and an NCI career development award (CA-10,316) to R.K.G.

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Loss of a Parathyroid Hormone–Sensitive Component of Phosphate Transport in X-Linked Hypophosphatemia

Abstract. *Mutant hemizygotes with X-linked hypophosphatemia lack a parathyroid hormone–sensitive component of inorganic phosphate transport in kidney; female heterozygotes retain a variable proportion of this type of transport. The residual mechanism for reabsorption in affected males allows inorganic phosphate efflux from the kidney to urine so that net "secretion" is sometimes observed; the latter is directly proportional to the serum concentration of inorganic phosphate. Calcium acts on the kidney tubule to enhance net reabsorption by this component of inorganic phosphate transport.*

The primary defect of X-linked hypophosphatemia has eluded clarification since the first descriptions of this form of vitamin D–resistant rickets (*1, 2*). Because of the impaired clinical responsiveness to vitamin D, and because of the discovery that intravenous calcium infusion could suppress the elevated renal clearance of inorganic phosphate (P_i) which accompanies hypophos-

phatemia (*2, 3*), attention was originally focused on vitamin D–dependent mineral metabolism in this trait. The primary defect was thought to involve impairment of calcium absorption in the intestine, or abnormal vitamin D metabolism, and secondary hyperparathyroidism was invoked as the basis for the renal loss of P_i (*2, 4*). These hypotheses are improbable because untreated patients with X-linked hypophosphatemia have normal concentrations of parathyroid hormone (PTH) in the blood (*5*).

An alternate hypothesis emphasizes a primary disturbance of P_i transport in kidney, and perhaps also in other tissues (*2, 6*). Hypophosphatemia is the single constant phenotypic trait in carriers of this X-linked mutant allele (*2*), suggesting that the allele primarily influences P_i metabolism. Replacement of P_i is particularly effective in correcting the mutant clinical phenotype (*7*), as would be expected if P_i metabolism is mainly affected in the disease.

We present evidence that the defect in X-linked hypophosphatemia concerns a PTH-sensitive component of P_i transport that is responsible for about two-thirds of the total net reabsorption of P_i in human kidney; this component is partially absent in female patients and completely absent in male patients. A residual component of P_i transport, capable of being saturated, is retained in male patients. This permits P_i flux from tubular lumen to plasma and also allows a flux in the reverse direction. Although this transport is insensitive to PTH it can be modulated by calcium ion. The net reabsorption of P_i is enhanced immediately upon intravenous infusion of calcium.

Tubular transport of P_i is saturable in normal human subjects (*8*). We examined the tubular reabsorption rate [TRP_i, micromoles per 100 ml of glomerular filtrate (GF)] and the maximum reabsorption rate (TmP_i, micromoles per 100 ml of GF, at saturation) at various concentrations of serum P_i (*9*) in six males (all children) and nine females (three children, six adults) with X-linked hypophosphatemia. Subjects with bone disease (six male and three female children) were on treatment regimens with P_i (1 to 3 g/day) and vitamin D_2 (10,000 to 50,000 unit/day) prior to investigation; each patient had a normal glomerular filtration rate (GFR).

The tubular reabsorption rate is lower in mutant hemizygotes than in female heterozygotes, and below the normal mean in all patients (Fig. 1); this finding confirms many earlier reports (*2*). The TRP_i in female heterozygotes overlaps the normal range, as predicted by the Lyon hypothesis (which implies random inactivation of one X chromosome early in development) if P_i transport in the kidney is closely coupled to the effect of an X-linked mutation. However, we could find no constant relation between the TRP_i and the degree of bone disease in heterozygotes.

A saturable component of P_i reabsorption is present in both hemizygotes and heterozygotes but is much lower

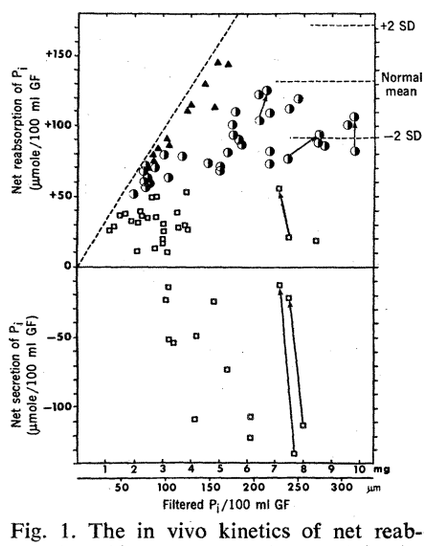

Fig. 1. The in vivo kinetics of net reabsorption of inorganic phosphate (P_i) by the kidney of normal subjects and these with X-linked hypophosphatemia. The substrate concentration (P_i) is varied in the glomerular filtrate by an intravenous infusion method (*8*). Reabsorption rate is calculated as described (*9*). Mutant hemizygotes are represented by squares, heterozygotes by half-filled circles and normal subjects by triangles. The mean ± 2 standard deviations (S.D.) for the normal maximal rate of tubular reabsorption (TMP_i) in man (*2*) is shown. The interrupted diagonal line (upper graph) indicates complete reabsorption of filtered phosphate; points falling below this line indicate incomplete net tubular reabsorption of phosphate. The net reabsorption rate (TRP_i), the difference between filtered and excreted P_i, is plotted upward as a positive value when the excreted P_i is less than the filtered P_i. Negative reabsorption (net secretion), found in several hemizygotes (excreted $P_i >$ filtered P_i), is plotted downward. Arrows indicate the change in P_i reabsorption 30 to 60 minutes after intravenous infusion of calcium (4 mg/kg) over a 15-minute period; this dose was sufficient to raise total calcium in serum by 2 mg/100 ml. A single patient is represented by the two studies of the effect of calcium on P_i secretion.

in the former (Fig. 1), confirming previous reports (*2, 10*). There is no unusual "splay" in the graph of P_i reabsorption, indicating no change in binding of P_i by the available transport system. The residual P_i transport, in mutant hemizygotes, is virtually saturated even at endogenous concentrations of plasma phosphate. This suggests the presence of more than one form of P_i transport, the residual form being unaffected by the X-linked mutation.

The renal excretion of P_i in mutant hemizygotes was often greater than its filtered load; this was observed over a threefold range of P_i concentration in serum (lower part of Fig. 1). These observations were monitored by inulin clearance, and the rate of net tubular secretion was found to be directly proportional to the P_i concentration in serum. When net reabsorption of P_i occurred, the serum calcium concentration in these subjects was 9.9 ± 0.9 mg/100 ml (mean ± standard deviation, 27 samples) and when there was net secretion of P_i, it was 9.9 ± 0.4 mg/100 ml (14 determinations).

We examined the effect of bovine PTH (*11*) on the endogenous P_i excretion in normal, heterozygous, and mutant hemizygous phenotypes. Because of prior phosphate therapy, the endogenous serum immunoreactive PTH (*12*) (IPTH) was above normal in one male and one female patient before bovine PTH infusion (*5*); the concentration was normal in the remaining subjects. Renal clearance of P_i relative to creatinine increased sharply in the four normal subjects after infusion of bovine PTH (Fig. 2). The GFR was not changed significantly by this procedure in any patient. The P_i excretion in three heterozygotes overlapped the normal response. On the other hand, each of the three mutant hemizygotes were less responsive to PTH than were the heterozygotes, and one male actually excreted less P_i after the PTH infusion. One of the hemizygotes (second from right, Fig. 2) was studied when his serum P_i had been raised to 8 mg/100 ml by the infusion method (*9*). His response to PTH was like that of the other two male patients who were studied when their concentrations of serum P_i were low (< 3 mg/100 ml). We reported previously (*5*) that a tenfold increase in endogenous serum IPTH does not influence TRP_i in mutant hemizygotes. We conclude, from the

present and the early studies, that the residual P_i transport, unmasked in the hemizygote with X-linked hypophosphatemia, is virtually insensitive to PTH.

Phosphaturia may not be a satisfactory index of the renal tubular response to PTH. Urinary excretion of adenosine 3′,5′-monophosphate (cyclic AMP) is said to be a more reliable index (*13*), and for this reason we measured cyclic AMP in the urine of the patients infused with bovine PTH (*14*). Urinary cyclic AMP was normal in all patients before infusion. The excretion rate of cyclic AMP in female heterozygotes, mutant hemizygotes, and normal subjects increased by 19 to 80 nmole/min in the first 30 minutes after bovine PTH injection; the response for mutant and normal subjects overlapped each other and all were in the normal range (*13*). We also measured the change in calcium excretion relative to creatinine, before and after PTH infusion. Calcium excretion dropped by 50 to 70 percent after PTH infusion in normal, heterozygous, and hemizygous subjects. Therefore, PTH acts in a normal manner on the renal tubule in X-linked hypophosphatemia but the hormone is unable to exert its usual effect on P_i transport.

Intravenous infusion of calcium enhances P_i transport in the mammalian kidney (*2, 15*). The effect is mediated both through a parathyroid-dependent mechanism and by a direct effect upon the tubule (*15*). The latter can be the only mode of action of calcium in male patients with X-linked hypophosphatemia who respond to calcium infusion with improvement in the net tubular reabsorption of P_i (*2, 3*), because P_i reabsorption is insensitive to PTH, and serum IPTH is not abnormally elevated in the untreated mutant hemizygote (*5*). Studies of the change in P_i reabsorption after calcium infusion in X-linked hypophosphatemia, often were performed with large amounts of calcium infused over several hours (*3*). We examined five patients (three female, two male) at 30 to 60 minutes after a calcium infusion (4 mg/kg over 15 minutes) that was sufficient to raise serum calcium by 2 mg/100 ml. Tubular transport of P_i was studied at concentrations of serum P_i sufficient to saturate the transport. Tubular reabsorption of P_i improved under these conditions in each subject (Fig. 1), whether net tubular reabsorption or net secretion of P_i was present initially. Although serum immunoreactive PTH fell by about 20 percent in all subjects after the calcium infusion, this response would not account for the change in TRP_i in hemizygotes.

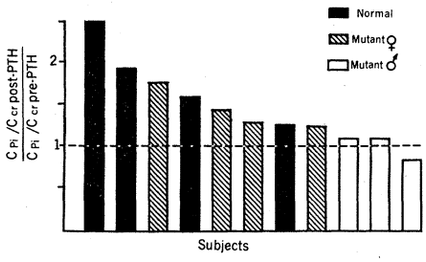

Fig. 2. Effect of an intravenous infusion of purified bovine parathyroid hormone (PTH) on phosphate excretion by mutant hemizygotes (open bars) and heterozygotes (hatched bars) with X-linked hypophosphatemia, and normal subjects (black bars). The ordinate is the ratio (C_{Pi}/C_{cr}) after PTH infusion to the ratio (C_{Pi}/C_{cr}) before PTH infusion, where C is the endogenous renal clearance rate of inorganic phosphate (P_i) and creatinine (cr) averaged for three 30-minute periods before and after PTH infusion.

Familial hypophosphatemia is a dominantly expressed X-linked disease (*2*) and, according to the Lyon hypothesis, such a disease will be expressed fully in male hemizygotes and variably in the females. We must conclude, however, that the loss of P_i transport in kidney is not complete even in mutant hemizygotes with X-linked hypophosphatemia. A component of P_i transport, with a saturable capacity for net reabsorption at about one-third the normal value, is still active in the male patient. Transport of P_i in females is between normal and hemizygous values; this is compatible with partial retention of the transport that is absent in males. We propose, therefore, that P_i transport in human kidney involves at least two components (*16*). One component accounts for about two-thirds (about 100 μmole per 100 ml of GF) of the total net reabsorptive capacity; this transport is modulated by endogenous parathyroid hormone. A second component accounts for all or most of the remaining uptake; its capacity is about 50 μmole per 100 ml of GF, and its P_i transport can be directly modulated by the calcium ion. These findings suggest that calcium may regulate P_i efflux (plasma to lumen) relative to net influx (lumen to plasma) by the second type of P_i transport. It is possible that whatever benefit has been achieved by massive vitamin D treatment in X-linked hypophosphatemia was actually derived from the effect of vitamin D on calcium metabolism which in turn modulated P_i transport by the calcium-sensitive system.

It is still not known whether the two (or more) components of P_i transport are separate binding sites (proteins) with different capacities and sensitivities to PTH and calcium, functioning in parallel at the brush border, or whether they function in series, perhaps at opposite poles of the tubular epithelial cell. The latter hypothesis implies a mediated brush border transport with high capacity, which is sensitive to PTH and calcium, and an antiluminal carrier capable of transporting P_i from cell to plasma and also from plasma to cell. The X-linked mutation presumably influences only one mode of transport ni the parallel hypothesis and there is precedence for this type of membrane transport mutation (*17*). In the series hypothesis the mutation concerns transport on the brush border site which allows P_i binding but not a coupled transport to allow accumulation of P_i in the cell; there is also precedence for mutations which "uncouple" substrate-specific transport (*18*). The X-linked mutation may affect the membrane system directly or it may affect another cell function which modulates membrane transport of phosphate.

We do not know whether other cells and organs in man have similar mechanisms for P_i transport. A number of the clinical features of X-linked hypophosphatemia, particularly in the female heterozygote, cannot be explained solely on the basis of an hereditary defect in P_i transport in kidney. Moreover, some nonsaturable but relatively inefficient P_i transport in cell membrane (*19*) is likely to be present since high concentration of P_i improves phosphate retention (*7*). We anticipate that mediated P_i transport may be impaired in other tissues in this disease; there is evidence for this in other inborn errors of membrane transport (*17*). The X-linked hypophosphatemia may be genetically heterogeneous and different types of defective P_i transport may be identified; such genetic heterogeneity has been found in the other inborn errors of membrane transport (*17*).

FRANCIS GLORIEUX
CHARLES R. SCRIVER
deBelle Laboratory for Biochemical Genetics, McGill University–Montreal Children's Hospital Research Institute, Montreal 108, Quebec, Canada

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Ultrathin Carbon Support Films for Electron Microscopy

Abstract. *Carbon support films only 4 to 6 angstroms thick have been made for use in electron microscopy. The determination of their thickness is based on geometrical calculation, electron scattering measurements, and elemental microanalysis.*

A method has been developed for the routine production of electron microscope specimen-support films believed to be only 4 to 6 Å thick. Such films are valuable in reducing background scatter in the examination of biological macromolecules by bright- or dark-field electron microscopy and by transmission electron diffraction.

Kleinschmidt and Vasquez (*1*) have reported the use of support films 30 to 40 Å thick, as measured by ellipsometry, while Crewe *et al.* (*2*) have deduced that films used by them were 20 Å thick. This conclusion was based on a comparison of measured electron scattering (*3*) with the scattering from films of different thicknesses calculated from equations derived by Lenz for a shielded Coulomb potential (*4*). Use of a carbon film 7 Å thick in dark-field microscopy has been reported (*5*), but it is not clear that such a film could be used as a specimen support.

The thin carbon films described here are deposited on freshly cleaved mica surfaces in a standard evaporating apparatus. A rotating shutter (240 rev/min) provided with a radial slot is placed 10.5 cm below the carbon arc; one piece of mica is positioned 0.5 cm below the plane of the shutter and another the same distance above it. The dimensions and shape of the slot are such that most of the lower mica surface should receive 5 percent as much carbon as does the companion upper one, but a small "tab" region at the outer edge of the lower mica piece receives 25 percent as much. The carbon arc is operated for about 10 seconds.

After deposition, the carbon film is floated off the mica by first inserting the thicker tab portion into a clean water surface. This portion is readily visible and helps to locate the almost invisible remainder of the film as it floats on the water. Portions of the film are then picked up by touching them from the air side with a reticulated collodion film on a 200-mesh copper grid (*6*). Films calculated as only 4.5 Å thick (5 percent of the companion film, measured as 90 Å thick) can be reliably manipulated in this manner.

The thickness of carbon deposited on the piece of mica above the rotating disk is determined by measuring the lengths of shadows cast by edges of broken fragments after they have been shadowed lightly with uranium. The fragments are obtained by floating the thick film on water and breaking it into small pieces, which are then picked up on a collodion support film. Polystyrene spheres are used to determine the local shadow angle. Film thicknesses ranging from 90 to 160 Å were measured with an estimated accuracy of ± 10 Å.

Measurements of the relative intensity of electron scattering by films of different thicknesses were made by dark-field electron microscopy at ×4000. In some instances portions of films were found to be folded (double thickness), and scattering from these was recorded. The electron microscope was operated at 80 kv and was equipped with one cold finger (decontaminator) located between the specimen and the objective aperture and a second one located above the specimen airlock. The intensity of primary illumination was measured and adjusted to a fixed value by use of a lithium-drifted silicon detector located in the final image plane (*7*). The dark-field images were recorded by use of tilted-beam illumination and an axially positioned,