*THE GENERATION OF LATENT-ION-TRANSPORT CAPACITY**

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Introduction.—Of the various hypotheses designed to explain the movement of ions from the environment into the plant cell, the most attractive suggests that ions traverse cell membranes in combination with an endogenously produced carrier substance.[1, 2] The hypothesis stems from the recognition that absorption kinetics are not first-order, but are, rather, best represented by a formulation analogous to that which describes the reversible combination of substrate and enzyme.[3] The carrier hypothesis is at least consistent with the known characteristics of the absorption process. Thus the remarkable specificity of the absorption process may be imputed to specificity on the part of the carriers; the requirement for respiratory energy may be related either to the production of carrier or to the degradation of ion-carrier complex; and the ability of ions to penetrate ion-impermeable cellular membranes may be ascribed to the formation of a permeating ion-carrier complex.

Cation absorption in plant tissues is characterized by an initial stage in which combination with a cellular component may be demonstrated directly. However, there is every indication that the observable first phase of cation absorption does not represent combination with carrier, but rather reflects an adsorption-exchange process in which the adsorption sites are the free carboxyl groups within the cell wall.[2] Compared to cation exchange sites, anion exchange sites are present in vanishingly low concentration.[4, 5] The existence of true carrier, sites both for cations and for anions has been deduced almost exclusively from kinetic analyses. In a study which is perhaps unique in attempting the direct determination of carrier concentration unobscured by extraneous adsorption processes, Hagen and co-workers[6] have estimated the concentration of phosphate-carrier sites in barley roots.

The concept of carrier-mediated ion transport is to be distinguished from the electrochemical hypothesis of Lundegårdh[7] in which the transient separation of electron and proton during the respiratory transport of hydrogen atoms is considered the prime mover in ion absorption. The carrier hypothesis allows for the possibility that metabolic events which have to do with ion transport may be separated in time from the actual absorptive act. An example of such separation is presented herein. Potato slices have been chosen for the experimental object for two reasons. First, considerable information exists regarding the accumulatory[8] and respiratory[9] characteristics of this tissue. Second, slices of potato, as well as of other storage organs, are transformed during aging from a tissue with little or no ability to absorb salt to one with a vigorous accumulating capacity.[10] The controllable change in physiological state may ultimately allow the characterization of those metabolic events which are particularly related to salt transport.

Materials and Methods.—Blocks of tissue, 1.5–2.0 inches to a side, were cut from the center of Russet Burbank potato tubers. A block was pierced six times through two-thirds of its depth with a No. 4 cork borer, the cores remaining in the block. Millimeter slices were removed with a hand microtome, and the disks (9 mm. in diam.) so formed were freed from the slice by gently dipping the latter into a dish of

ice water. The disks were briefly rinsed, transferred to an 80-mm. Buchner funnel, and bathed for 20 minutes with 3 liters of distilled water, which passed by gravity flow through the stem of the funnel and out over the funnel walls. The disks were thereupon used directly, or transferred to a liter Erlenmeyer flask (10 gm tissue/100 ml 10^{-3} M $CaSO_4$) to be gently swirled at room temperature on a New Brunswick Gyrotary shaker. Except for the experiment described in Figure 1, all disks were aged for 22–24 hours with three changes of $CaSO_4$ solution.

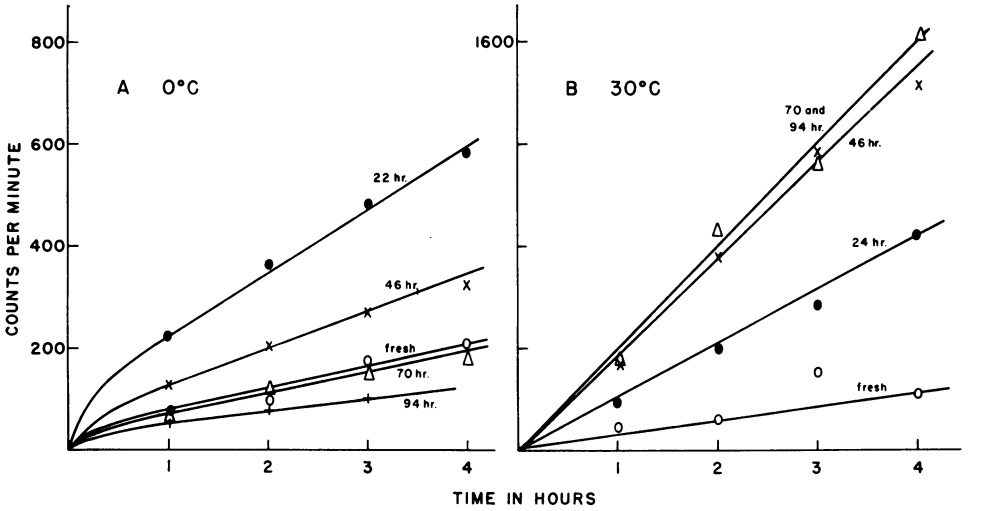


FIG. 1.—The time-course of chloride absorption as a function of disk age. *A*, 0° C.; *B*, 30° C.

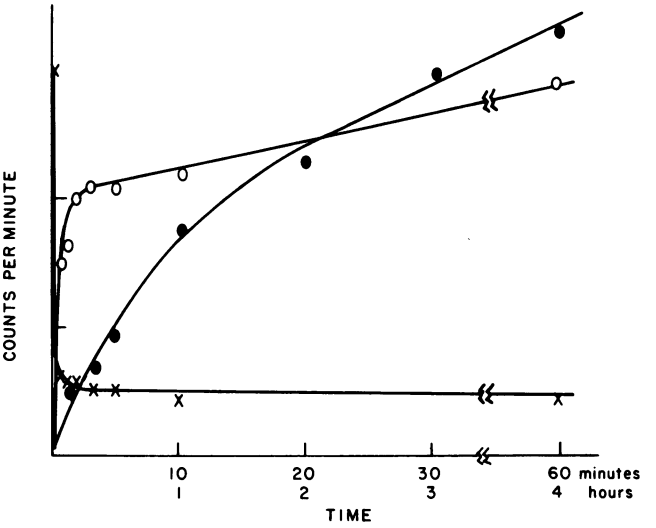


FIG. 2.—The time-course of AFS penetration compared with that for absorption. *Solid circles*, absorption progress as indicated by activity of H_2O-rinsed disks as a function of time (hours) in Cl^{36}. *Open circles*, AFS penetration, as indicated by total activity of unrinsed, blotted disks as a function of time (minutes) from immersion in Cl^{36}. *Crosses*, loss from AFS, as indicated by activity as a function of time in H_2O of disks preincubated for 1 hr. in Cl^{36} and surface-dried without rinsing before transfer to H_2O.

Seven disks (0.44 gm. fresh wt.) were placed into 10-ml. KCl^{36} solution in 50-ml. Erlenmeyer flasks. The flasks were shaken in a Dubnoff metabolic shaker, when absorption was measured at 30° C. For absorption studies at 0° C., flasks were shaken on an Eberbach reciprocal shaker, in a specially constructed insulated box which was filled with ice. At the end of an absorption period, disks and solution were rapidly poured onto a circle of filter paper held in a 40-mm. Buchner funnel

fixed to a water pump through a side-arm flask. Disks were then either blotted dry and prepared for counting at once—as in the experiments involving diffusion kinetics shown in Figure 2—or rinsed with a stream of water from a wash bottle and placed in 20 ml. ice water for an additional 20 minutes' washing on the shaker at 0° C. Then 0.2 ml. of 3.6 per cent polyvinyl alcohol (Elvanol, grade 72-51, Dupont) was spread on a 30-mm. planchet, and the seven surface-dried disks were arranged on the planchet with neighboring disks touching. After being dried overnight in an oven at 80–100°C., the radioactivity of the disks was determined with a gas-flow micromil window detector, in conjunction with an automatic sample changer (Nuclear-Chicago). Determinations in Figure 1 are in triplicate; all others are in duplicate. Self-absorption is approximately 19 per cent. HCl^{36} was received from Oak Ridge as a 1.64 N solution with a specific activity of 0.586 mc/gm Cl. The

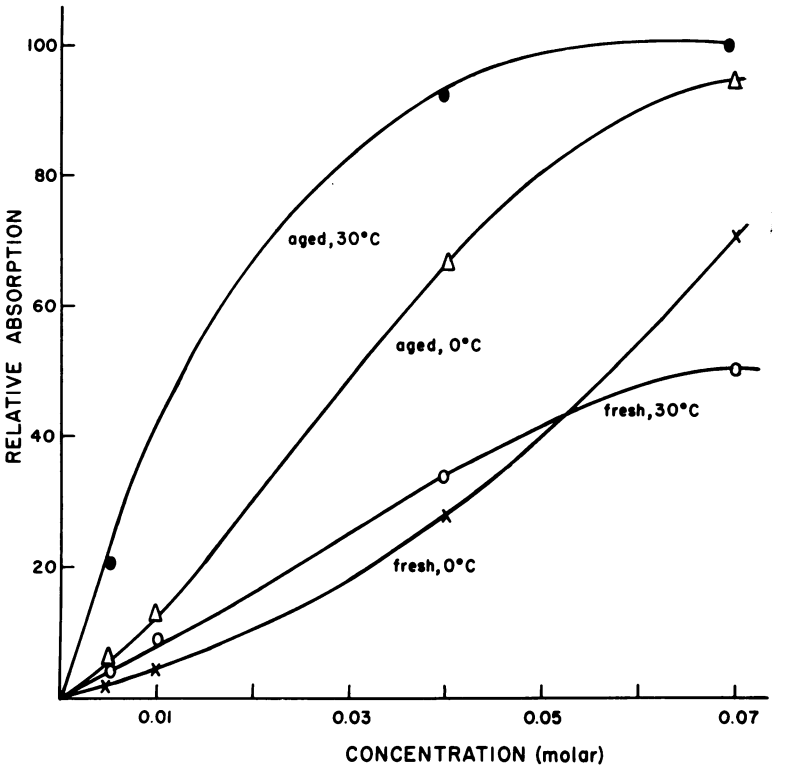


FIG. 3.—Absorption as a function of KCl concentration. Fresh disks used after 20 min. washing. Aged disks incubated 40 hr. at room temperature. To compare actual rates, the scale for aged tissue at 30° C. must be taken as 5 times that for the others.

solution was diluted as desired, as much stable Cl as necessary was added as KCl, and the solution brought to pH 6.0 with KOH. Experimental solutions contained 20 μC/l, which, as measured, gave 24,000 counts per minute per milliliter. Except for the experiments of Figure 3, all solutions were 0.04 M KCl.

Experimental Results.—The time-course of Cl^{36} absorption at 0° C. by fresh or aged disks transferred from room temperature is depicted in Figures 1, A, and 2. Figure 1, B, describes the time-course at 30° C. It is proposed to call the initial

rapid phase of uptake at 0° C. the "absorption shoulder." At no time during the absorption period can the shoulder be abolished by washing the tissue either in water or in nonradioactive KCl. The shoulder is thus thought to represent bona fide uptake into the cell and in this sense is to be distinguished from shoulders which owe their origin either to penetration of ions into the apparent free space (AFS)[11, 2] or to adsorption exchange on surface or extracellular binding sites.[2] In Figure 2 the kinetics of AFS penetration are compared with absorption kinetics at 0° C. Whereas penetration into the AFS is half-completed in perhaps 1.5 minutes and is essentially independent of temperature, the absorption shoulder achieves half its maximum value in approximately 20 minutes at 0° C. and is not demonstrable at 30° C. (Fig. 1, *B*). The relationship between absorption and external concentration is shown in Figure 3. The remarkable exponential relation noted especially with fresh tissue at 0° C. will not be discussed here. Suffice it to say that the aberration from the standard type of absorption isotherm (e.g., Fig. 3; aged tissue, 30° C.) is due more to the low temperature than to the age of the tissue.

The fact that a shoulder is manifested only when disks are transferred from a higher to a lower temperature (Fig. 1) has led to the following hypothesis, which is evaluated below. It is assumed that a cellular metabolite which positively affects carrier synthesis has a steady-state concentration which varies directly with the

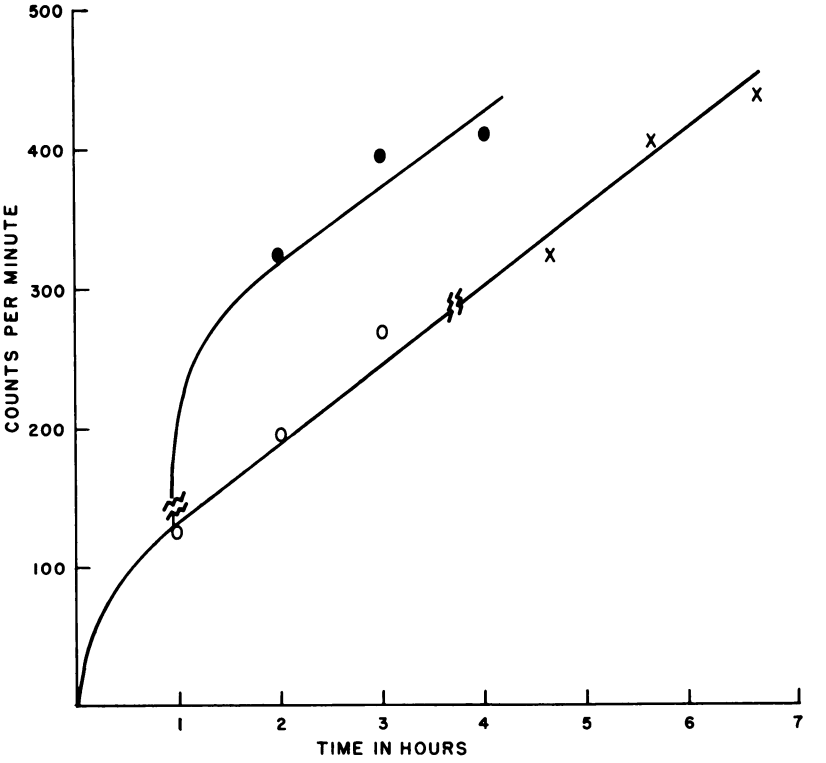


FIG. 4.—The regeneration of the absorption shoulder. *Open circles*, disks placed at once in Cl^{36} at 0° C. *Closed circles*, disks placed in Cl^{36} at 0° C. for 1 hr., transferred to H_2O at room temperature for 1 hr., then back to 0°C.Cl^{36}. *Crosses*, disks maintained in 0° C. Cl^{36} solution for 3.5 hr., then into 0° C. H_2O for 1 hr., and back into 0° C.Cl^{36} solution.

temperature. For convenience, the metabolite will be termed a "carrier precursor," which is to be construed only in an operational sense. The steady-state level of precursor typical of the elevated temperature constitutes a relative excess at the lower temperature. When disks are transferred to 0° C., the extra precursor is utilized in the first 1–1.5 hours, and the steady state typical of the lower temperature is gradually achieved. If the foregoing hypothesis is valid, it should be possible, once the precursor has been expended, to produce another interval of high absorptive activity at 0° C. by raising the tissue to room temperature for some time. As may be seen in Figure 4, a brief period in water at room temperature subsequently evokes a new shoulder at 0° C. which is as great as the initial shoulder. When the absorption period is interrrupted by an hour in H_2O at 0° C., no shoulder is formed upon return to a solution of Cl^{36} at 0° C., and absorption continues essentially unaffected. Actually, the establishment of the steady state at room temperature is rapid, 5-minute exposures resulting in almost maximal shoulder development. For this reason, disks may not be handled at room temperature when transferred from one cold solution to another, and the technique which has been adopted is to transfer the disks with a perforated stainless steel spoon, dipping them into appropriate ice-cold rinsing solutions where necessary.

It might be anticipated that if disks are kept for approximately 1.5 hours in nonradioactive chloride at 0° C. and then transferred to a solution of the radioisotope at the same temperature, the time-course of Cl^{36} absorption will at once be linear. Such in fact is the case. Interestingly, however, the same effect can be achieved by maintaining the disks in 0° C. H_2O in a preincubation period. Figure 5 indicates the extent to which the shoulder is diminished as a function of

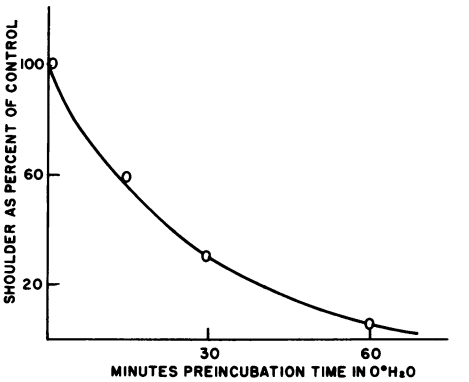


FIG. 5.—The effect of preincubation in H_2O at 0° C. on the height of the absorption shoulder. The control value is taken as the shoulder height observed in disks placed directly into Cl^{36} at 0° C.

preincubation in 0° C. H_2O. The implication of the foregoing is that precursor is destroyed whether or not transportable ions are present. In each case H_2O-treated disks were rinsed in 0° C. Cl^{36} before being transferred to the absorption vessels.

Since it is recognized that respiratory activity and presumably respiratory energy are the *sine qua non* of active salt absorption, it would be expected that an increase in the salt-absorbing capacity, as exemplified by the development of a new shoulder, would depend upon the normal respiration. Figure 6, *B*, indicates that when the coupling of respiratory energy to physiological processes is prevented by 2,4-dinitrophenol (DNP),[10] there is no shoulder formation in response to an incubation period at 30° C. Disks were first preincubated in H_2O at 0° C. to do away with

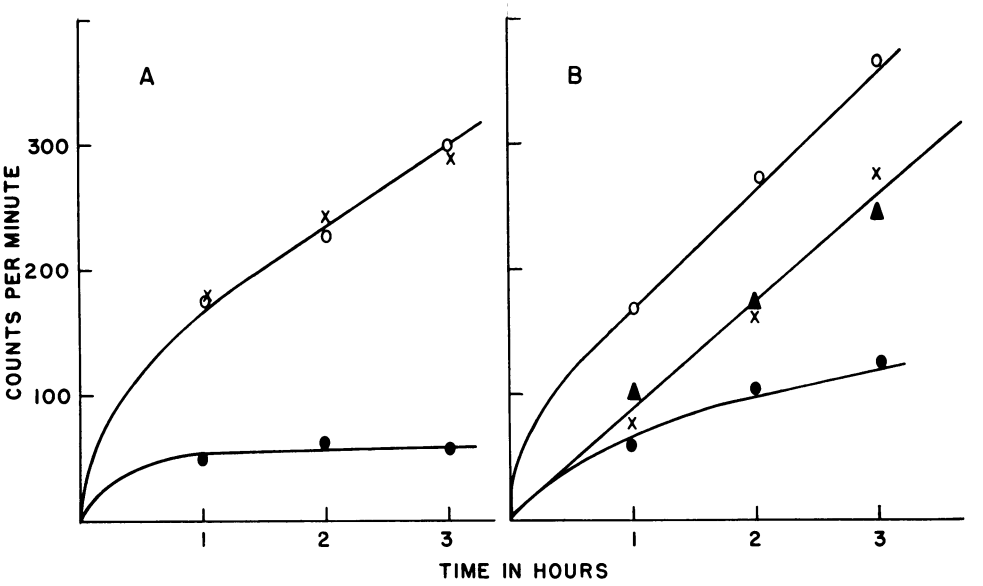


FIG. 6.—The effect of DNP on steady-state uptake and on shoulder generation. *A: solid circles*, disks placed at once in Cl^{36} and 10^{-4} *M* DNP. *Open circles*, disks in Cl^{36} from t_0. *Crosses*, disks in Cl^{36} plus DNP at 0° C. for 3 hr., followed by 1 hr. in H_2O at 30° C. before transfer to 0° C. Cl^{36} solution. *B: solid circles*, disks in Cl^{36} and DNP from t_0. *Open circles, triangles, and crosses*, disks preincubated for 1.5 hr. in 0° C. H_2O following which: *triangles*, disks placed at once in 0° C. Cl^{36} solution; *open circles*, disks 15 min. in 30° C. H_2O before transfer to 0° Cl^{36}; *crosses*, disks 5 min. in 0° C. DNP followed by 15 min. in 30° C. DNP, two rinses in 0° C. H_2O, and transfer to 0° C. Cl^{36}.

the shoulder, after which they were given 15 minutes at 30° C., either in H_2O or in 10^{-4} *M* DNP. In the latter case disks were first passed through DNP at 0° C. to allow diffusion into the AFS to be completed before the temperature was raised (cf. Fig. 2). The DNP was thoroughly removed with several rinses of 0° C. H_2O before disks were finally dipped in 0° C. Cl^{36} and transferred to the experimental solution. Evidence that the effect of DNP is quite reversible is given in Figure 6, *A*. It may be noted, first, that DNP abolishes the steady-state absorption of Cl^{36} and considerably diminishes the shoulder as well. When disks are kept in Cl^{36} plus DNP at 0° C. for 3 hours and then bathed in H_2O at 30° C. for 1 hour, they subsequently display a new shoulder at 0° C., and a steady-state absorption rate equal to the control. Cyanide (10^{-3}–10^{-2} *M*) fails to affect steady-state uptake at 0° C. and is without effect, as well, on the regeneration of shoulder at 30° C. in disks preincubated at 0° C. However, cyanide frequently abolishes the shoulder when presented together with chloride at 0° C. (Fig. 7). The peculiar effects of cyanide are discussed below.

Discussion.—Compared with the substantial kinetic evidence for the carrier hypothesis, there has been little direct experimental verification of carrier existence. Russell *et al.*[12] have postulated the presence of phosphate carriers in barley roots on the basis that, at very low phosphate concentrations, azide fails to affect absorption. The rationale offered is that the actual combination of phosphate ion with carrier is azide-resistant and that, at sufficiently low phosphate concentrations, enough carrier exists to combine with, and hence to transport, all the phosphate

present. Hagen *et al.*,[6] in turn, have deduced the presence of phosphate carrier in barley roots by noting that in brief absorption periods the graphical regression of the time-course of phosphate uptake intersects the ordinate. The intercept has been taken as a measure of the amount of carrier present, which was shown to be on the order of 10^{-5} *M* on a fresh-weight basis (cf. ref. 13). Lundegårdh[14] has recently reported chloride absorption shoulders, both in wheat roots and in potato slices, equivalent to several μmoles of chloride per gram fresh weight. The magnitude proved independent of the temperature, and the shoulders were taken to represent an initial chloride-binding reaction. Anion-binding sites have heretofore been considered virtually absent in these same materials,[4, 5] and since, in the cited experiments, chloride uptake was measured by noting its disappearance from the external solution, the significance of the shoulders in this case is uncertain.[2]

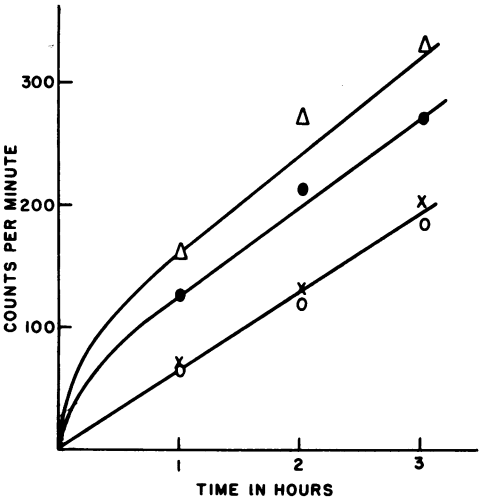


Fig. 7.—The effect of cyanide on steady-state uptake and shoulder generation. *Open circles*, disks placed at once in Cl^{36} plus 10^{-2} *M* cyanide, pH 6.0. *Crosses, solid circles, triangles*, disks preincubated in 0° C. H_2O for 1.5 hr., following which: *crosses*, placed in 0° C. Cl^{36} at once. *Solid circles*, placed in 30° C. H_2O for 30 min. before transfer to 0° C. Cl^{36}. *Triangles*, placed in cyanide solution at 0° C. for 5 min., followed by 30 min. in KCN at 30° C., followed by water rinse at 0° C., and transfer to Cl^{36} at 0° C.

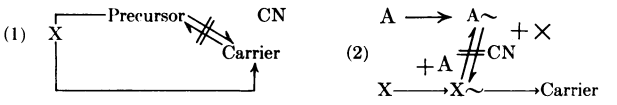
The experiments described herein indicate that, under selected conditions, metabolic activity occurring at one time can produce a unit of ion-absorbing capacity which can be manifested at another time. The foregoing phenomenon is to be distinguished from the well-known influence of the prehistory of storage organ slices on their subsequent steady-state absorption behavior.[10] Absorption during the initial stage—represented by the shoulder—is considered of a piece with steady-state absorption. The relationship of absorption to concentration at 0° C. is the same whether the shoulder height or the steady-state slop be taken as a measure of the rate of uptake. Figure 3 depicts the total absorption in a 4-hour period as a function of concentration. However, the curves would look the same if uptake in the interval between 1.5 and 4 hours were plotted as a function of concentration or if the shoulder heights were so plotted. All in all, the observations which have been made are inconsistent with the concept that salt transport is effected by a cytochrome-mediated electron pump,[7] but they permit the deduction that ion uptake depends upon metabolically produced carriers.

The assumption was made above that the absorption shoulder reflects the utilization of a unit of absorptive capacity—which, for convenience, has been termed "carrier precursor"—and not of carrier per se. The basis for this contention stems from a consideration of the kinetics of shoulder diminution in the presence or ab-

sence of chloride, and from the effect of cyanide on each of the absorptive phases. First, it is evident that there is no immediate binding of a quantity of chloride equivalent to the absorption shoulder—followed by its transport into the cell. The quantity of radioactive chloride in the AFS is the same in untreated disks as in disks wherein the shoulder has been removed by preincubation in 0° C. H_2O. By the same token, there is no exchangeable chloride at any time during absorption at 0° C., and chloride uptake at 30° C proceeds linearly from the origin. That is, there is no perceptible intercept attributable to combination of chloride with carrier (cf. refs. 6, 13). Thus it is not the transport of ion-carrier complex into the cell which controls the absorption rate.

It is pertinent to recall that the duration of the shoulder (Fig. 2) is the same as the time required in H_2O at 0° C. to remove the shoulder (Fig. 5). Since the rate of shoulder diminution is independent of the presence of chloride ion, it hardly seems likely that the combination of carrier with chloride determines the kinetics of the first phase. If, alternatively, the spontaneous degradation of carrier controls the rate of shoulder depletion, then the quantity of carrier present initially must be much greater than estimated from the extent of the shoulder, for the carrier utilized in transport would then be but a fraction of the total. Since the estimated chloride equivalent of the shoulder is already quite high, being *ca.* 5×10^{-4} M on a fresh-weight basis, it is unlikely that the actual carrier concentration is in fact considerably higher (cf. Hagen *et al.*[6]). A more reasonable hypothesis is that the rate-determining event involves the conversion of precursor to carrier, the latter breaking down at a rate independent of the presence of chloride. In this view the fraction of the total available carrier which is used in transport will be determined by the external salt concentration. If the only path of precursor degradation is through carrier, spontaneous carrier breakdown and carrier utilization through transport will not be distinguishable. Shoulder duration will then be independent of the presence of salt, and the magnitude of the shoulder at high external salt concentrations will be a rough indication of the total amount of precursor.

The most compelling reason for considering that the shoulder does not represent carrier per se is that cyanide frequently wipes out the shoulder, while leaving the steady-state absorption rate at 0° C. unaffected. If cyanide in any way impeded the utilization of carrier, it must perforce inhibit steady-state absorption. Lundegardh[14] offers an entirely similar example in his Figure 2, which is ascribed to the combination of cyanide with cytochrome sites which are presumed to bind anions as the first step in transport. However, his explanation is inconsistent, since in the same figure it is evident that cyanide fails to affect steady-state uptake, which, in Lundegårdh's view, is certainly considered to be mediated by the cyanide-sensitive cytochrome oxidase system. A tentative and tenuous hypothesis is offered in the following alternative representations:



In the first scheme the precursor is a structural progenitor of the carrier; in the second, the precursor essentially represents an energy supply for carrier synthesis,

the curlicue being a formalized representation of a high-energy state.[10] As will be seen below, the direct path of carrier formation appears linked to a cyanide-sensitive respiration. An indirect contribution to the absorptive capacity may be made along a path which is at least in part CN-resistant. It is not understood why cyanide, when present during the experimental period, does not invariably obliterate the shoulder. An example in which cyanide does remove the shoulder is given in Fig. 7 both because of the pertinence of this observation to the argument just adduced, and because Lundegårdh[14] has independently observed precisely the same cyanide effect.

It has been reported that as the respiration rate of potato slices rises with incubation, the sensitivity to CN and to CO diminishes markedly.[9] Cyanide-resistant absorption might thus be anticipated in aged disks, although Griffiths and Hackett[15] have observed that phosphate uptake by potato slices remains CO-sensitive at a time when the respiration is largely CO-resistant. The extent to which cyanide sensitivity varies with age in potato slices depends upon the incubation conditions.[16] Under the conditions obtaining in this study the resporation of day-old disks at 30° C. was inhibited almost 90 per cent by 10^{-2} *M* CN. At this temperature chloride absorption was inhibited to the same extent. On the other hand, at 0° C. where chloride absorption was essentially unaffected by CN, the respiration was diminished less than 30 per cent. The failure of CN to inhibit Cl absorption at 0° C. appears to be due to the relative ineffectiveness of CN as a respiratory inhibitor at this temperature. It is pertinent that in the presence of cyanide, chloride uptake at 0° C. is at least three times that at 30° C.

Finally, there is no obvious relation between the magnitude of the shoulder at 0° C. and the steady-state rate at 30° C., which is only to say that the rate of steady-state uptake depends on the rate of carrier turnover. The shoulder magnitude, on the other hand, depends on the relative rate constants for precursor synthesis and degradation. The balance of the latter processes is markedly determined by temperature in relation to age (Fig. 1). Since the absorptive capacity at 30° C. continues to increase through 70 hours (Fig. 1), while a respiratory maximum is reached in about a day,[9, 10] there must be metabolic transformations relating to the development of absorptive capacity which are not related alone to the magnitude of respiration.

Summary.—1. The absorption of chloride ion at 0° C. by potato disks transferred from 30° C. proceeds in two stages. The initial stage of rapid uptake—which has been termed the "absorption shoulder"—lasts approximately 1 hour, and is taken to reflect absorption capacity generated at the higher temperature.

2. At any time after the first stage has been completed, a new shoulder can be created by transferring the tissue to 30° C. for as little as 5 minutes. The original shoulder can be dissipated by an hour's preincubation in H_2O at 0° C.

3. DNP inhibits both steady-state uptake and the generation of a new shoulder. Cyanide affects neither, but wipes away the original shoulder when presented simultaneously with chloride at 0°C.

4. It is considered that the first stage of absorption is of the same type as that which constitutes the steady state. Reasons are offered for presuming that the shoulder represents a relative excess of carrier precursor and not of carrier per se.

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*A THEOREM ON ZERO-FIELD SPLITTINGS**

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Hutchison and Mangum[1] have recently observed a zero-field splitting of the paramagnetic resonance of the phosphorescent triplet state of the naphthalene molecule. Such studies on this and other aromatic molecules are of considerable theoretical interest, since the zero-field splittings almost certainly arise from a first-order simple magnetic dipole-dipole interaction between electron spins rather than from highly complex spin-orbit interactions that are largely responsible for zero-field splittings in metal ions[2] and other atoms of large nuclear charge. Experimental information on zero-field splittings may then lead to useful information on the correlation of electron spins in molecules. On the other hand, since the dipole interaction is proportional to the inverse cube electron-electron distance, one might expect that the problem of interrelating observed zero-field splittings and approximate molecular electronic wave functions would be especially difficult because most approximate wave functions are poorest in their description of electron-electron close approaches, where the inverse cube distance is largest. The purpose of the present work is to point out that, although the calculated energy of any particular state will be uncertain because of this close approach of the electronic magnetic dipoles, differences in energies corresponding to zero-field splittings do not involve energy terms arising from close approaches due to an antisymmetry requirement of the space part of the wave function which automatically introduces some correlation tending to keep the electron spin magnets apart. This then leaves the hope that approximate wave functions may be