in this fashion can be combined with the Gauss-Cadazzi equations and the previous results to yield the remaining unknowns $b_{\alpha\beta}$ and $x_\alpha{}^A$. If one proceeds in this manner, cognizance of certain intrinsic properties of the bounding surface Σ can be included in a straightforward manner. For instance, if it is known that the hypersurface Σ admits a group of motions, this fact can be combined with (9.3) so that the resulting a's will exhibit such properties (see sec. 6 of ref. 1 for a particular example). In the particularly simple case in which $\phi = 0$, we can take $Q_{\alpha\beta} = 0$. The system (9.3) then shows that the space Σ^* is a three-dimensional analogue of an Einstein-Riemann space with incoherent matter.

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THE ROLE OF SODIUM IONS IN THE ACTIVATION OF ELECTROPHORUS ELECTRIC ORGAN ADENOSINE TRIPHOSPHATASE

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A phosphorylated enzyme intermediate has been proposed to explain the exchange kinetics of the Na^+-activated adenosine triphosphatase of crab nerve.[1] A reaction with similar activation characteristics[2] is catalyzed by a small particle fraction derived from electric organ tissue of *Electrophorus electricus*. We have recently reported that these particles will incorporate radioactive phosphate upon brief incubation with ATP^{32}.[3] This and related observations are presented in greater detail in the present communication, together with some observations on the possible correspondence of the mechanisms of ATP hydrolysis and sodium ion transport.

Materials and Methods.—(a) *Preparation of particle fraction containing sodium-activated ATPase:* Electric organ tissue is dissected from freshly killed eels, minced with scissors, and disintegrated in a stainless steel blendor in 10 volumes of 0.05 *M* Tris buffer, pH 7.5. After straining through gauze, the homogenate is centrifuged 20 min at 9,000 × *g*. The precipitate is resuspended in 10 volumes of buffer and sonicated for 3 one-min periods, maintaining the temperature of the suspension at 6°C; after centrifuging as above, the combined supernatant is centrifuged for one hr at 50,000 × *g*. The sonication and extraction of the 9,000 × *g* precipitate may be repeated one or more times to increase the yield.

The 50,000 × *g* precipitate containing the ATPase is resuspended to a protein concentration of approximately 10 mg/ml in 0.1 *M* Tris pH 7.5, divided into small aliquots, and rapidly frozen by immersion in dry ice-acetone. The quick-frozen particles can be stored for several months at −20°C with little loss of ATPase activity.

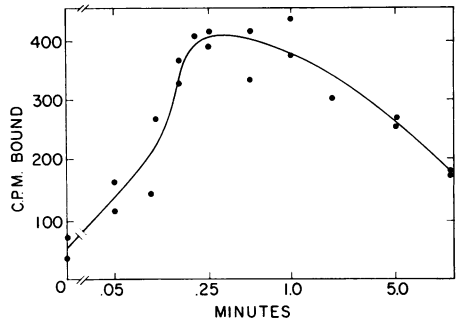

FIG. 1.—Kinetics of ATP^{32}-binding by ATPase fraction. Reaction mixture is described in Table 1 with the following exceptions: 0.003 *M* $MgCl_2$; 0.001 *M* ouabain; 200,000 cpm ATP^{32} (1 mμmole) was added together with 0.5 μmole P to initiate the binding reaction.

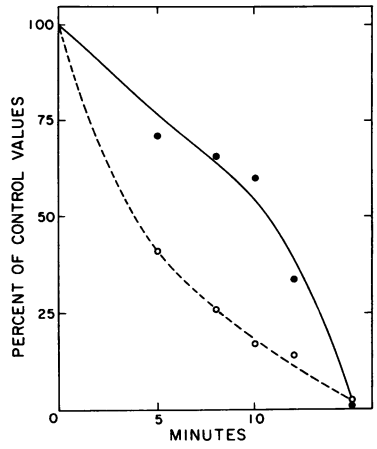

FIG. 2.—Rate of heat inactivation of ATP^{32}-binding activity and of ATPase activity at 47°C. Incubation mixture as for Fig. 1. Solid circles are P^{32}-binding data; open circles indicate relative rates of hydrolysis.

(b) *Preparation and assay of ATP^{32}:* The ATP^{32} used in this study was prepared from carrier-free P^{32} (Oak Ridge) and pyridine ADP according to the procedure of Lowenstein.[4] The nucleotide was isolated according to a modification of the method of Morell *et al.*[5] The ATP concentration was determined using firefly luciferinase.[6] The extent of γ-labeling was determined by hydrolysis with ATPase and separation of the radioactive orthophosphate by thin layer chromatography.[7]

(c) *P^{32} incorporation experiments:* The enzyme was incubated with ATP^{32} in small Pyrex tubes (3 × 60 mm) in an ice bath. Additions were made with specially designed Lang-Levy pipettes and mixed by "buzzing."[8] After 10% TCA denaturation and centrifugation at 1,500 × *g* for 10 min in a refrigerated centrifuge, the supernatant was removed. The precipitate was resuspended in cold washing solution (5% TCA containing 0.05 *M* Na_2ATP and 0.05 *M* KH_2PO_4) and again centrifuged. After 3–4 washings the radioactivity remaining in the final precipitate was counted by inserting the tube in the well of a plastic phosphor block adapted to the Packard Tri-Carb scintillation spectrometer.

Results and Discussion.—(a) *Characteristics of the incorporation of radioactive phosphate from ATP into the particles:* Maximal incorporation of P^{32} is attained within 15–30 seconds at 0°C (Fig. 1). This bound radioactivity is stable to repeated washing with cold trichloroacetic acid containing orthophosphate and ATP. Only slight diminution of the labeling results when orthophosphate is present in 1,000-fold excess of the ATP (Table 1). This phosphorylation is heat-sensitive (Fig. 2). The level of phosphorylation is increased in the presence of Mg^{++} (Table 2*A*). At the optimal Mg^{++} concentration there is a further increase

TABLE 1

Additions	Cpm (15″-0)
0.1 mμmole ATP^{32} (72,000 cpm)	317
0.1 mμmole ATP^{32} + 0.16 μmole Tris PO_4	274
0.1 mμmole ATP^{32} + 0.16 μmole Tris ATP	1

Comparison of effects of dilution of ATP^{32} by orthophosphate and ATP on amount of P^{32} incorporated: The incubation volume of 70 μl contained 200 μg ATPase protein; 5.4×10^{-3} *M* $MgCl_2$; 1.8×10^{-3} *M* ouabain; 0.06 *M* Tris-HCl, pH 7.5. The radioactive ATP (76% γ-labeled) was added simultaneously with the nonradioactive ATP or orthophosphate. Incubations were terminated after 15 sec at 0° by adding 8 μl 50% TCA. In the case of zero time controls, the order of addition of TCA and ATP^{32} was reversed. The acid precipitates were washed 3 times with a solution containing 5% TCA, 0.05 *M* Na_2ATP and 0.05 *M* KH_2PO_4 before counting.

TABLE 2A

EFFECT OF CATIONS ON PHOSPHORYLATION OF ELECTRIC ORGAN PARTICLES

Additions	Bound radioactivity cpm (25″-0)
None	92
Mg^{++}, 0.003 M	145
Mg^{++}, 0.003 M + Na^+, 0.06 M	345
Mg^{++}, 0.003 M + K^+, 0.025 M	114
Mg^{++}, 0.003 M + Na^+, 0.06 M + K^+, 0.025 M	264
EDTA, 0.002 M	94

The incubation volume of 50 μl contained enzyme, the indicated concentration of cations as chlorides, 0.02 M Tris phosphate pH 7.5, and 13 mμmoles ATP^{32}, 6 $\times$ 10^5 cpm.

TABLE 2B

EFFECT OF HIGH CONCENTRATIONS OF NaCl ON PHOSPHORYLATION OF ELECTRIC ORGAN PARTICLES

NaCl	Bound radioactivity cpm (25″-0)
0.06 M	833
0.24	1,449
0.50	3,315
1.00	4,360

Conditions as in 2A except that 0.003 M $MgCl_2$ was present in all tubes. The 13 mμmoles of ATP contained 1.4 $\times$ 10^6 cpm.

in extent of phosphorylation in the presence of Na^+. K^+ superimposed upon Mg^{++} and Na^+ reduces the amount of labeling. The extent of labeling as a function of ATP concentration follows Michaelis kinetics in the presence of Mg^{++} and ouabain (Fig. 3). The apparent K_m under these conditions is 1.2 $\times$ 10^{-4} M. This compares with K_m = 8 $\times$ 10^{-5} M for hydrolysis.

(b) *Characteristics of the isotope exchange reaction:* The electric organ ATPase is similar to that of crab nerve[1] in catalyzing the equilibration of labeled ADP with ATP (Table 3). At concentrations which maximally activate the hydrolysis,

TABLE 3

I		II	II/(I + II)
(a) Kinase exchange C^{14}-ADP	→	ATP	0.170
(b) Adenylate kinase C^{14}-AMP	→	ATP + ADP	<0.003
(c) Adenylate deaminase C^{14}-AMP	→	IMP	<0.003
(d) Adenylate cyclase C^{14}-ATP	→	3′, 5′-AMP	<0.001

Nucleotide interconversion reactions catalyzed by electric organ particles: All incubations were of 20 min duration at 26°C with 2.5 μg enzyme; 3 μmoles Tris-HCl, pH 7.2; 0.1 μmole $MgCl_2$; 45 μl total volume. (a) 0.3 μmole Tris ATP; 0.003 μmole C^{14}-ADP. (b) 0.28 μmole C^{14}-AMP; 0.36 μmole ADP; 0.36 μmole ATP. (c) 0.1 μmole C^{14}-AMP; 0.15 μmole IMP. (d) 0.12 μmole C^{14}-ATP, 0.2 μmole 3′, 5′-AMP.

After deproteinization with 4% formic acid in methanol, the nucleotides were separated by thin layer chromatography[7] and counted by liquid scintillation technique.

Na^+ and K^+ have no appreciable effect on the exchange rate. P^{32} does not measurably exchange into ATP.

(c) *Comparison of the properties of the hydrolytic, exchange, and particle phosphorylation reactions:* Since the particulate preparation may contain enzyme activities other than the ATPase, it is important to establish the extent to which the three reactions under study may be facets of the same catalytic entity. There is no detectable hydrolysis of ATP by this preparation unless both Mg^{++} and Na^+ are present.[2] Adenylate kinase is not detectable as measured by the complete absence of incorporation of C^{14}-AMP into ADP (Table 3), and hexokinase is not detectable by spectrophotometric assay.

However, other kinases may contribute to the observed phosphorylation and exchange reactions. The initial rate of equilibration of C^{14}-ADP with ATP may be equated with the rate of phosphorylation of the enzyme protein: ATP + E $\rightleftarrows$ ADP + E − P. Under conditions of suboptimal Mg^{++}, this step can be made

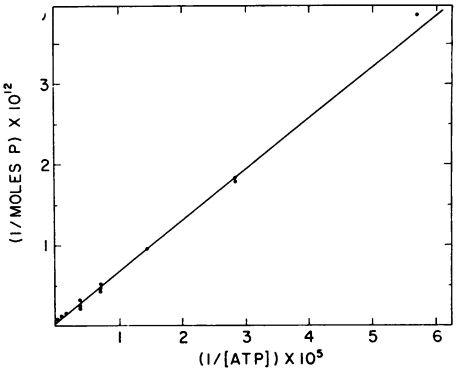

FIG. 3.—Effect of ATP^{32} concentration on amount of radioactive phosphate bound. ATPase fraction equivalent to 0.30 mg protein was incubated in 54 μl containing 0.01 *M* Tris phosphate, pH 7.2; 0.003 *M* $MgCl_2$; 0.001 *M* ouabain. Samples denatured and washed as described in Table 1.

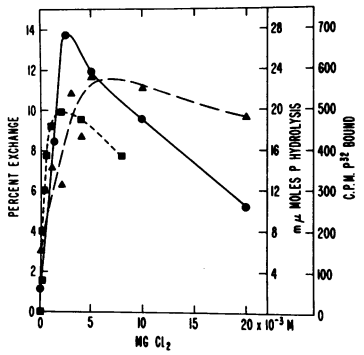

FIG. 4.—Mg^{++} activation. (●———●) ATPase activity at optimal Na^+ and K^+ concentrations; (■———■) C^{14}-ADP exchange into ATP, no alkali metal ions; (Δ———Δ) P^{32} incorporation from ATP^{32} into particles, no alkali metal ions.

rate-limiting in the ATPase reaction. Rate of exchange and rate of hydrolysis will be direct and inverse functions, respectively, of the ADP concentration, so that a simple quantitative comparison is not valid. However, under our assay conditions, the measured rates of isotope equilibration and of hydrolysis are of the same order of magnitude. The ATPase reaction must therefore account for a large part of the observed exchange reaction.

All three reactions, while requiring Mg^{++}, are inhibited at higher concentrations. The optimal Mg^{++} concentrations are similar for the three reactions (Fig. 4).

The small effects of sodium and potassium ions on the exchange reaction led Skou to ascribe their principal role in the ATPase mechanism to an activation of the hydrolysis of the phosphorylated enzyme.[1] The effect of K^+ on particle phosphorylation is consistent with this (Table 2*A*). However, the increment of phosphorylation in the presence of Na^+ constitutes a qualitative distinction in the roles of these two cations. These results are in agreement with those observed for the labeling of kidney particles by ATP^{32}.[9] The opposing effects of Na^+ and K^+ upon the level of phosphorylation, when considered in the light of the mutual competition of Na^+ and K^+ in the hydrolytic reaction,[10] led to the expectation that very high concentrations of Na^+

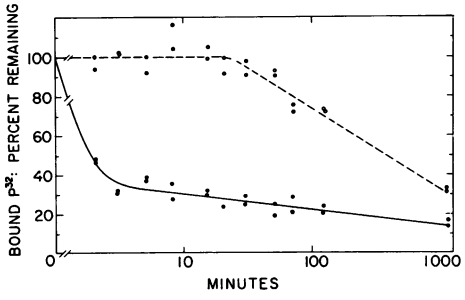

FIG. 5.—Release of bound radioactivity by treatment with pepsin. P^{32}-labeled denatured ATPase fraction was prepared under the conditions of Table 1 except on a larger scale: 7 mg protein was incubated in 1.0 ml. The denatured preparation was rehomogenized after 8 washes. One half was taken for incubation in 1.0 ml 0.01 *N* HCl containing 10 mg pepsin. The remaining half was incubated in 0.01 *N* HCl as a control. Incubation was at 25°C. Aliquots were withdrawn at the indicated intervals, added to small tubes containing $^1/_{10}$ volume 50% TCA, and counted before and after removal of the TCA soluble supernatant.

might increase the extent of labeling still further by blocking dephosphorylation at the K^+ site. This effect was obtained (Table 2*B*).

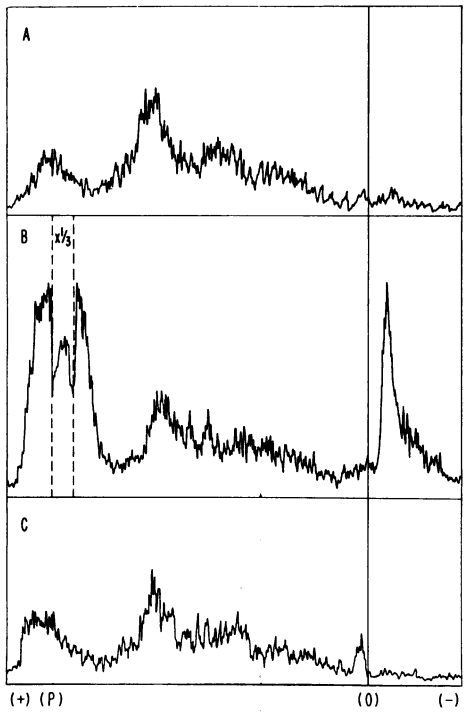

FIG. 6.—Effect of Na^+ on electrophoretic pattern of radioactive fragments released by pepsin. Approximately 1 mg of particles were labeled by incubating at pH 7.5 in 0.02 *M* Tris HCl containing 44 mμmoles ATP^{32} (330,000 cpm/mμmole) and 3×10^{-3} *M* $MgCl_2$. In addition, *B* contained 1.0 *M* NaCl, and *C* contained 1.0 *M* LiCl. Incubation was for 25 seconds at 0°C. The washed, acid-denatured precipitate was incubated with 1% pepsin, pH 2, for 10 min at 25°C. After precipitation with 20% TCA, the supernatant was extracted with 5 volumes of ether to remove the TCA before lyophilizing. Electrophoresis conditions: pH 3.5, 0.05 *M* ammonium formate; 5,000 volts; 90 min; 12°C. (*P*) indicates mobility of orthophosphate.

(d) *Enzymatic degradation of P^{32}-labeled particles:* Although the particle-bound P^{32} is stable to repeated washings with cold acid, brief alkaline treatment or prolonged incubation at room temperature releases most of the radioactivity in the form of orthophosphate. Brief incubation of P^{32}-labeled particles with pepsin in 0.01 *N* HCl also results in the release of about 70 per cent of the P^{32} in acid-soluble form (Fig. 5). However, only a small fraction of this is in the form of orthophosphate in the peptic digest derived from Mg^{++}-activated particles (Fig. 6*A*). At pH 3.5 the electrophoretic pattern of this material consists of three major peaks in addition to orthophosphate, all on the anodal side. The corresponding pattern from particles labeled in the presence of both Mg^{++} and Na^+ consists of the same series of peaks and an additional peak on the cathodal side (Fig. 6*B*). Moreover, the orthophosphate peak is greatly increased. When additional precautions are taken to reduce the exposure to acid, the cathodal peak is increased relative to the orthophosphate (Fig. 7).

(e) *Possible mechanism for the sodium-activated ATPase:* The foregoing observations may be reconciled by the following proposed mechanism:

$$(1)\quad \mathrm{ATP} + XEY \overset{Mg^{++}}{\rightleftharpoons} \mathrm{ADP} + \mathrm{P} \sim XEY$$

$$(2)\quad \mathrm{P} \sim XEY \overset{Na^+}{\rightleftharpoons} XEY - \mathrm{P}$$

$$(3)\quad XEY - \mathrm{P} + H_2O \overset{K^+}{\rightleftharpoons} XEY + \mathrm{P}$$

$$(4)\quad \mathrm{ATP} + H_2O \xrightarrow{Mg^{++},\ Na^+,\ K^+} \mathrm{ADP} + \mathrm{P}$$

The particles are considered to contain three distinct catalytic activities which constitute the ATPase reaction in sum: a Mg^{++}-activated kinase, a Na^+-activated transferase, and a K^+-activated phosphatase. This scheme is not meant to specify the number of protein subunits which may be associated with these reactions, nor does it rule out the participation of intermediates other than phosphorylated proteins. However, in several attempts, we have been unable to remove any appreciable fraction of the particle-bound P^{32} by extraction with ethanol, butanol, or chloroform-methanol.

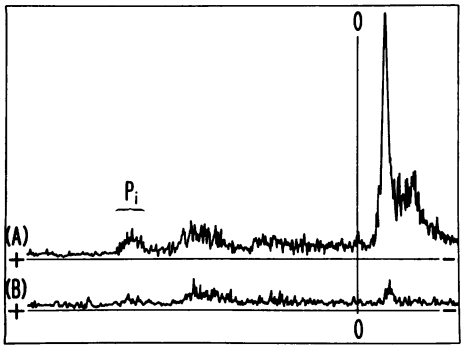

FIG. 7.—Electrophoretic pattern of radioactive acid-soluble fragments after pepsin. Labeling in the presence of 1.0 *M* NaCl and 0.003 *M* $MgCl^2$ (*A*) and 0.003 *M* $MgCl^2$ (*B*). Conditions as in Fig. 6, except that the exposure time to TCA was minimized by extracting the peptic digest with ether immediately after addition of TCA.

It seems reasonable to associate the sodium-augmented component of Figure 7 with the $Y - P$ component of the enzyme. The increment of P_i^{32} labeling in the presence of high $Na^+ + Mg^{++}$ may be 5–10-fold of that attained with Mg^{++} alone (Table 2; Fig. 7). For this reason, the structure of the ATPase unit may be better represented as XEY_n. As discussed below, the theoretical requirements for a cation-selective transport mechanism make it attractive to consider that a *series* of Na^+-dependent transfer reactions may intervene between the kinase and phosphatase steps:

$$(-Y_j - P) + (-Y_k) \xrightarrow{Na^+} (-Y_j) + (-Y_k - P).$$

(f) *Relation of the proposed mechanism to a scheme for active transport:* Any hypothetical scheme for active transport must provide (1) a sufficient source of energy, (2) a mechanism for countergradient translation, and (3) the appropriate specificity. In addition, it must be reconciled with whatever homeostatic controls and pharmacologic sensitivities have been demonstrated to apply to the physiological process.

The proposed elemental reactions of the Electrophorus ATPase can be incorporated into a mechanism for active transport of sodium ions which satisfies most of the preceding criteria.

(1) The energy requirements of active sodium transport have received considerable study,[11–14] but are still in doubt. The upper limit for transfer of sodium by any carrier system consisting of esterified phosphate is 2 Na^+ per P. If the chemical potential of the system decreases in the successive configurations ($P \sim XEY$) → ($XEY - P$) → (XEY, P), the energy released in these transformations may be utilized in the transport process.

(2) An orientation of the kinase, transferase, and phosphatase reactions, in that order, from the inner to the outer surface of the cell membrane (Fig. 8) would result in the translocation of phosphate from within the cell to the outer region of the cell membrane. If sodium is specified as the counterion in this process, its

transport would be the coupled, energy-consuming element of the sequence of reactions.

(3) The specificity of the transferase for sodium ions is therefore the essential factor in obtaining work from the system. In the model under consideration, phosphate is visualized as migrating down a free-energy gradient which drives it across the cell membrane through an environment which selects for sodium as the counterion. That a sufficient degree of selectivity is operant in the particles is evidenced by the activation kinetics of ATP hydrolysis. Eisenman has presented an excellent analysis of the factors which enter into the cation specificity of fixed anionic sites.[15] Some experimental support for the concept of a sodium-catalyzed phosphate migration may be derived from the observations of Taborsky[16] on the metal-catalyzed migration of phosphate in phosvitin.

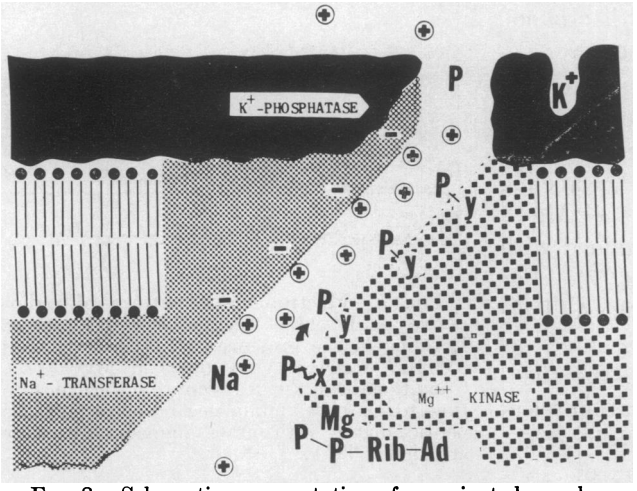

FIG. 8.—Schematic representation of an oriented membrane ATPase. Transphosphorylation from the primary phosphorylation site (P∼X) to P—Y can only occur in the presence of sodium ions, ⊕. Other counterions are rejected by the environment of fixed charges within the membrane.

In common with that of Hokin and Hokin,[17] the present model does not explicitly account for the return of phosphate into the cell. One function of the fixed negative charges at the external cell surface, which are believed to consist chiefly of sialic acids[18, 19] may be to screen the escape of phosphate ions. A back diffusion of phosphate set up in this manner, if channeled through a potassium-selective environment, could account for the coupled transport of K^+.[20, 21]

Summary.—Labeling of electrophorus electric organ Na^+-ATPase particles by ATP^{32} is stimulated by Mg^{++}. The sites available for phosphorylation are increased severalfold when Na^+ is also present. K^+ decreases labeling and Li^+ is without effect. Brief peptic digestion of "Mg^{++} sites" releases 3 major radioactive fragments, in addition to orthophosphate, which migrate anodally at pH 3.5. The electrophoretic pattern from "Mg^{++} + Na^+ sites" contains an additional slowly migrating cathodal component which readily breaks down to release orthophosphate.

Since Mg^{++}, but not Na^+, activates the ADP-ATP exchange reaction, Na^+ sites are evidently phosphorylated secondarily to the Mg^{++} sites. These observations suggest that Na^+ transport may be a consequence of oriented transphosphorylations across the cell membrane through an environment which selects for Na^+ as the counterion.

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*DEMONSTRATION OF TWO POPULATIONS OF CELLS IN THE HUMAN FEMALE HETEROZYGOUS FOR GLUCOSE-6-PHOSPHATE DEHYDROGENASE VARIANTS**

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In 1932, Muller coined the term "dosage compensation"[1] to account for the equality of phenotypic expression in males and females for most genes located on the X chromosome. Over the years several explanatory theories for this phenomenon have been considered, and these have been recently reviewed by Stern[2] and by McKusick.[3]

In 1961, a unique hypothesis was developed by Lyon[4] and by Russell.[5] The hypothesis, often referred to as the "Lyon Hypothesis," proposes that in each somatic cell of the female, one of the two X chromosomes is genetically inactive. The inactivation must occur early in development, and it is a matter of chance whether the maternal or paternal X is inactivated. Once an X chromosome is inactivated in a developing cell, all progeny of that cell presumably maintain the same inactive X.

Examination of single cells in a female who is heterozygous for an X-linked gene(s) with a measurable effect would provide direct evidence bearing on the hypothesis. In such a case, the female would be expected to produce a mosaic of X chromosome activity. In some of her cells, one allele would be active; in the remainder, the other allele would function.