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Debunking the Alleged Resurrection of the Sodium Pump Hypothesis

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Abstract: A detailed debunking of the alleged resurrection of the sodium-pump hypothesis which was disproved more than 35 years ago.

1. The Origin of the Membrane Pump Hypothesis and Its Disproof 35 Years Ago

A worthy scientific hypothesis is almost always the creation of a protagonist, an author. In this, it is not different from creations in literature, music and the arts. The sodium-pump hypothesis, in contrast, seems to have no real author in the true sense of the word.

It is true that Robert Dean has been cited often as the founder of the sodium-pump hypothesis. Yet, the contribution Dean made appears to be not much more than a passing comment, to wit: "It is safer to assume that there is a pump of unknown mechanism which is doing work at a constant rate excreting sodium as fast as it diffuses into the cell" (Dean, 1941, p. 346). Dean did not provide a mechanism for the sodium pump. Nor did he provide or cite experimental evidence in support of the existence of the sodium pump. Then there is the question of priority.

In 1839 Theodor Schwann, widely accredited as the originator of the Cell Theory, expressed the view that control of cell activities was in the cell membrane, which possesses "metabolic power"—a power or, in German, "Kraft" reminiscent of, if not the direct outgrowth of, the vitalistic "Lebenskraft" of his mentor and close associate, Johannes Müller—by which the cell can regulate the chemical composition of the fluids inside and outside the cells. In 1898 E. Overton proposed his famous "lipoidal membane theory" (Overton, 1898). Since a lipid membrane is impermeable to salts and yet salts accumulate within cells, he suggested that the salts (ions) were ferried across the cell membrane by "adenoid" or secretary activities (see Collander, 1959, p. 9). Ralph Lillie—well-known for

his "Iron Wire Model" of conducting nerves—wrote in 1923: "Either the (sodium) salts do not diffuse across the membrane [a concept soon proven wrong (Wu and Yang, 1931; Kaplanski and Boldyreva, 1934; Heppel, 1939; Cohn and Cohn, 1939; Steinbach, 1940)] or some active physiology factor is at work which opposes or compensates for the effect of diffusion . . ." (Lillie, 1923, p. 117). Note that Lillie's comment cited here is not very different from Dean's comment cited above.

An age-old idea, sporadically retrieved to serve as an argumentative last resort in order to preserve the membrane theory—according to which cells are membrane enclosed sacs of dilute solutions (for the history of how this idea began in error and became entrenched, see Ling, 1997c)—was more or less what I discovered in 1948, when as a graduate student at the Department of Physiology of the University of Chicago, I was getting ready to give a seminar on the sodium pump. Having found little substance in the sodium pump hypothesis nor experimental data affirming its existence, I apologetically told the audience that the only thing I could say firmly about the sodium pump is that nobody seemed to know much about it.

Right after the lecture, two of my professors, who had become good friends as well as teachers, each individually took me aside and said to me privately in more or less the same tone, even the same words: The sodium pump is a "Holy Cow." Stay away from it. Nothing would be gained by making yourself a martyr.

I thanked each most heartily for their kindness and concern. However, I thought that they were overly worried. Why would famous scientists in power pay any attention to the opinion of a lowly graduate student? But then I began to think that maybe what we need is some bread-and-butter laboratory work. With good luck, perhaps the next time I give a seminar, I might have something less apologetic to report.

So shortly after that, I did some very simple experiments, taking advantage of the marvelous freedom I enjoyed as a graduate student. I reasoned that if there is indeed a sodium pump in the cell membrane—which by its ceaseless activity at the expense of metabolic energy keeps the level of Na^+ in the cell lower than in the bathing medium and the level of cell K^+ higher than in the bathing medium—then cutting off the energy supply with metabolic poisons should bring about a prompt gain of cell Na^+ and loss of cell K^+. What I observed was not what I expected. The concentrations of both K^+ and Na^+ in the poisoned muscles remained unchanged for as long as the duration of my experiment.

This result puzzled me and excited me at the same time. It looked to me as if ion distribution in living cells was more mysterious and perhaps more fundamental than the cellular electrical potentials, which had been my exclusive interest up to that time. Before long I became deeply immersed in an attempt to see if the sodium pump hypothesis was really tenable and even to fantasize what may be an alternative—although, to be honest, it was then more like trying to catch an unmarked fish swimming somewhere in the Atlantic Ocean. I strongly suspected that it must be there, that it must be some simple physical mechanism and even went around asking my fellow-theoretical physics friends for ideas. But I got nowhere.

And before you knew it, three years went swiftly by. All this time I could not stop thinking about these problems even though in the meantime I had left Chicago and moved to a new laboratory at Johns Hopkins Medical School in Baltimore.

My earliest publications in K^+ and Na^+ distribution consisted of a short article in the *American Journal of Physiology* (Ling, 1951) and a much longer one in the *Symposium on Phosphorus Metabolism* (Ling, 1952). Described in these two papers are the firm estab-

lishment that both K^+ and Na^+ in frog muscles remain unchanged hours after their metabolic energy had been cut off—by the combined action of pure nitrogen (which blocks respiration, which converts sugar into carbon dioxide) and sodium iodoacetate (which stops glycolysis, which converts sugar into lactic acid) (Ling, 1952, Table 5; for later more extensive confirmatory data, see Table 8.4 in Appendix 1). Furthermore, the experiments were carried out at 0°C, a temperature which, like the metabolic poisons, slows down outward pumping of Na^+ (if the pump does exist)—as pumping, being a chemical reaction, must have a higher temperature coefficient—more than it slows down inward leakage of Na^+ into the cells—a process which is physical with a lower temperature coefficient. Therefore, low temperature would further enhance the action of the poisons.

However, according to the common belief at that time, respiration and glycolysis are not the only energy sources of the frog muscle. A third energy source comprises the reserves of what has been known as the "high-energy-phosphate-bond" compounds, notably creatine phosphate (CrP) and adenosine-tri-phosphate (ATP). I had also given thought to the possible existence of a fourth or even fifth source of energy, but eventually I was able to muster evidence that no additional energy source existed in frog muscle (and most likely not in other tissues either) beyond respiration, glycolysis and the high-energy-phosphate bonds (Ling *et al.*, 1973, pp. 11–12; Ling, 1984, p. 125; also see below for the cataclysmic discovery in regard to the very existence of the high-energy-phosphate-bond energy).

I also found that during the hours of maintained normal K^+ and Na^+ levels in the nitrogen-IAA poisoned frog muscles, the contents of creatine phosphate and ATP changed little (Ling, 1952, pp. 766–767, including Figure 4). From the quantitative data I could gather, a rough estimate was made. *The result showed that the minimum energy needed for the postulated sodium pump is at least four times higher than, or 400% of the maximally available energy to the muscle cell, even if (1) the muscle spends all its energy on pumping sodium, and even if (2) all the essential energy conversion and utilization processes operate at 100% efficiency* (Ling, 1952, p. 766–767, Figure 4).

In summary, observations reported in this pair of early articles squarely contradict what one would have expected if the K^+ and Na^+ concentrations in muscle cells really depend on the ceaseless activity of an energy-consuming sodium pump—and they left little doubt in my mind that the sodium pump hypothesis is not tenable. But that was only part of the story.

One day somewhere in 1950 I was sitting in one of the little cubicles in the Welch Library reading and thinking as usual. Suddenly an idea came up—an idea which had to be modified considerably before being presented at the Phosphorus Syposium held in Baltimore in 1951 (Ling, 1952, pp. 769–781). The central idea is that of a molecular mechanism for the selective accumulation of K^+ over Na^+ in living cells which requires no continual energy expenditure. To the best of my knowledge, a mechanistic theory of this nature—based on the fundamental principle of the branch of physics called statistical mechanics—had not been proposed before in the history of cell physiology. I shall return to this early theory and its further development below.

While I felt confident that the gist of my conclusion against the membrane-pump theory was correct, I would admit that some of the experimental methods I used could stand improvements and that, for a study of this singular importance, not enough repeat experiments had been done. Refinements of the methodology followed (parts of the new improved methods are described in Appendix 2). And I repeated the final version of the refined experiments many times over. Four years later in 1956, the definitive experimental data

were ready for publication. Six more years were to pass before they were published in my first monograph, "A Physical Theory of the Living State: the Association-Induction Hypothesis" (Ling, 1962, Chapter 8).

This monograph introduced not merely my definitive experiments against the sodium pump hypothesis on energy grounds, but also the association-induction hypothesis (AI Hypothesis)—which has grown beyond the molecular mechanism for the selective accumulation of K^+ over Na^+ in living cells into a general, unifying theory of cell physiology. However, the part of the AI Hypothesis on cell water was not included until three years later (Ling, 1965a). Because of its extreme importance, it bears pointing out here that a unifying general theory—not necessarily this one, although no other unifying theory to my knowledge existed then or has existed since—is the only remedy to heal the grave illness of intense fragmentation of the inherently coherent and indivisible science of cell physiology (see Ling, 1997b, also Rothschuh, 1973, pp. 348–351).

In the 30 years following, I wrote two more monographs: "In Search of the Physical Basis of Life" (Ling, 1984) and "A Revolution in the Physiology of the Living Cell" (Ling, 1992). Each volume describes the AI Hypothesis during its continued growth as well as the gathering of supportive evidence for this hypothesis.

While the two later books are still in print, my first monograph, "A Physical Theory of the Living State" is not. But all is not lost, since the copyright of the book now belongs to me and accordingly I have the right to reproduce part or the entirety of my out-of-print volume. Since the disproof of the sodium pump hypothesis on energy grounds was published nowhere else except in the 8th Chapter of the book—and this dispoof has been under attack (see below)—I decided to reproduce the entire 8th Chapter as Appendix 1 in this communication. (The assay methods originally used in work described in Appendix 1 were presented as Appendix D in the monograph; it too is reproduced here—as Appendix 2).

The work described in Appendix 1 culminates in Table 8.9. The data shown in this Table confirm my earlier rough estimate of a large disparity between energy need and energy available. However, with improvements made on all aspects of the work, the minimum energy needs of the postulated sodium pump have grown from 4-times to respectively 30-times (or 3060%), 15-times (or 1542%) and 18-times (or 1800%) of the maximally available energy—that is, if all energy available to the muscle cells is devoted exclusively to pumping Na^+, and if each and every intermediate step involved is 100% efficient.

A disparity of this magnitude between energy supply and demand leaves no room for loose arguments. In the decade or so following the publication of my first monograph, "A Physical Theory of the Living State," the gist of my disproof of the sodium pump hypothesis has been twice confirmed (Jones, 1965) (Minkoff and Damadian, 1973; for rebuttal of criticism on the 1973 paper, see Minkoff and Damadian, 1974). Meanwhile no one had challenged in print either my conclusions in regard to the disproof of the sodium pump theory or the experimental approaches taken to reach my conclusions. Thus, on energy grounds alone, the sodium pump hypothesis has been unequivocally disproved. The time was 1956, if one considers the time the definitive experiments were completed. It would be 1962, if one relies on the time of publication.

But the disproof of the membrane-pump hypothesis (including the sodium pump hypothesis) is far from being limited to the disproof on energy grounds alone. For the other independent disproofs of the membrane-pump hypothesis on *non-energy* grounds, including the failure to demonstrate selective K^+ and Na^+ distribution in a cell-membrane preparation

containing only a functional and anatomically intact cell membrane but no cytoplasm (i.e., the materials filling up the inside of a cell); and the success in demonstrating precisely the same in a cell preparation containing intact cytoplasm but no functional cell membrane and (postulated) pumps, see Ling (1978), Ling (1992, p. 20; pp. 51–55, for summary, p. 324–325), also Ling (1997a).

However, as will be made clear below, the case against the sodium pump hypothesis on energy grounds alone is even farther beyond dispute—if one assumes that as possible—than that presented by these 15- to 30-fold disparities between energy available and energy needed. First, the sodium pump is but one of *many pumps* needed, all requiring energy from the same source already far-gone in bankruptcy supporting just one (sodium pump). Second, the largest source of energy counted on in the study described above has evaporated into nothing. Or put more precisely, *that source has never really existed* except in an attractive but mistaken theory, and in the minds of those who, for one reason or another, have been tardy in coming to grips with the revolutionary discovery against the so-called high-energy-phosphate bond concept. All these and more will be made clear in a forthcoming section.

2. A Flabbergasting Discovery Twenty Years Later

Then suddenly in 1976, 14 years after the publication of the disproof of the sodium-pump hypothesis, a fledgling reporter for the *Science* magazine, Dr. Gina Kolata published in her widely read magazine, an article entitled "Water Structure and Ion Binding: A Role in Cell Physiology?" (Kolata, 1976). In this article she announced that two scientists, Drs. Jeffrey Freedman and Chris Miller had produced "crucial experiments and calculations . . . that provide strong evidence for the existence of pumps" (p. 1220). When this announcement belatedly came to my attention, I was absolutely dumfounded.

2.1 Where and how did Kolata get this stuff?

I was dumfounded because the same Dr. Gina Kolata had earlier, in 1975, sent to me and several other scientists a pre-publication manuscript on the same subject. We all thanked her for her courtesy. Each then responded to various aspects of what she wrote, and the responses all appeared in the Letters to the Editor Column of a later issue of *Science* (Ling, 1976). Yet neither my recollection of the content of Kolata's pre-publication article, nor that of my response, indicate that her article has a section describing the so-called "crucial experiments and calculations"—allegedly invalidating scientific work that took me and my coworkers years to accomplish. The question rose: Did Kolata send me an earlier version of her manuscript (and I responded to it), but put in print later a different version containing this "crucial experiment and calculations" statement absent in the earlier version?

I raised this issue in my letter to her dated June 21, 1996. She answered me on another subject (see below) but not on this one. On November 5, 1996, I wrote her a second letter addressing this issue once more and exclusively. Again I got no response. On February 5, 1997 I sent her a third letter, asking for a yes-or-no answer; this time the letter carried a return slip (which was duly acknowledged and returned to me). But still no answer.

Whatever the reason for her unwillingness to respond to my simple question, the statements she made in her article could very well have produced then and in the twenty some years hence the impression that I was not able to rebut the "crucial experiments and calculations"—utterly contrary to the truth (see below).

Feeling at that time that I had *already answered all* the relevant criticisms she raised in

her pre-publication manuscript, I turned to other pressing tasks to keep my laboratory going (see Ling, 1997, under Section entitled "Absolute Power Corrupts Absolutely"). Thus I was not to learn about Kolata's "crucial experiment and calculations" until twenty years later when a friend, Dr. Gerald Pollack, brought it to my attention.

Since Jeffrey Freedman and Chris Miller, the authors of the alleged "crucial experiments and calculations," were once my graduate students, the fact that I did not respond promptly to their broadcasted "crucial experiments and calculations" might also have been misconstrued, innocently or by design, as evidence that the *en masse* departure of my former graduate students from my laboratory (Ling, 1997) was not for what I believe to be its real cause (i.e., jobs and research grants were all totally under control of my scientific opponents) but purely for legitimate scientific reasons—a misconception that could be made into a deadly weapon against me and my work in the hands of those who would prefer that my NIH-supported work be put to an end, as it was (see Ling, 1997 again, under the section cited above and the succeeding section entitled "Twenty Morons at the Right Places Can Kill a Science [Erwin Chargaff]."

As my laboratory was being forcibly closed in 1988 at the height of its productivity by the withdrawal of all public support, I would have been thrown out of scientific research then and there, were it not for Dr. Raymond Damadian (and his Fonar Co.), who has offered shelter and support for myself and two close associates ever since (see Ling, 1997 under section entitled "Noah's Ark"). Did Kolata's pronouncement of "crucial experiments and calculations"—and related doing of my former students Miller and Freedman—play a role in the forced closing of my laboratory and in the state of de facto excommunication vested upon all those who oppose the sodium-pump theory? This is a question I only began to ask very recently.

2.2 Where were the alleged "crucial experiments and calculations" published?

Where did Jeffrey Freedman and Chris Miller publish their alleged "crucial experiments and calculations" which would, in Gina Kolata's opinion, make the membrane pumps energetically feasible and thus disprove my disproof of the sodium pump theory from energy consideration? And what are these "crucial experiments and calculations"?

Neither Freedman nor Miller had conducted any experimental studies on the energy need of the sodium pump in frog muscle while they were working in my laboratory. Could they have carried out new experimental studies elsewhere unbeknown to me after they left my laboratory? To find the answer, I went to the Citation Index and did a thorough search for all Freedman's and Miller's scientific publications. I could not find a trace of what could be regarded as the source of Kolata's "crucial experiments and calculations." I then wrote to all three: Kolata and my two former students, asking each to provide me with a copy of the published source(s) of the alleged "crucial experiments and calculations".

Freedman never answered my letter or another follow-up letter I sent later. It is conceivable that he had moved and the letters never got to him. But my letters were not returned. Kolata did answer and here is what she wrote in her letter dated July 16, 1996:

> "I am sorry to say that I can no longer remember where I got that information about Dr. Freedman and Dr. Miller's conclusions. I'm especially chagrined because Dr. Miller and Dr. Freedman never published their results and Dr. Miller says I never even interviewed them. In retrospect, that seems hard to believe, but I was very

> young then and maybe I really was so inexperienced that I included that statement about their work without calling either Dr. Miller or Dr. Freedman. . . . "

This explanation notwithstanding, what she reported to her world-wide audience on Freedman's and Miller's "crucial experiments and calculations" was and has remained the centerpiece of her whole article, refuting my definitive work. And the validity of her claims had not been challenged until just now (Ling, 1997, under "Misled from Day One by a Wrong Theory" and in particular, Ling, 1997b). Miller's letter dated June 28, 1996, corroborated the fact that Kolata did not interview him or Freedman. Strangely, Miller also could not or would not provide me with a copy of the published source of Kolata's "crucial experiments and calculations". This is the relevant part of what Miller wrote:

> "When I was a postdoc at Cornell and Jeff was one at Yale, the two of us, still fresh from the tumultuous experiences in your lab, wrote a manuscript on analyzing Na^+ efflux data in muscle—a sort of literature review—taking into account compartmentation effects. It was an attempt to show, among other things, that your apparently fast efflux data could be reconciled with a multicompartment membrane theory. (Remember, though, that I also considered the rates measured in your zero-degree experiments too high, as I discussed at length in my thesis.) Jeff and I had fun writing the paper, and we sent it to *J. Membrane Biology*, I believe, where it was immediately rejected . . . for not having any new data—going over the same old ground once again. We didn't try to publish it after this rejection. . . . But we may have circulated it around to friends, etc. So maybe she heard about it in the grapevine. That's just conjecture . . . As for citing it, that's impossible: never having passed through the fire of peer-review, it doesn't exist, and so it isn't part of the literature—nothing for you to argue with. I don't have a copy of the paper, having thrown out the manuscript as useless junk over a decade ago."

This letter has made it abundantly clear that neither Miller nor Freedman carried out experimental study on the (postulated) sodium pump *after* they left my laboratory—just as they had never studied it *before* leaving my laboratory. Therefore Kolata's alleged "crucial experiments" never existed. Who then made up the story of "crucial experiments"? Kolata? Miller and Freedman?

What about Miller and Freedman's alleged "crucial *calculations*"? Did they exist? Before attempting to answer the questions, let us get a bearing on what we do and where we stand. Let us begin with the fundamentals of the intellectual pursuit we call Science.

In his book, "The Search," C.P. Snow wrote: "The only ethical principle which has made science possible is that the truth shall be told all the time . . . And of course a false statement of fact, made deliberately, is the *most serious crime* a scientist can commit" (Snow, 1959). Even earlier, Charles Babbage, the English mathematician who invented the first mechanical calculator, gave names to the three kinds of scientific frauds, including "cooking," i.e., "to select (for publication) those only which agree or very nearly agree" (Babbage, 1830, p. 178).

Yet *not telling the truth* seems to have become fashionable among the most prestigious cell physiologists, beginning with the first-of-its-kind review on the subject of "The Sodium Pump."

In this review I.M. Glynn and S.J.D. Karlish (1975) from the Cambridge Physiological Laboratory, Cambridge, England, cited 245 articles all supporting the sodium pump hypothesis and none against the hypothesis, including all of my own on the subject. And the new "style" Glynn and Karlish thus introduced, was imitated in a stream of reviews on (more or less) the same subject in years following. All in all, this tactic of telling "half truth" by influential review-writers has worked wonders in accomplishing what legitimate scientific experimentations and open debate could never have done—silencing the opponents of the long-ago-disproved membrane-pump hypothesis (for details of this unbelievable transgression and desecration of Science, see my home page, Ling, 1997, section entitled "Absolute Power Corrupts Absolutely").

It is truly depressing but hardly surprising that a young scientist trying to find his way in such an environment learns soon how to practice the art of a magician—creating the right kind of illusion for the right audience.

The success of a magician lies in his/her ability to make you see and believe what the magician wants you to see and believe—something deceptive and untrue. This is usually done with smoke and mirrors. But as you will discover below again and again, in the cell physiological science in its current state, it may be easier. All you need to do is to assume the role of an impartial specialist and tell the part of the study you want them to hear and withhold the rest— after making sure that the story you want them to hear happens to be what those in power also like to hear.

3. Game of Illusion: Part 1

Even though Miller now is unable or unwilling to present me with a copy of what he and Freedman once composed in fun, he did, in his June 28, 1996, letter cited above, mention that he had already rejected the association-induction hypothesis while still in my laboratory, and that he has given the reasons for this rejection *at length* in his Ph.D. Thesis (italics mine).

However, before opening the pages of Miller's Ph.D. Thesis, let us first construct a bird's-eye view of the whole domain of asymmetrical ion and non-electrolyte distribution across the surfaces of living cells. Only with such a complete 360 degree vision of the background knowledge are we in a position to evaluate judiciously Miller's (and Freedman's) attempt to resurrect the sodium pump hypothesis for what it really is.

3.1. The sodium pump is but one of many pumps, some already postulated and others not yet postulated but which must be postulated.

The sodium pump hypothesis is nothing much more than just a name, a rephrasing of one *arbitrarily*-chosen observation out of context of a much larger and broader problem. It was an ad-hoc make-shift device to patch up a crumbling *sieve membrane theory* (Boyle and Conway, 1941), when the supposedly impermeant (large hydrated) Na^+ turned out to be fully permeant (Wu and Yang, 1931; Kaplanski and Boldyreva, 1934; Heppel, 1939; Cohn and Cohn, 1939; Steinbach, 1940).

As far back as 1955, I have clearly pointed out (Ling, 1955, p. 94, paragraph 2) and repeated again and again afterward (Ling, 1962, p. 216; 1969, p. 6; 1992, p. 17; Ling *et al.*, 1973, pp. 8–10) that if the membrane pump hypothesis is offered as a *bona fide* replacement of the now disproved Sieve Membrane Theory, it cannot be limited only to this arbitrarily chosen Na^+ ion alone. Instead, "we must have 'pumps' for all these ions" which are, like the

Na^+, also permeant to the cell membrane, and also do not follow the predicted (Donnan) distribution ratio according to the membrane theory. Nor can one postulate pumps only for ions. Pumps must be postulated for nonelectrolytes, chemicals bearing no net electric charges; many of which do not follow the equal distribution pattern predicted by the membrane theory from its basic tenet that a living cell represents a membrane-enclosed dilute water solution.

Thus while it is entirely legitimate and meaningful to choose just one pump to ***disprove*** *the membrane pump theory on energy ground—as I did in 1962. To argue that the membrane pump theory is tenable energetically—as Freedman and Miller attempted to do, and as Kolata's alleged "strong evidence for the existence of pumps" was supposed to substantiate [Kolata, 1976, p. 1220], much more is required.*

(1) *One must take into account not just one, two or even three but* ***every single pump*** *which must be postulated to keep the living cell afloat, and then explain why no more pumps are yet to be added. Only when that question has been unequivocally answered, can one draw up a truly complete list of all the pumps needed. Indeed, it would be a meaningless waste of time to proceed to the next step before such a truly complete list is on hand.*

(2) *Based on that truly complete list of required pumps, one can then show that the* ***total*** *energy needs of all these pumps added together must fall well within the limit of the energy available.*

(3) *I must also add that the defenders of energy solvency of the membrane pump theory cannot—as its challenger can—assume without proof that the cell has no other energy need than pumping ions, non-electrolytes and other solutes, but must account in detail what these extra energy needs are, and the indisputable reason why no more additional ones might arise, and add all these non-pumping energy needs to the energy needs for all truly complete lists of pumps in arriving at the final energy balance sheet.*

(4) *I must also point out that the defenders of the energy solvency of the membrane pumps cannot—as the pump challengers can—assume that all the energy conversion and utilization processes are indeed carried out with 100% efficiency without proof. Rather the defenders must find out what the efficiency of each and every step involved is, and produce concrete proof that indeed they are not otherwise. And then take the departure from 100% efficiency also into account in the final energy balance sheet.*

From Miller's letter cited above, the reader can see that Miller (and presumably Freedman too) did not do all that is required to resurrect the sodium pump hypothesis. However, the question arises: "Could Miller (and Freedman) be unaware of the need for *many* other pumps at the time he wrote his Thesis?"

The answer is a resounding No. At the time when Miller was preparing his Ph.D. thesis—in which he announced his rejection of the AI Hypothesis,—there were at least 20 pumps already published. Some of them are not single pumps but long lists of pumps like the various sugar pumps and various free-amino-acid pumps. In fact, a list of all these pumps was collected from the literature and put together as a table—by none other than Miller himself.

Furthermore, this table, reproduced here as Table I, was later published in a review article, which Miller co-authored with Ochsenfeld and myself (Table 2 in Ling, Miller and Ochsenfeld, 1973). I may mention that we will return to this review article again and again below.

TABLE. I. A Partial List of Postulated Membrane Pumps*

Solute	Direction	System	Reference
Na, K	coupled	many cells	169
Ca^{++}	outward	RBC, striated muscle	170, 171
Mg^{++}	ouward	frog sartorius	172
$Choline^{+}$	inward	RBC	173
Amino acids	inward	RBC, muscle, tumor	174–176
D-xylose	inward	rat diaphragm	177
D-xylose	outward	rat diaphragm	178
Na^{+}	inward	frog sartorius	179, 180
Noradrenaline	inward	vascular smooth muscle	181
Prostaglandins	inward	mammalian liver	182
Curarine	inward	mouse diaphragm	183
Br^{-},I^{-},ReO_4^{-}, WO_4^{-}	outward	Ascites	184
Cu^{+2}	inward	Ascites	185
Aminopterin	inward	Yoshida sarcoma	186
Cl^{-}	inward	squid axon, motor neurons	187, 188
Mn^{++}	inward	*E. coli*	189
Cl^{-}	outward	*E. coli*	189
Sugars	inward	*E. coli*	189
Amino acids	inward	*E. coli*	189
Tetracycline	inward	*E. coli*	190

* Data collection was more or less arbitrary and not intended to be comprehensive. (For sources of references, see Ling *et al.*, 1973.)
(From Ling *et al.* (1973) by permission of the New York Academy of Sciences.)

All these pumps Miller had gathered from the literature are pumps postulated to exist at the cell membrane, also called plasma membrane. As I pointed out repeatedly, pumps are also needed at the membranes of the subcellular organelles because the ion and nonelectrolyte distributions across their surfaces are also as a rule asymmetrical (Ling, 1988, p. 873; 1992, pp. 17–20). One example of such subcellular organelles is the *sarcoplasmic reticulum* (SR). In frog muscle cells, the SR has a total surface 50 times larger than the plasma membrane (Peachey, 1965). Since the energy need of the membrane pump (otherwise the same), varies directly with the surface area of the organelle, a similar pump at the surface of the SR would consume 50 *times* more energy than that at the cell membrane.

Then we must also recognize that the SR is not the only subcellular organelle in need of pumps. Mitochondria is another one. The mitochondria in rat liver, for another example, also need pumps and have a surface area 20 times that of their plasma membrane (Lehninger, 1964, Chapter 2) and a correspondingly large appetite for energy to run all the pumps.

Not only do sodium ion, magnesium ion, chloride ion, bicarbonate ion, sugars, free amino acids etc., etc. found in the cells' natural environment require pumps, exotic solutes as a rule also do. Among these exotic solutes are those chemicals *synthesized for the first time by organic chemists* (Ling, 1988, p. 873; 1992, p. 18). The need of pumps for these man-created chemicals poses what I believe to be two more difficult if not insurmountable problems for the defenders of the membrane-pump theory:

First, since the living cell's genome had never been exposed to these new molecules in past history, how could the cell evolve genes for pumps in *anticipation* of their future synthesis by humans?

Second, since there is no limit to the number of new chemicals organic chemists can create, how is the limited space of the plasma membrane and the subcellular particle membrane able to accommodate an infinity of pumps?

All these daunting, if not downright insoluble problems, in addition to the equally hopeless task of resolving the vanishing energy source problem to be described next, must be solved before one can claim that the sodium pump hypothesis is energetically feasible.

3.2 Vanishing energy source in nitrogen-IAA poisoned muscles

In my computation of the maximum energy available to nitrogen-IAA poisoned muscle cells in 1962 (see Appendix 1), that energy came almost entirely from the "high energy phosphate bonds" of ATP and phosphocreatine initially present in the muscle cells. However, by the time my work was published, Podolsky and Kitzinger (1955), Podolsky and Morales (1956) and George and Rutman (1960) had conclusively and unequivocally demonstrated that the high-energy-phosphate-bond concept itself was a mistake. *There is no usable high-energy in the phosphate bonds of ATP and of phosphocreatine to speak of.* (For a discussion on the possible source of error in the original overestimated enthalpy of hydrolysis of ATP, see long footnote on page 195 of Appendix 3, which reproduces Section 7.1 of Ling, 1962).

Among the many major impacts of this momentous discovery, was that the maximum energy need I computed for the sodium pump must be revised. As a result, the figure of –22.97 cal/kg/hr for the total available free energy of poisoned frog muscle as given in my original Table 8.5 in Appendix 1—a sum of the contribution from the decomposition of creatine phosphate (–21.57 cal/kg/hr), of ATP (–0.64 cal/kg/hr), of ADP (–0.18 cal/kg/hr) and of residual glycolysis producing lactate (–0.56 cal/kg/hr)—must now be replaced by the free energy from the trivial residual lactate production alone. This, of course, is equal to a measly –0.56 cal/kg/hr. *A forty-fold (40 times) reduction* of the maximum available energy is the result.

With this corrected maximum available energy, the disparity between minimum energy need and maximum energy available would become even wider from 30.6 times, 15.4 times and 18.0 times (Table 8.9, Appendix 1) to *1224 times, 616 times, and 720 times* respectively (Ling, 1992, pp. 12–16). However, due to partial adsorption of intracellular Na^+ in muscle cell, there is a need for a two-fold reduction of these figures to *612 times, 308 times, and 360 times* respectively (see below). The 15–30 times figures, powerful and forbidding as they are, are already obsolete.

All of these new revelations of an endlessly rising number of energy-demanding pumps and vanishing energy source to power them are what a scientist seriously trying to resurrect the membrane-pump theory in general and the sodium pump hypothesis in particular, must deal with. Doing less—no matter how innocently and under what difficult circumstances—and claiming that the sodium pump is energetically feasible would have exactly the same impact on Science and Humanity, which must in the long run depend for its survival on a sound and healthy Science, as that of deliberate deception.

The sad truth is: Miller ignored all the long list of pumps he himself collected from the literature. He made no mention at all of the revolutionary discovery since 1956–1960 that the so-called high-energy phosphate bond contains no high energy.

4. A Decade-long Investigation Revealing that the Accepted "Na^+ Pumping Rate" is Fully a Whole Order of Magnitude Too Slow

Gina Kolata wrote on page 1222 of her report (Kolata, 1976):

> *"They (Freedman and Miller) report that Ling's analysis of his data led him to assume that the sodium was being transported out of the muscle cells at least 20 times faster than the rate accepted by muscle physiologists"* (Kolata, 1976, p. 1221).

Offhand, I might simply retort: "So what?" Taking into consideration that the issue of vanishing energy source alone has raised the energy discrepancy 40-fold, a 20 times over estimation of the pumping rate—*even if it were true*—would still leave the pump as dead as dead can be.

But I choose an alternative way of responding, not forgetting that my definitive disproof of the sodium pump hypothesis of 1956 (and reproduced in Appendix 1), augmented by the vanishing energy source (and list of many pumps) has already made this detailed nickel-dime hassling of no significance whatever. The pump has been dead since 35 years ago and it remains dead.

But I am not a trial lawyer. My objective is not limited to winning a debate—important as it is. As a scientist, I also owe to all my (worthy) fellow scientists living and yet to come, my evaluation of the validity of every accusation directed at my work—no matter how trivial and how spurious. And to do it by subscribing to an exactly opposite approach to what we have witnessed with mixed disbelief, disgust and sadness: telling half-truths. Namely, by providing you with *all* the relevant information which I know on the subject as well as references to what I think may be relevant but too bulky to include. At the end you yourself will find that in contrast to what Kolata had claimed, that I *overestimated* the Na^+ pumping rate by a factor of 20, it was the majority of so-called "muscle physiologists" who has *underestimated* the Na^+ "pumping rate" by a factor of 10 or even higher.

4.1 A major discrepancy between the size of the fast fraction and the size of the extracellular space

After radioactive sodium isotopes (e.g., ^{24}Na) became available, Levi and Ussing (1948) reported the historical first Na^+ efflux studies from living cells—efflux meaning outward flow or movement, while influx means inward flow. These workers first immersed an isolated frog sartorius muscle for two hours in a Ringer's solution containing radioactively-labeled Na^+. (A sartorius muscle is a thin sheet of a muscle found on the under surface of each thigh of a frog. It is usually 2.5 cm to 3.0 cm long, half a cm wide and half a mm thick, comprising about a thousand or so, parallel-arranged, thread-like muscle cells or fibers.) Levi and Ussing then washed the labeled-Na^+-loaded muscle in a non-radioactive Ringer's solution and recorded the falling labeled-Na^+ concentration in the muscle, $[Na^*]_{in}$, as the washing continued.

The logarithm of $[Na^*]_{in}$ remaining in the muscle at different times, after washing began (as ordinate) was then plotted against the time of washing, t (as abscissa). This semilogarithmic plot is curved but can be readily resolved into two "straight-line" fractions, one fast (with a steeper slope) and one slow (with a flatter slope). Levi and Ussing assumed that the fast fraction came exclusively from labeled Na^+ trapped in the *interfibrillar* or *extracellular space*, the space between the 1000 or so string-like individual muscle cells or fibers making

up the sartorius muscle. *They also assumed that the slow fraction represented the surface-, or membrane-limited efflux of labeled Na^+ emerging from within the muscle cells referred to as the Na^+ efflux from the muscle cells.* The rate of the Na^+ efflux from the muscle cells was determined from the slope or half time of exchange ($t_{1/2}$) of the slow fraction. ($t_{1/2}$ is the time it takes for the radioactivity in this fraction to fall to half of its initial value.) This assignment of the sources of the fast and slow fraction seemed reasonable at the time and has been accepted by virtually all the workers in the field. But not all.

4.1.1 A clue from the incongruously small size of the extracellular space

I began to have doubts about this assignment long before I carried out the definitive energy-balance study before and during 1956. The main reason for my doubts then was that the size of the fast fraction of labeled Na^+ leaving the muscle—which is obtained by extrapolating the fast fraction to zero t—is much larger than the size of the extracellular space. If Levi and Ussing's assignment were correct, these two sizes should match. But they don't. And the discrepancy is not so small that one can ignore it.

I was not alone in noticing this discrepancy. For example, Levi and Ussing themselves saw it in their own data. Six assays of theirs yielded fast fractions equal to 27.0%, 34.6%, 28.0%, 29.6%, 32.2% and 32.1%, averaging 30.1% of the muscle weight (Levi and Ussing, 1948, Table 1). And they remarked: "These values are all much higher than the generally accepted 13%" (for the extracellular space of frog muscle) (Levi and Ussing, 1948, p. 242). (For Levi and Ussing's explanation of this discrepancy, see Section 5.2.1 below.) As another example, Johnson also measured the fast fraction of Na^+ efflux of the sartorius muscle and found an even larger fast fraction at 37.2% ± 2.9% (s.e.) (Johnson, 1955).

Since there was general agreement on the (incompatibly) large size of the fast fraction, one asks: "Could the extracellular space be underestimated?" While the definitive answer was to come many years later, there was enough evidence even then to show that Boyle *et al.* (1941), responsible for this 13% figure, did not underestimate the size of the extracellular space. Indeed, if anything, they overestimated it (see below).

Table II taken from Ling *et al.* (1973) shows the chloride-ion-distribution data I had collected from the literature many years ago. By adopting a set of more or less arbitrary standards (individual assays must exceed 10, and the standard error of the mean must be 5% or smaller of the mean), I was able to select 4 sets of mutually-supporting data from the 7 sets I could find in the literature. These four sets surviving the test yield an average chloride-ion content of 9.8 μmoles per gram of fresh muscle. The chloride-ion concentration in frog plasma is 76.8 μmoles/ml. (Fenn, 1936). Dividing 76.8 into 9.8, one obtains a 12.8% "chloride space" in the frog sartorius muscles.

On the surface, this 12.8% value agrees with Boyle *et al.*'s 13% figure. But 12.8% should be recognized as an *upper limit* of the extracellular space because not all the chloride ion seen in the muscle could be in the extracellular space. Some of that chloride is bound to be within the muscle cells (Boyle and Conway, 1941).

These chloride-space data further strengthened my doubt that the fast fraction of Na^+ efflux originates exclusively from the extracellular space and that the slow fraction originates exclusively from within the muscle cells—as Levi and Ussing originally suggested. This doubt urged me to look for a new and more accurate way to measure the (presumed) Na^+ pumping rate of frog muscle for my energy-balance study in the 1950s. I shall describe the new way I adopted in Section 4.2 below.

TABLE II. Chloride Ion Content of Frog Muscle*

	Katz	Urano	Meigs and Ryan	Maurer	Cho	Fenn	Boyle and Conway
Number of detns.	2	2	2	23	94	79	13
Cl content, μmole/g (± S.E.)	11.2 not included	12.5 not included	18.6 not included	15.8 ± 0.8 not included	10.4 ± included	8.4 ± 6 included	10.5 ± 1.4 included
Average						9.8	

* Criteria for choosing data: number of determinations must exceed 10, and the S.E. must be smaller than 5% of the mean. The first four sets of data were excluded for one or the other of these reasons. (For sources of references, see Ling *et al.*, 1973.)
(From Ling *et al.* (1973) by permission of the New York Academy of Sciences.)

Between 1967 and 1975, I and my coworkers introduced four new methods to determine the extracellular space of frog sartorius muscle. Using these methods and a modified version of an old method (inulin probe method), we were able to establish unequivocally that the extracellular space is slightly below 10%: low-concentration-inulin probe (10.3%) (Ling and Kromash, 1967), poly-L-glutamate probe method (8.9%) (ibid), single-muscle-fiber-sucrose-space method (9%) (Ling *et al.*, 1969), ^{86}Br-analysis method (8.2%) (Ling, 1972) and the centrifugation method (9.4%) (Ling and Walton, 1975a). The average extracellular space of frog sartorius muscle is *9.2% ± 0.69%* (mean ± s.d.). With this full assurance from the future, let us return to the fifties again.

The striking discrepancy between the size of the fast fraction of the Na^+ efflux and the size of the extracellular space led me to the following working hypothesis some time in the fifties:

> *The true surface-, or membrane-limited efflux of labeled Na^+ from within the cytoplasm of the muscle cells makes up a part of the fast fraction.*

As mentioned, in the course of the next 30 years or so, I and my coworkers developed four new methods to determine the true surface-limited Na^+ flux rate, which will be presented below. Before doing that I must mention another seemingly minor but in truth highly significant error in the conventional method of determining the Na^+ efflux rate, an error that I began to correct since the time I was engaged in the work described in Appendix 1.

4.1.2 CTE: A widely overlooked source of error

Overlooked by most workers in the study of Na^+ efflux of frog muscle are what I call the "connective tissue elements" (CTE). Or perhaps one should say that the few who did recognize a possible impact of the components of CTE (e.g., sarcolemma) did not evolve a

method to combat it, so that in the end, the CTE contribution was ignored in virtually all work on the subject.

These "connective tissue elements" or CTE consist of small nerve fibers, blood vessels often on the under-surface and also elsewhere in the muscle as well as blood cells trapped in the blood vessels. By far the most important component of the CTE are the various types of connective tissues making up the parts and parcels of the muscle as a tissue. They include the tendons at the tibial end of the muscle, the fascia covering its dorsal surface, and the sarcolemma enclosing every muscle cell. Like the muscle cells themselves, these CTE components also take up and release labeled Na^+ in their own ways and thus subvert the validity of conclusions drawn from the efflux study of a sartorius muscle as representing efflux from muscle cells only.

To correct for the error in Na^+ efflux data of frog sartorius muscles caused by the CTE, we must first find out how much CTE is present in a sartorius muscle and then reproduce the CTE's own Na^+ efflux curve so that their contribution to the Na^+ efflux of a sartorius muscle can be quantitatively subtracted to reveal the true Na^+ efflux from the pure muscle cells. The method adopted for this correction was presented for the first time and exclusively in Chapter 8 of my 1962 monograph reproduced here as Appendix 1 (Table 8.8).

A microscopic examination of a sartorius muscle reveals that the CTE just described usually run continually to merge with thin sheets of loose connective tissues covering parts of the surface of the thighs and the calves. Furthermore, these loose connective tissue sheets can be readily peeled off (preferably from the same legs providing the sartorius muscle or semitendinosus muscle, for explanation of its preference to the sartorius muscle, see below) were obtained. This anatomical continuity offers justification for regarding the isolated connective tissue sheets as models for the CTE in the muscle.

Using the alkaline digestion method of Lowry *et al.* (1941), I then estimated the pure collagen contents of both the sartorius muscle and the connective tissue sheets from the same legs. Comparing these collagen contents with each other, I arrived at the conclusion that on the average, the CTE makes up 9.09% of the fresh weight of the muscle. I then studied the Na^+ efflux of such a piece of connective tissue sheet in the same way I studied the Na^+ efflux of a sartorius muscle. Figure 1, taken from Ling and Walton (1975b), shows the "efflux curves" of two pieces of loose connective tissue sheets after 43 min of incubation at 25°C.

Note on one hand the general resemblance of these curves in Figure 1 to the muscle efflux curves (to be shown below in Figures 2A,2B and 4), and on the other hand, how much higher is the initial labeled Na^+ contents of the CTE (ca. 70 μmoles/gram of fresh tissue) compared to that of a normal sartorius muscle (ca. 20–30 μmoles per gram). In contrast, a similar piece of connective tissue sheet after a short exposure of only 4 min and 24 seconds in the radioactive labeled solution reveals an initial labeled Na^+ content of less than 20 μmoles/gram of fresh tissue(Appendix 1, Figure 8.6A). This large difference seen between the initial Na^+ contents of the two sets of connective tissue data shows that the duration of incubation in a radioactive-Na^+-containing solution plays a highly significant role in determining the level of contaminations of the CTE to the labeled Na^+ content as well as apparent efflux rate of a sartorius muscle. Another seemingly trivial but in fact important difference worth mentioning is that after long exposure to the labeled Na^+-containing solution, the later part of the CTE efflux curve often appears much flatter than the corresponding part of efflux curves from muscle cells, especially after correction for CTE had been made, see Figure 6.

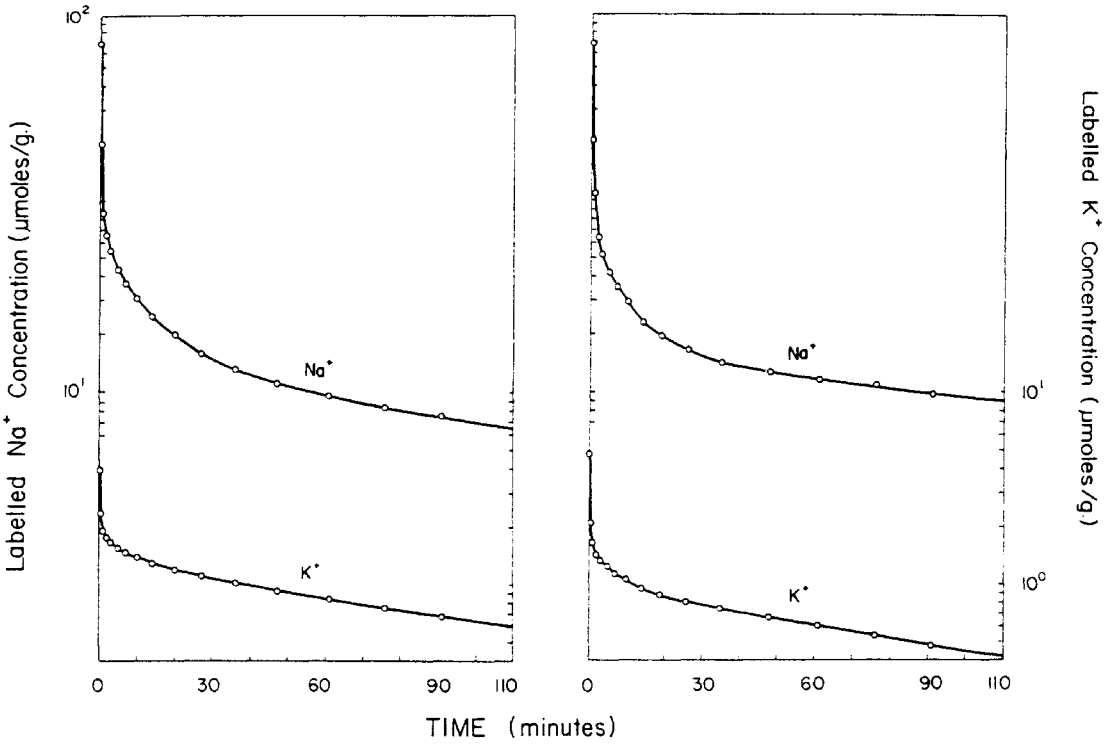

FIGURE 1. The time course of labeled Na^+ and labeled K^+ efflux frog connective tissue. The connective tissue sheets were isolated and incubated at 25°C with both labeled K^+ and labeled Na^+ for a total of 43 min and centrifuged at 1000 g for 4 min to remove the centrifugation-removable fluid. They were then washed in successve tubes of Ringer's-phosphate solution at 25°C. Data are given as μmoles per gram of fresh tissue. (From Ling and Walton, 1975a, by permission of *Physiol. Chem. Phys. & Med. NMR.*)

[The CTE makes up 9.1% of the fresh muscle weight (Table 8.8 in Appendix 1). However, as pointed out by Ling *et al.* (1969), it contains water which is accessible to the extracellular-space probe, inulin and therefore constitutes a part of the water in the extracellular space measured. This means that the 9.2% extracellular space correction must be appropriately reduced after a CTE correction has been made. For a more elaborate and precise method of correcting for both the extracellular space and the CTE components, see Ling and Walton, 1975.]

Ignoring the contribution of CTE, as in the conventional way of studying ionic fluxes from frog muscle tissues, leads to *inaccuracy. The most serious inaccuracy created is a spurious slowing down of the rate constant (or lengthening of the half time of exchange, $t_{1/2}$) of the slow fraction from the muscle cells.* This inaccuracy becomes especially serious (1) if the muscle has been soaked in the radioactively labeled solution for a long period of time as it was routinely done in many laboratories; (2) if the later portion of an efflux curve from a muscle is employed to estimate the time constants of Na^+ efflux from the muscle cells and (3) if the experiment is done at a low temperature, say 0°C. Due to the much slower rate (than muscle cells) at which CTE releases its (adsorbed) labeled Na^+ after prolonged washing especially at low temperature (compare the later parts of the plots in Figure 1 and Figure 4), the contamination due to the contribution of the CTE becomes progressively larger as the

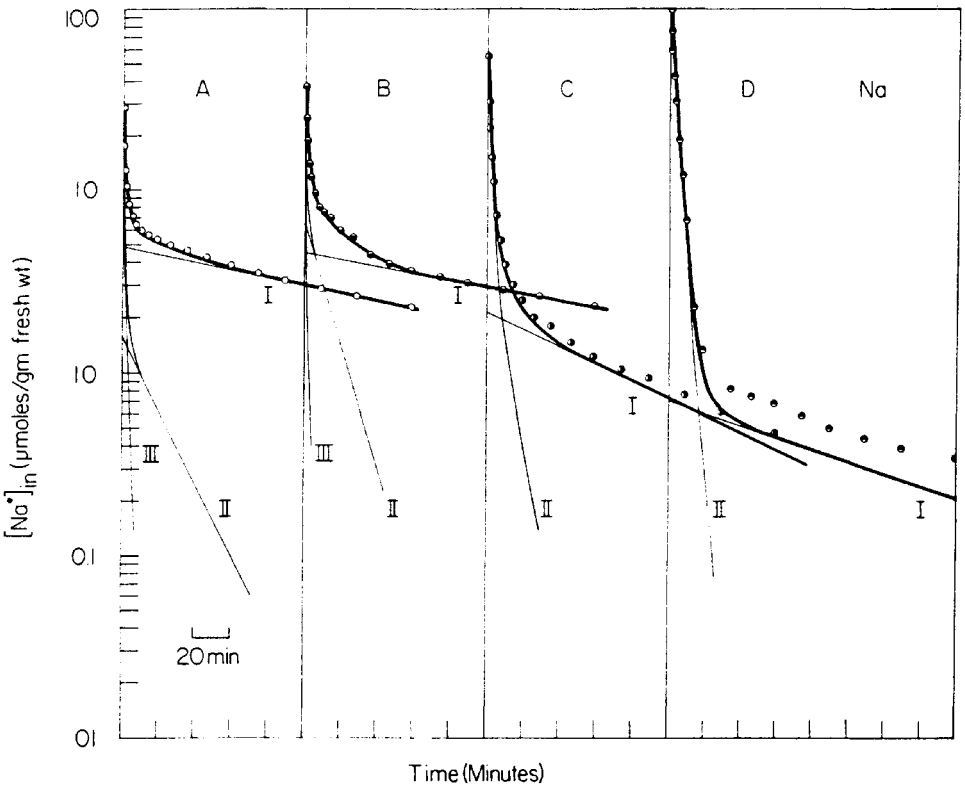

FIGURE 2A. Successive efflux curves of labeled Na^+ in a dying frog muscle. The sartorius muscle was repeatedly loaded with labeled Na^+ and washed in a Ringer's solution containing 0.05 mM sodium iodoacetate. In this and the following figure, curve A was the first washing curve, curve B, the next and so on. Heavy solid lines are the best fitting curve to the experimental points after subtracting the connective tissue contribution similarly loaded wih labeled Na^+ and exposed to IAA. These corrected curves were then resolved into a slow fraction (I) and a fast (II) or even a third fast fraction(III). Note that the slow fraction remained unchanged in magnitude—as indicated by its zero time intercept on the ordinate—until it falls to even lower values. It is the fast fraction (II or II + III) which rose steadily until it reached the concentration in the loading solution (100 mM). (From Ling *et al.* (1981) by permission of the *J. Cellular Physiology*.)

duration of washing increases. At worst, what the investigator thinks that he/she is measuring (membrane-limited efflux from (pure) muscle cells) may be largely the desorption rate of Na^+ from the CTE.

This CTE correction was routinely applied in all my work described in Table 8.6 and 8.8 and elsewhere in my 1962 monograph (Appendix 1). Curiously, Miller who knew this CTE correction well, saw nothing wrong in the data of Keynes and Steinhardt—who seemed entirely oblivious of this serious source of error—but cited their 1976 publication as an exemplary piece of work. Indeed, it was on the basis of this uncorrected data of Keynes and Steinhardt, Miller then passed the judgment that my data were about 20 times too fast. However, Keynes and Steinhardt's error was far more than merely ignoring the CTE contribution as will be made clear below.

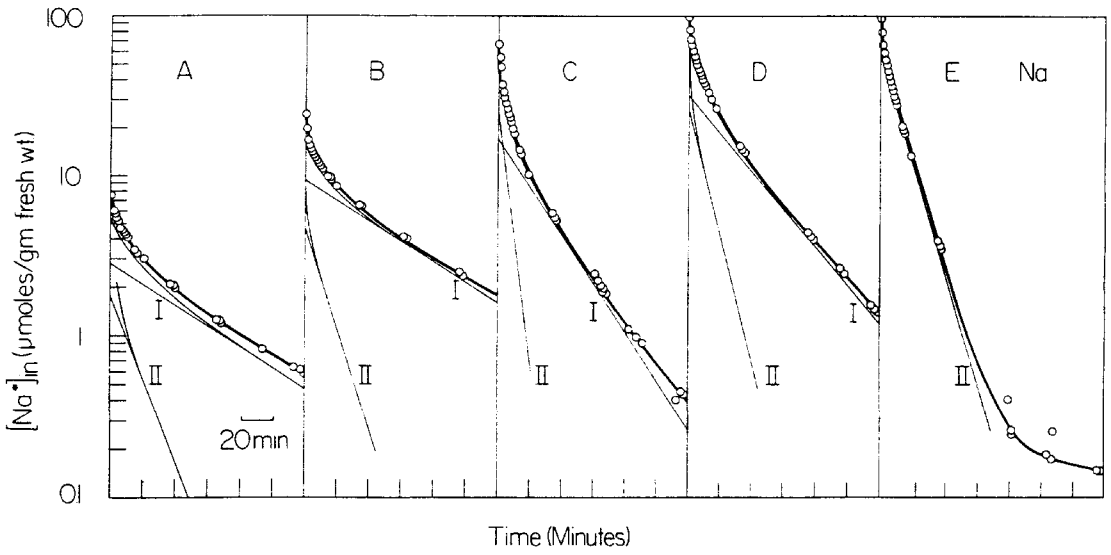

FIGURE 2B Successive efflux curves of Na^+ from a dying frog sartorius muscle. For experimental details and source, see legend of Figure 2A.

4.2 The correct membrane-limited-Na^+-efflux rate (i.e., "pumping rate") is some 10-times-higher than the value subscribed to by the majority

What follows is a brief review of four independent sets of studies published between 1970 and 1980 which in a mutually complimentary fashion demonstrate that the conventional assignment of the *slow* fraction of Na^+ efflux curve as representing the cell-surface-limited efflux from muscle cells is wrong, and that it is a part of the *fast* fraction that represents this surface-limited efflux of labeled Na^+ from the muscle cell cytoplasm, or sarcoplasm. The four sets of experiments in general and the one described under section 4.2.4 in particular have also provided for the first time unadulterated surface-, or membrane-limited Na^+ efflux rate from frog muscle cells at room temperature.

4.2.1 From dying muscles

When frog muscle is exposed to a low concentration of the poison, iodoacetate (0.2 mM IAA), the muscle slowly deteriorates until it dies. In this dying process, the total Na^+ concentration in the muscle cells rises slowly from 20 to 30 μmoles per gram of fresh muscle cells to approach the Na^+ concentration in the bathing Ringer's solution (ca. 100 mM or 100 μmoles per gram) (Ling *et al.*, 1981).

Isolate a frog sartorius muscle and immerse it for some time in a Ringer's solution (called Solution A) containing both IAA at a low concentration and radioactively labeled Na^+, to be followed by washing the muscle in a Ringer's solution containing only IAA but no radioactivity (call it Solution B). This way one can obtain a Na^+-efflux plot of the poisoned muscle. If at the end of say one hour of washing in Solution B, one soaks this muscle in Solution A again, and then starts washing it again in non-radioactive Solution B, one obtains a Na^+-efflux plot of the muscle in a more advanced state of poisoning. The cycle of soaking and washing can be repeated a number of times, each time producing yet another Na^+ efflux

plot of the muscle in a more advanced state of poisoning. (In order to minimize cell deterioration during the reloading steps, various methods were used, for details see original article.)

Each of these plots of Na^+ efflux curves can be resolved into a slow and a fast fraction. Now if the conventional assumption is right and it is the slow fraction which represents the membrane-limited efflux of intra-cellular Na^+, one would expect that this slow fraction should grow bigger and bigger as the cells deteriorate until the Na^+ concentration represented by this fraction reaches that of the incubating solution when the cells die. But we did not see that. What we did observe was quite the opposite.

As the cells deteriorated, the size of the slow fraction in more than half of the cases observed remained at 10 μmoles/gram or lower from the time the muscle was virtually normal until it was dead (Figure 2A and Figure 3A). In other cases, the slow fraction went up momentarily to some 20 μmoles/gram or so before falling down to lower values again (Figure 2B and Figure 3B). In either case, the slow fraction never rose to approach the high concentration of Na^+ in the bathing medium at 100 μmoles/gram.

Instead, it was the *fast* fraction of Na^+ efflux which steadily rose in size with each re-immersion in the labeled poison solution. When the cells died completely, the labeled sodium ion in the *fast fraction* approached the high concentration in the soaking Solution A (Figures 2A, 2B and 3). These experiments demonstrate that it is a part of the fast fraction from the muscle that represents the Na^+ efflux from within the muscle cells (Ling *et al.*, 1981). But the fast fraction seen here is a mixture of labeled Na^+ from inside the muscle cells and from the extracellular space. In the next approach, we eliminated the fraction from the extracellular space.

4.2.2 *From whole sartorius muscles after removal of their labeled Na^+-containing interfibrillar fluid*

In 1975 Ling and Walton (1975a) developed a new centrifugation technique to remove quantitatively *all* the *extracellular space fluid* from a frog sartorius muscle. They first exposed an isolated sartorius muscle to a Ringer's solution containing radioactive Na^+ isotope and then removed the labeled Na^+ caught in the extracellular space of the muscle by this technique—before washing the muscle in a non-radioactive Ringer's solution (Ling and Walton, 1975b).

However, as shown in the four sets of data presented in Figure 4 (in which the simultaneous efflux of labeled K^+ is also shown, in which a more elaborate CTE correction was made on both the Na^+ and K^+ efflux curves and in which labeled Na^+ in the extracellular-space fluid had been removed by prior centrifugation), the CTE-corrected Na^+ efflux curves still retain both a fast and a slow fraction, confirming the hypothesis once more that a part of the fast fraction represents the fast fraction which as shown above represents labeled Na^+ emerging from within the muscle cells.

However, a minor departure from the usual was also noted in the resolved Na^+ efflux curves shown in Figure 4. The fast fraction does not appear as a simple straight line any more. Instead, it now shows a bend.

The simplest explanation is that even though the original labeled Na^+-containing extracellular space fluid has been removed by the centrifugation procedure, the extracellular space thus vacated must soon be filled with the non-radioactive Ringer's washing solution

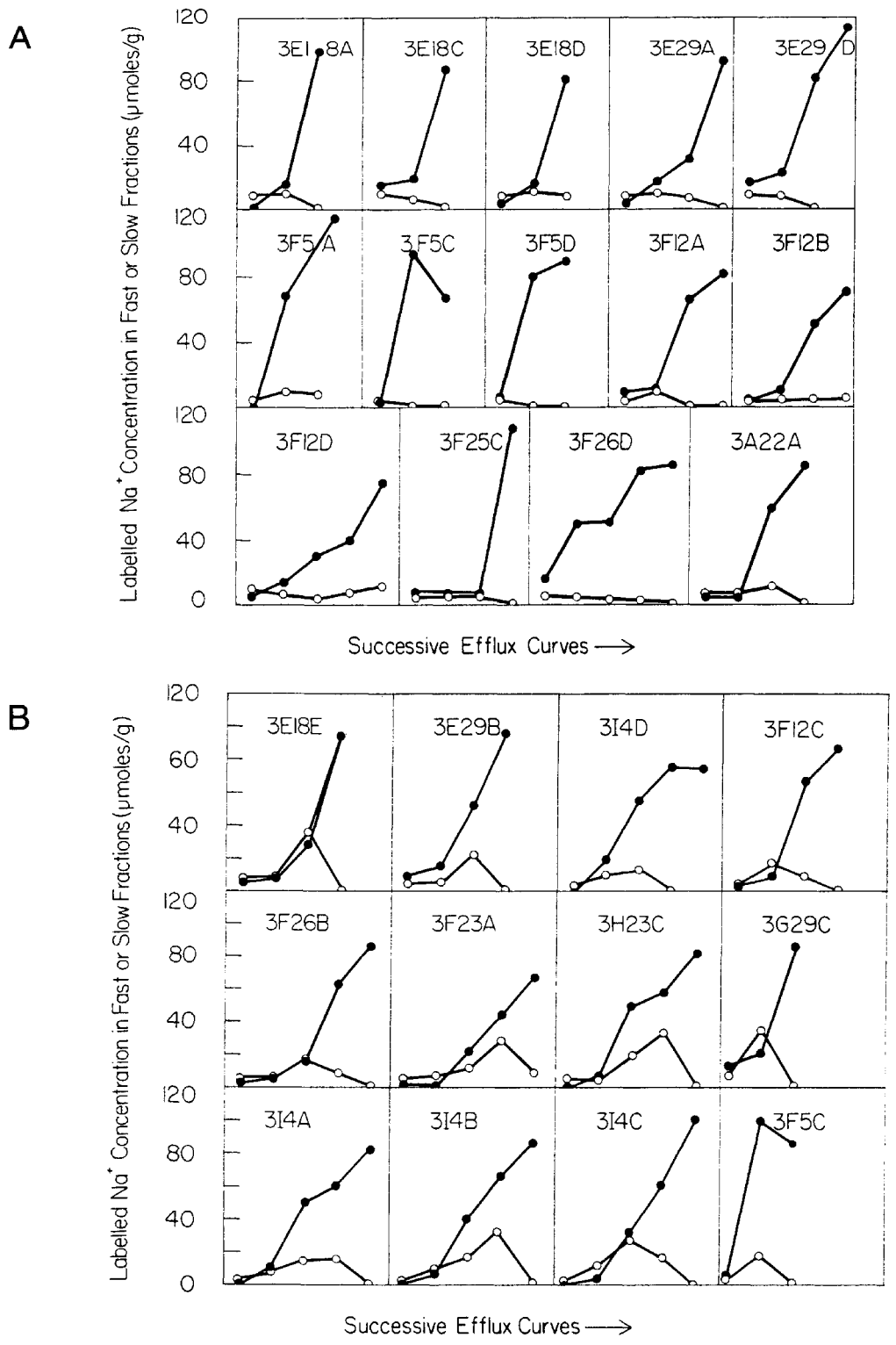

FIGURE 3. A summary of all experiments conducted on the Na^+ efflux of dying frog muscle. All data show rising fast fraction (solid circles) of labeled Na^+ with cell deterioration and death, reaching the level of labeled Na^+ in the soaking solution as the cells approached death. The slow fraction (empty circles) on the other hand, either show no change (A) or a transient rise before falling to still lower levels (B). (From Ling *et al.* (1981) by permission of *J. Cellular Physiology.*)

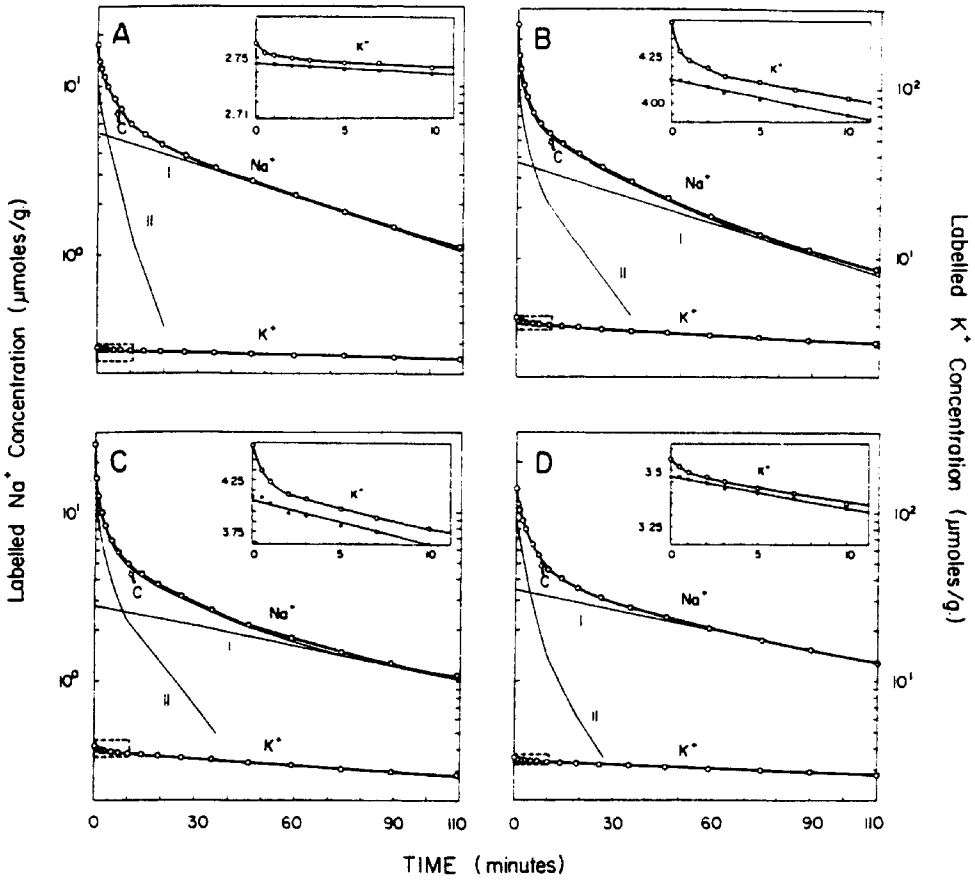

FIGURE 4. The time course of simultaneous Na^+ and K^+ efflux from centrifuged frog sartorius muscles. The muscle in A was incubated overnight at 25°C with labeled Na^+. Labeled K^+ was added to the loading solution for the final 33 min of incubation. The muscles in B, C and D were incubated at 25°C with labeled Na^+ and labeled K^+ for 33 min. The muscles were exposed to the incubating solution for 10 more mintues while wrapped and centrifuged (at 1000 g for 4 min) in the air-tight centrifugation packets, which increased the total exposure time to 43 min. Washing was carried out successively in tubes of Ringer's phosphate solution maintained at 25°C. Correction for connective tissue was made on the basis of a composite curve of ^{22}Na efflux from similarly incubated connective tissue sheets. Line 1 was obtained from a 1.9% connective tissue correction in A, a 7.0% correction in B, a 6% correction in C, and a 1.9% correction in D. An enlargement of the first 10 min of K^+ efflux for each muscle is illustrated with the connective tissue correction, so that the corrected efflux curve will not become negative in value at any time. (From Ling and Walton (1975b) by permission of *Physiol. Chem. Phys. & Med. NMR.*)

as soon as washing began. As a result, labeled Na^+ emerging from within the cells during the washing process must travel through the thickness of this water-filled space before reaching the outside bathing solution. If this interpretation is on the right track, working with single muscle fibers or a cluster of a few muscle fibers—which do not have the kind of deep extracellular space as in whole sartorius muscle—should produce a fast fraction without such a bend. Let us find out next if this prediction is confirmable.

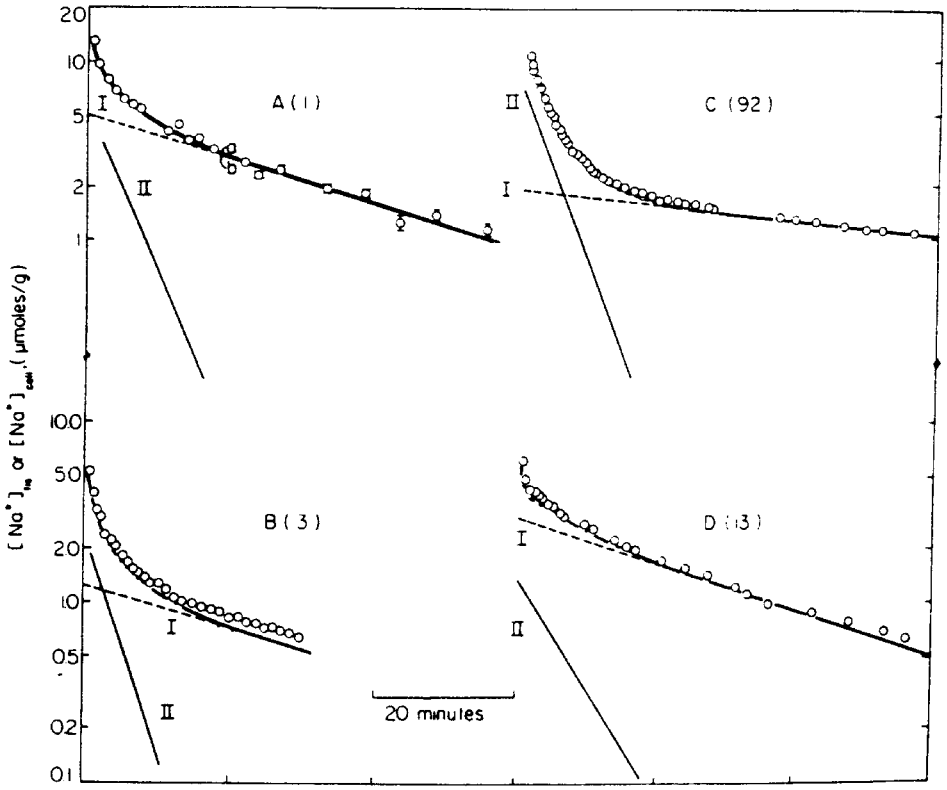

FIGURE 5. Time course of labeled Na^+ efflux from a single muscle fiber and small multiple muscle fiber bundles. Number of fibers is indicated on Figure. All fibers were washed in normal Ringer's phosphate at 25°C except B, which was washed at 0°C. Experimental points are in units of μmoles per gram of fresh tissue weight. Correction for a 5% connective tissue contribution was made on the basis of a composite curve of ^{22}Na efflux from similarly incubated connective tissues from four frogs. (From Ling (1970) by permission of *Physiol. Chem. Phys. & Med. NMR.*)

4.2.3 From single and multiple muscle fiber studies:

Early in the 1950s, I put together an apparatus-assembly for monitoring the loss of radioactively labeled Na^+ and other isotopes from frog muscle while the muscle was being continually washed (Ling and Schmolinski, 1956). The key component is a well-type γ-scintillation counter illustrated in Figure 8.5B in Appendix 1. In this simple setup, the sensing element encloses the radioactively-labeled sample and thus allows radiation in almost all directions to be recorded. As a result, more readings are recorded within a given period of time than the older methods can register—in which the sensing element sits a distance below the sample and "sees" only a much smaller portion of the emanating radiation.

With a special adaptation of this new apparatus-assembly, I studied the Na^+ efflux from a single isolated muscle cell and small bundles of a few muscle cell(s) isolated not from a sartorius muscle but from the two-bodied cylindrical semitendinosus muscle (for reason, see

below). In all cases here, a 5% (rather than 9.1%) correction for the CTE was applied, on account of the expected lower CTE contents in these single or multiple cell preparations because, unlike an intact sartorius muscle, the single cell or cell cluster does not have a covering fascia. (While seemingly all right in most cases, in some cases where a larger piece of tendon might be included, the correction might be too small or in other cases too large. For a way of correcting, see legend of Figure 4.) The results from studies on these single and multiple muscle cells preparations are reproduced here as Figure 5 (Ling, 1970).

The data show that even though I was measuring efflux from a *single* cell or a few cells (which has no interfibrillar or extracellular space to speak of. The superficial layer of radioactive solution on the cell surface is instantly washed away and hence *free* from the complication due to the presence of radioactivity trapped in a deep interfibrilar or extracellular space), the efflux cure remains curved and—like that from an intact sartorius muscle containing a thick stack of some one thousand muscle cells—also easily resolvable into the two fractions, one fast and one slow. Furthermore, the fast fraction plot is definitely straight and shows no bend—as we had hoped to see.

This new set of data confirms the hypothesis that surface-, or membrane-limited Na^+ efflux occurs at a much faster rate than widely believed and is represented by the fast fraction from the single and multiple cell preparations. Table III summarizes much of the data with my single and multiple fiber experiment carried out in 1966 and earlier. Though some of the data are partially presented in the 1970 paper, this tabular data is presented for the first time here. All data have been corrected for CTE.

Two aberrations were noted in some of the data: (1) Exceptional short $t_{1/2}$ of the fast fraction (e.g., 1 min or so) in Experiments 66G26 and 66G15. I reason that these extra-fast fast fractions seen are due to the inclusion of one or a few injured cells in the muscle fiber bundle studied. These injured muscle cells as a rule have higher labeled Na^+ contents and they efflux at a fast rate (see later sets of efflux curves in Figure 2A and 2B for example). As a result, they exercise a camouflaging effect on the normal efflux from intact muscle cells with low radioactively labeled Na^+ concentration and slower effluxing pace. (2) Extraordinarily long $t_{1/2}$ in the slow fractions. This could be due to injury or other causes which have increased the relative adsorption energy of the Na^+ on the β- and γ-carboxyl groups. This phenomenon has occasionally been seen following an early stage of injury or poisoning (see the extra slow slow-fractions of the first two efflux curves marked A and B in Figure 2A—not found in the slow fractions of the efflux curves of Figure 2B).

Making use of the new knowledge acquired above and adding a modified version of the simple short-dip technique for studying the Na^+ efflux of muscle cells—the prototype of which I first introduced in the fifties—we will be in a position to conduct the definitive study on the Na^+ efflux of frog muscle cells. But before closing this section, I want to call attention to the fact that for the ease of dissection and lesser propensity toward injury, the single and multiple fiber preparations used in this set of study and the one following were not obtained from the sartorius muscle but from the thigh muscle, known as the semitendinosus muscle. Evidence will be presented that the muscle cells in the sartorius muscle and in the semitendinosus muscle provide quantitative data indistinguishable from one to the other. This similarity is not surprising since muscle fibers in these as well as other voluntary muscles are all made up of similar finer units called myofibrils.

TABLE III. Rate constants (k) and half-time of exchange ($t_{1/2}$) of labeled-Na^+ efflux from single and multiple-muscle fiber prepartions (25°C).

Expt No	Muscle Fiber No	Fast Fraction		Slow Fraction
		$t_{1/2}$ (min)	k (min^{-1})	
55K12	3	2.5	0.28	26
56K2	92	2.8	0.25	70*
66G2	18	4.3	0.16	105*
66G6	14	4.0	0.17	72*
66G7	22	3.4	0.20	32
66G8	1	4.0	0.17	40
66G15	10	1.0*	0.69*	30
66G26	13	4.9	0.14	23
66G26a	24	1.2*	0.58*	22
66G27	14	3.8	0.18	24
66G27a	15	2.0	0.35	20
66G28	13	4.0	0.17	24
66G29	6	3.8	0.18	21
66H1	1	3.2	0.22	24
mean ± s.e.		3.6 ± 0.78	0.27 ± 0.43	26 ± 5.6

Single and multiple muscle fiber preparations were isolated from the semitendinosus muscles of leopard frogs (Rana pipiens pipiens, Schreber), loaded with radioactive ^{22}Na and washed in a continuous stream of non-radioactive Ringer's solution containing 2.5 mM K^+ and 100 mM Na^+. A 5% connective-tissue-element correction was applied to all data. For more details see legend of Figure 5. Partly taken from Ling (1970) but includes much more data than given in that publication. (By permission of *Physiol. Chem. Phys. & Med. NMR.*)

4.2.4 A definitive technique for measuring the surface-, or membrane-limited Na^+ efflux rate of frog muscle cells

The modified simple new technique, called the "Small-muscle-fiber-bundle—Short-dipped and—Centrifuged with—Closely separated-data-points in its efflux-curve and after-CTE-correction" or *SSCCC technique* to be described next:

Isolate from a frog semitendinosus muscle a small muscle-fiber bundle containing a few tens of muscle fibers or cells. Expose the fiber bundle for an accurately measured short period of time, say 3.0 min to a labeled Na^+-containing Ringer's solution containing high

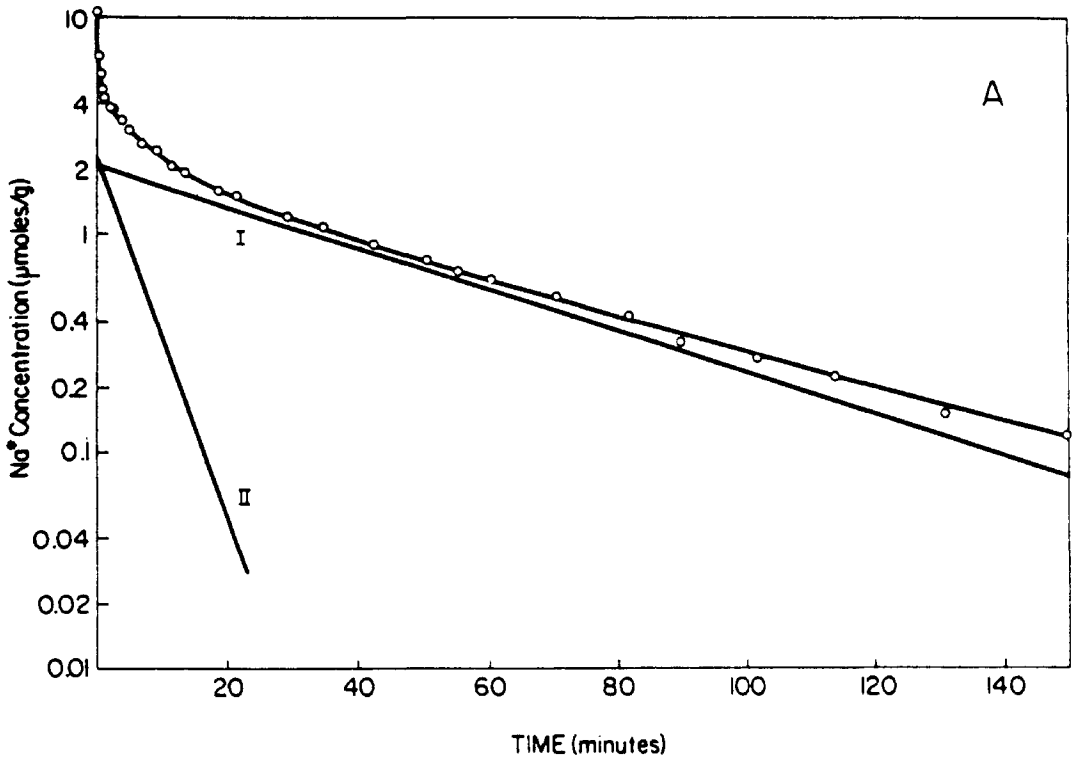

FIGURE 6. Na^+ efflux from a small muscle fiber bundle freed of extracellular space fluid by centrifugation. Incubation in tagged solution lasted 16 hours at 4°C before another 4 hours of incubation in the (same) incubation solution at 25°C. Correction for a 5% connective tissue contribution from the efflux curve of a piece of connective tissue sheet isolated from the same frog following identical treatments. Note how the curved later part of the efflux curve straightens out after connective tissue correction. Muscle 1 of 11/18/77 of Table 4A. (From Ling (1980) by permission of *Physiol. Chem. Phys. & Med. NMR.*)

enough radioactivity to yield an efflux curve with closely-spaced data points. Remove the labeled Na^+-containing extracellular space fluid by the centrifugation method of Ling and Walton (1975a). And then conduct the usual washout in a well-type scintillation counter arrangement as illustrated in Figure 8.5B in Appendix 1. Plot the Na^+ efflux curve on semilogarithmic graph paper. Make corrections for the CTE contribution after obtaining a CTE efflux curve under identical conditions on a piece of connective tissue sheet taken from the same leg from which the muscle fibers bundles were obtained. Extrapolate from the corrected efflux curve of the muscle bundle to zero time to find the total initial labeled Na^+ concentration in the muscle cells. Divide this initial, CTE-corrected labeled Na^+ concentration by the time of exposure (e.g., 3.0 min) and the true weight in grams of the muscle cells, one obtains the labeled Na^+ influx rate in μmoles/gm/hr.

Since during the entire time period of soaking and washout, there is no change in the total Na^+ concentration, this labeled Na^+ *influx rate* is equal to the labeled Na^+ *efflux rate*. And this value will be what I believe to be the most accurate way—up to this time—of

determining the Na^+ efflux rate of (pure) frog muscle cells. In the context of the membrane pump model, this correct influx rate is within an error of less than 1%, equal to the Na^+ pumping rate.

In Figure 6 an example of the uncorrected and corrected efflux curve of labeled Na^+ from a centrifuged small muscle fiber bundle (muscle E_6 of 11/23/1977) is shown. It contains 40 muscle fibers, and was exposed to labeled Na^+ for 3.25 min. All these and additional data can be found in Table IVA taken from Ling (1980). Obtained by this SSCCC procedure, each efflux curve obtained can be neatly resolved into a fast fraction and a slow faction. Both appear as straight lines.

Now each one of these straight lines provides two sets of data to estimate how much labeled Na^+ has entered the cells (influx) during the initial 3.0-min exposure to the radioactively-labeled Ringer's solution. One set comes from the extrapolated intercept already described which yields the directly measured surface-, or membrane-limited Na^+ *influx rate.* The other set comes from the slope of the straight line in the semilogarithmic plot, representing the rate of *efflux* (*out*ward flow), but which must equal the rate of *influx* (*in*ward flow) since, as just mentioned, the total Na^+ concentration does not change during the experiment.

The important point is this: *only the fraction which truly represents the surface-, or membrane-limited intracellular-extracellular exchange can yield two values of the influx rate—one directly from the intercept and the other indirectly from the slope—that agree with each other.* (Or put a little differently, only in the correct fraction would the *influx rate* determined from the intercept equal the *efflux rate* determined from the slope.) In contrast, the fraction which does not represent the true intra-, extracellular exchange will produce as a rule two "influx" rates that disagree with each other.

In a series of studies on 22 experiments (21 were on small-muscle-fiber bundles and a single experiment was on a whole sartorius muscle) in 8 sets of studies carried out between October 27, 1977 and November 23, 1977, the ratio of the intake of labeled sodium obtained from the intercept of the fast fraction over that from the slope of the fast fraction is close to unity or 100% from the fast fraction {107% ± 3.6%, (mean ± s.e.)}.

In contrast, the same ratio from the *slow fraction* is far from unity, or 100%, at 481% ± 39.5%.

This piece of work, in my view, provided our final and definitive proof of the hypothesis that the true surface-, or membrane-limited Na^+ efflux from frog muscle cells is that represented by the fast fraction (after removal of labeled Na^+ trapped in the extracellular fluid) from the small muscle fiber bundles (Ling, 1980).

From 21 experiments on small-fiber bundles only taken from Table IVA, column 11, and listed in Table IVB—along with other parameters often used to measure the rate of Na^+ efflux data—, the average *half time of exchange* ($t_{1/2}$) of the fast fraction is 3.34 ± 0.21 min and that of the slow fraction, given in column 9 minus that of the data from the whole sartorius muscle is 24.0 ± 1.10 min (not shown in Table IV). The data show that the fast fraction is 7.18 times faster than the slow fraction. The data are also in reasonable accord with the set of data obtained from single and multiple muscle fibers—after eliminating from averaging the two exceptionally fast fast fractions and three exceptionally slow slow fractions, both marked by asterisks in Table III—yield a ratio of the $t_{1/2}$'s of the slow and fast fraction equal to 26.0/3.56 = 7.30.

TABLE IVA.

Date	Muscle No	Muscle Weight (mg)	Muscle Number of fibers	Exposure time (min)	$[Na^+]$ In,eq Slow fraction (mM)	$[Na^+]$ In,eq Fast fraction (mM)	Slow fraction Intercept (mM)	Slow fraction $t_{1/2}$ (min)	Fast fraction Intercept (mM)	Fast fraction $t_{1/2}$ (min)	Fast fraction k (min^{-1})	Influx rate (mmoles/liter × min) Slow fraction From slope (A)	Slow fraction From intercept (B)	Slow fraction B/A (%)	Fast fraction From slope (A)	Fast fraction From intercept (B)	Fast fraction B/A (%)
10/27/77	I	8.6	54	4.0	5.9[a]	8.8[a]	2.1	33	4.2	6.0	0.12	0.12	0.44	3.67	1.02	1.05	103
10/31/77	I	75.5[b]		3.0	7.6	8.2	1.5	56	4.0	3.0	0.23	0.094	0.49	521	2.20	1.79	81
11/10/77	I	8.73	40	4.0	8.8	11.9	5.3	19.5	6.0	3.4	0.20	0.32	1.34	419	2.43	2.74	113
11/11/77	I	11.8	60	4.5	8.8	11.9	7.3	30	3.3	4.4	0.16	0.20	0.75	375	1.87	2.37	127
	II	10.1	60	4.5	8.8	11.9	3.5	21.5	4.9	3.7	0.19	0.28	0.52	186	2.57	2.19	85
	III	8.7	40	4.5	8.8	11.9	3.3	28	6.1	4.8	0.14	0.21	0.74	352	2.05	2.36	115
	IV	12.0	50	4.5	8.8	7.8	4.0	21.5	5.8	3.7	0.19	0.29	0.88	303	2.22	2.16	97
11/18/77	I	10.7	60	4.5	4.9	6.8	3.0	31	3.0	3.7	0.19	0.11	0.67	609	1.24	1.34	108
	II	4.6	28	4.5	4.9	6.8	2.4	17	5.8	1.5	0.46	0.20	1.11	555	3.19	2.61	82
11/22/77	I	6.4	40	4.0	5.0	6.7	2.4	32.5	3.3	4.1	0.17	0.11	0.61	555	1.12	1.44	129
	II	8.9	50	4.0	5.0	6.7	2.7	26	3.8	2.6	0.27	0.14	0.68	486	1.78	1.64	92
11/27/77	D1	10.3	35	5.0	6.1	10.6	4.9	23	10.6	2.9	0.24	0.11	0.97	882	2.54	3.10	122
	D2	10.1	35	5.0	6.1	10.6	6.1	22	7.3	3.2	0.22	0.20	1.21	605	2.30	2.68	117
	D3	12.2	50	5.0	6.1	10.6	6.2	26	7.0	2.6	0.27	0.17	1.25	735	2.84	2.64	93
	D4	13.0	60	5.0	6.1	10.6	5.3	24	3.5	4.3	0.16	0.15	1.06	707	1.72	1.76	102
	D5	4.1	15	5.0	6.1	10.6	6.4	23	6.4	3.1	0.22	0.18	1.28	711	2.46	2.55	104
11/28/77	E1	8.0	25	3.25	9.1	9.4	4.7	26	5.9	3.1	0.22	0.24	1.49	621	2.10	2.64	126
	E3	10.1	50	3.25	9.1	9.4	5.2	14	5.8	2.8	0.25	0.46	1.29	280	2.33	2.74	118
	E4	5.0	30	3.25	9.1	9.4	1.12	22	4.6	3.5	0.20	0.29	0.96	331	1.87	1.73	93
	E5	7.4	30	3.25	9.1	9.4	5.3	22	12.1	2.3	0.30	0.29	1.08	372	3.65	5.32	146
	E6	6.6	40	3.25	9.1	9.4	3.6	26	6.1	2.3	0.30	0.24	1.12	467	2.84	2.99	105
	E8	3.7	14	3.25	9.1	9.4	3.3	16	7.3	2.1	0.33	0.40	1.03	258	3.10	3.27	105
Mean								25.5 ±		3.32 ±	0.229 ±	0.218 ±	0.95 ±	486 ±	2.25 ±	2.41 ±	107 ±
± SE								1.76		0.71	0.016	0.02	0.06	37.5	0.14	0.18	3.5

Rate constants (k) and half-time-of-exchange ($t_{1/2}$) of Na^+ efflux from small muscle fiber bundles after removal of labeled Na^+-containing solution in its extracellular space by the centrifugation technique of Ling and Walton (1975a). Correction for the contribution of the connective tissue elements (CTE) on the basis of 5% CTE in bundle weight. Mean and S.E. were calculated from all 22 sets of data including the single set from an intact sartorius muscle (10/31/77). For average values from small-muscle-fiber bundles data only, see Table IVB. The added rate constants (k) were calculated from $t_{1/2}$ by the relationship: $k = \ln 2/t_{1/2} = 0.696/t_{1/2}$. Near equality of the Na^+ influx rate obtained from the intercept (B) and the Na^+ efflux rate from the slope (A) establishes that it is the fast fraction of the efflux curve which represents the surface-, or membrane-limited intra-, extracellular exchange of the Na^+. (Data from Ling (1981) by permission of *Physiol. Chem. Phys. & Med. NMR.*)

TABLE IVB. Rate constants (k), half-time-of-exchange ($t_{1/2}$), flux rate (ϕ) and specific permeability constant (κ) of Na^+ efflux from muscle cells at 25°C.

$t_{1/2}$	k	ϕ		κ
(min)	(min^{-1})	(μmoles/cm^2hr)	(moles/cm^2sec)	(cm/sec)
3.34 ± 0.21	0.23 ± 0.016	0.247 ± 0.013	6.87×10^{-11} $\pm 0.36 \times 10_{-11}$	6.67×10^{-6} $\pm 0.47 \times 10^{-6}$
(21)	(21)	(42)	(42)	(21)

Data taken from Table IVA after eliminating that from the single intact sartorius muscle (10/31/77). ϕ's are obtained from both the influx (from intercept) and efflux data (from slope) of the fast fraction and the relationship $\phi = (V/A)kC_i$, where V/A is the volume (V)/surface (A) ratio of muscle fibers equal to (1/570) (from Ling, 1962 or Appendix 1, Table 8.7). C_i is the equilibrium concentrations of Na^+ in cells and given in Table IVA. κ is equal to (V/A)k. Number in parenthesis at the bottom of each column refers to number of studies performed.

Since the slow fraction really represents the rate of desorption of Na^+ from adsorption sites, the ratio of the $t_{1/2}$'s is itself of limited interest. It becomes of greater interest because in the conventional practice, the $t_{1/2}$ of the slow fraction has been regarded as representing the membrane-limited Na^+ efflux rate (or "pumping rate") from the muscle cells. But since the $t_{1/2}$'s from the majority of workers in this field were obtained without correcting for the CTE contamination, and the CTE contamination further slows down the apparent $t_{1/2}$ of the slow fraction, the $t_{1/2}$ is no longer in the range of 24 to 26 min—as correctly determined by the SSCCC technique—but much longer. And accordingly, the $t_{1/2}$ ratio of the slow and fast fraction would also end up much higher. The next section will show just how much higher.

In Table V, I have collected from the literature several sets of data on the $t_{1/2}$'s of frog sartorius muscles obtained from the slope of the slow fraction of the Na^+ efflux curves at the higher temperature ranges between 18.5° and 25°C and the lower one of 0° to 1.5°C. Note that in none of the work listed (including that of Ling *et al.*, 1973) was correction for the contribution of the CTE made. The average $t_{1/2}$ is for the higher temperature range between 34 to 39 min, averaging 37 min. Dividing this number by the $t_{1/2}$ of 3.34 min from the fast fraction that was obtained after short-dip and CTE correction, one obtains a ratio of 11.1.

The data of Keynes and Steinhardt at 21.5° to 21.7°C are even higher at 40 to 52 min. At the low temperature of 0° and 1°C, the data from Levi & Ussing and Harris range from the shortest at 33.7 min to 96 min. Again the low temperature range data of Keynes and Steinhardt is much longer at 202 min. One possible explanation for their exceptionally slow efflux rates is their choice of obtaining the time constants after unusually long washing of the labeled muscle. Such a practice, as pointed out above, exaggerates the contaminating influence of the (uncorrected) CTE contribution, which Keynes and Steinhardt were apparently oblivious of, or at least made no effort to correct for its confounding influence.

The four sets of independent studies described under Section 4.2.1 to 4.2.4 when taken together leave no doubt that a minor revolution on the method in the way of ascertaining the surface-, or membrane-limited Na^+ efflux rate has been completed within the major

TABLE V. Na^+ efflux rate from frog sartorius muscles from several groups of workers.

	Temp. (°C)	No. of Trials	$t_{1/2}$(min)	
			mean ± s.e.	range
Levi & Ussing (1948)	20	18	34	26–47
	1	4	70 ± 7	
Harris (1950)	18	6	34.2 ± 2.7	
	0	5	79 ± 16	
Keynes & Steinhardt (1968)	21.5		40	39–41
	21.7		52	46–58
	1.5		202	170–230
Ling *et al.* (1973	25	14	37.4 ± 2.7	

Data, given as half-time-of-exchange ($t_{1/2}$), all obtained from the slope of the slow fraction, including the data of Ling *et al.* (1973). In none of these cited data was correction made for the connective tissue element or CTE contamination. Only those data from Table 1 of Harris were chosen where the (semilogarithmic) efflux curve does not show a change of slope and marked with the symbol × in Harris' table. Keynes and Steinhardt's 21.5°C data were taken from the initial part of their efflux curve shown in their Figure 1 in which the muscle was washed in a Ringer's solution containing 10 mM K^+ and 111 mM Na^+. Their 21.7°C data were taken from the initial part of their Figure 2, in which the washing solution was free of K^+. $t_{1/2}$'s were obtained from their rate constants, k's by the relationship $t_{1/2} = 0.693/k$. The data of Ling *et al.* (1973) were taken from those at 25°C in their Table 4.

revolutionary transition from the defunct membrane pump theory to the AI Hypothesis. The conventional assignment of the slow fraction to represent the cell-surface, or membrane-limited Na^+ efflux, is wrong. Instead, it is a fast fraction which represents the cell-surface-limited Na^+ efflux. One consequence of this iconoclastic discovery is that the true Na^+ efflux rate is some 10 or more than 10-times faster than what has been accepted by most muscle physiologists and other workers in this field to this day.

Just as important, the last set of data given in Table IV in columns 2 and 3 from the left, are, in my opinion, the most accurate data up to now on the surface-limited influx rates (or efflux rates since they are equal) from frog muscle cells to this date. When the two sets of data from the intercepts and the slopes of the fast fraction are pooled we obtain from the 42 pieces of data a flux rate of 0.247 ± 0.013 *mmoles/cm².hr* (mean ± s.e.). This data can also be converted into units of moles/cm².sec by utilizing the average volume/surface (V/A) ratio given in the top part of Table 8.7 in Appendix 1 which can be averaged to yield a value of 17.5 μm, equivalent to an A/V ratio of 570 cm^{-1}. The result is the correct, uncontaminated Na^+ flux rate equal to $6.87 \times 10^{-11} \pm 0.36 \times 10^{-11}$ *moles/cm².sec* (mean ± s.e.) for the (pure) frog voluntary muscle cells at approximately 23°C.

I want to emphasize that the iconoclastic change both in the value of the efflux rate and the method to obtain it may seem a subject of limited provincial interest to specialists. In fact it is not so at all. As one example, it may be mentioned that this 10-fold increase in the

Na^+ efflux rate when cast as a new Na^+ permeability, P_{na} in the Hodgkin-Katz-Goldman equation would predict a resting potential of frog muscle equal to only 27.1 millivolts—in contrast to the 80–90 millivolts actually observed (Ling and Gerard, 1949). In contrast, the data are in harmony with the new surface-adsorption theory of cellular electric potentials as part of the AI Hypothesis (Ling, 1960; Ling, 1962, Chapter 10; 1984, Chapter 14; and 1992, Chapter 11).

What this tells us is that the living cell is a self-consistent coherent system. No area of speciality really exists as a totally insulated area of knowledge immune to iconoclastic change in other areas. And the disproof of the membrane pump hypothesis is far more than the subject of interest to a few specialists who study Na^+ effluxes but an earthquake right in the most vital center of all living cell functions.

4.3 Why the "pumping rate" used in my 1962 energy calculations was sound then, is sound now and will remain sound in the future

Let us now go back to the mid-fifties when I was doing my original energy balance studies of the Na^+ pump—to be published one day in Chapter 8 of my first monograph (Ling, 1962), and now reproduced here in Appendix 1. The first key issue I faced was how to estimate the minimal energy need of the postulated Na^+ pump. For that, I needed to know how fast the Na^+ is being pumped out. In the context of the sodium pump hypothesis, this pumping rate is virtually the same as the Na^+ efflux rate (see Section 5.1.1 below for discussion on a negligible passive exchanging component). I did not obtain a correct Na^+ rate the conventional way from the slope of the slow fraction. For reasons known to me even then (i.e., the large disparity between the size of the extracellular space and the size of fast fraction of Na^+ efflux) I believed that it would be a fatal mistake to do so—a belief and strategy by now proven sound beyond doubt.

In theory, I should have chosen either the slope or the intercept or slope of the fast fraction to obtain the true surface-, or membrane-limited Na^+ efflux rate which, as I have repeatedly mentioned, in the context of the membrane pump theory is equal to the Na^+ pumping rate. This is what I would do now. Unfortunately, this decision was made more than 40 years ago. At that time, the methods for sorting out the fast fraction from muscle efflux curves without contamination from extracellular space fluid and CTE were not developed yet.

I did develop and use the "short-dip" part of the SSCCC technique—described above under section 4.2.4—but obtained the initial influx rate by extrapolating from the *slow fraction.* This technique used, entirely new at the time, is *perfectly sound* for the specific objective of my study then, which was to find out if the muscle cells have enough energy to operate the hypothetical sodium pump. Thus even though I already knew that what I got with my new method was to obtain a pumping rate (equal to the efflux rate from the muscle cells) *slower* than what it truly is, it did not matter.

It did not matter because even this admittedly slower pumping rate led to an energy requirement far beyond the maximum energy the cells command. And that was what I set out to find out at that time. (To determine the true efflux rate was the objective of an entirely different project and it was tackled many years later as the reader of Section 4.2 above now knows.)

Though the majority of workers in this field and I hold different views on certain key issues, we agree on others. Since the belief that the slow fraction of the Na^+ efflux represents labeled Na^+ coming from within the muscle cells is thus shared, I started with this shared

belief . . . even though the majority of workers and I disagree on what the rate-limiting step for the slow fraction of Na^+ efflux is. They believe the rate-limiting step to be permeation through the cell membrane, while I believed then and believe now that it is the rate of desorption from β-, and γ-carboxyl groups of intracellular proteins (notably myosin) on which about half of the intracellular Na^+ is adsorbed (Ling and Schmolinski, 1956; Ling, 1966, Figures 14 and 15; Cope, 1967; Ling and Cope, 1969; Edelmann, 1986, Figure 4; see also Ling, 1992, Chapter 4 and also Ling and Ochsenfeld, 1991). But that difference of opinion on the rate-limiting step of the slow fraction is of no consequence to what I intended to do as stated above.

I exposed a small muscle fiber bundle—isolated from frog semitendinosus muscle—for 3.0 min in a Ringer's solution maintained at 0°C and containing labeled Na^+ with and without metabolic poisons. I also treated in a similar manner a piece of connective tissue from the same frog legs providing the muscle fiber bundles. I then did a standard washout study of the muscle fiber bundles as well as its CTE model, plotted their efflux curves semilogarithmically and made the CTE corrections on the muscle fiber bundle efflux curve before resolving the corrected efflux curve into a fast fraction and a slow fraction.

Next I extrapolated to zero time the *slow fraction*—which, I repeat, the majority of workers on this subject and I agree to represent Na^+ coming from within the muscle cells—and which I chose to use because at that time there was no better alternative, nor the need for a better alternative. I then obtained from the ordinate intercept of the slow fraction what amounts to a lower limit of labeled Na^+ which has entered the muscle cells during the 3.0-min exposure to the labeled solution. Dividing that amount of labeled Na^+ in the cells by 3.0 min— after correcting for the CTE contribution—and the corrected muscle-fiber weight in grams yielded the rate of *influx* into a gram of muscle cells per min. Examples of these efflux curves are shown in Figure 8.6 of Appendix 1 and the numerical data in Table 8.7. The concentration units used were in moles/kg.hr (Column 5 in bottom part of Table 8.7) or in units of moles/cm^2 sec (Column 8).

Since during the experiment there was *no significant change in the total Na^+ concentration,* the influx rate and efflux rate of Na^+ must be equal. Therefore the *influx* estimated from the intercept of the slow fraction gives me an estimate of a lower limit of the Na^+ *efflux* rate, i.e., pumping rate in terms of the membrane pump hypothesis.

I also pointed out clearly that the method I chose for estimating this Na^+ rate does not yield the true initial labeled Na^+ content after a three min exposure, but a "deliberately underestimated initial ^{22}Na content for the muscle fiber." (Line 6 from bottom of the legend of Table 8.7 in Appendix1), implying clearly that the true surface-, or membrane-limited efflux rate is *even faster*—as subsequent studies, especially the definitive Na^+ flux rate data given in Table IVB and averages cited above have established without ambiguity.

But as far as arriving at a definitive answer on the feasibility of the membrane-pump hypothesis—which was my only purpose then—there was no need to pursue the more perfect higher Na^+ rate further. The data obtained from this admittedly underestimated pumping rate, when augmented by the vanishing energy source of the poisoned muscle, would give a discrepancy between minimum energy need and maximum energy available to between 300 times and 600 times, is all I care to spend time on. As I said before, the membrane-pump hypothesis has been dead for more than 35 years. And in my view will remain so in the foreseeable future.

5. Miller's Attack on My Disproof of the Na+ Pump Hypothesis on Energy Grounds

I now remind the reader once more that an "at length" if not exact copy of what Miller and Freedman once circulated around might still exist, containing what Kolata called "crucial calculations" even though we know by now what she called "crucial experiments" had never been done. This "at length" copy is Miller's Ph.D. Thesis. And it did not take long to find what we are looking for. Thus on page 31 of the Thesis (Miller, 1974), one discovers the following:

> "In that reference, (Ling, 1962; *the relevant part of which, is reproduced here as Appendix 1—added by GL*), one is immediately struck by the extremely large Na^+ pump rate used by Ling, in the order of 100 μmole/gram-hr. This is about 20 times higher than the flux measured under normal conditions at 20°C by Keynes and Steinhardt. Clearly it is this choice of flux which is the primary factor causing the large energy discrepancy . . . "

This paragraph is followed by a page-long description of how I determined the Na^+ pumping rate in poisoned frog muscle (already described in full here under Section 4.3) followed by his criticism of the technique I used in these words:

> "At any rate, whatever the explanation for the *flux anomaly*, it may be said that Ling used the anomaly itself to arrive at a large Na^+ flux. If he had done the calculation of pump flux in the usual manner by using the efflux directly, the energy requirement would have been in the manageable range of 20%."

And on page 36, Miller made the following concluding remarks;

> "The conclusion I wish to draw from this review is not that Ling's energy calculation for the Na^+ pump is wrong, but simply that it is equivocal enough that it should not be taken as disproof of the membrane theory."

Together these passages constitute the center-piece of this allegedly "at length" attack on my work on the energy balance study—which, I maintained then and maintain now, has disproved the membrane-pump hypothesis unequivocally. These paragraphs and other relevant parts of Miller's Thesis also confirm once more what I had gathered from his letter. Namely, he made no new experimental study on the subject, he has totally disregarded the role of the expanding list of energy-demanding pumps and the vanishing energy source of the poisoned muscles—that which I have listed as the Game of Illusion: Part 1.

I shall now give an in depth analysis of all his evidence cited to support his conclusion and the other claims cited above and to leave no doubt that all these statements in their entirety (with a single expression) have no validity whatsoever. In anticipation of what is to come, I may add that *all that he considered as reasons to make my disproof of the sodium-pump hypothesis equivocal was achieved by withholding critical information establishing the opposite, thereby creating the illusion of a dead and mummified hypothesis made alive and breathing again.*

I shall first dispose of three minor criticisms he cited against my disproof of the

membrane pump hypothesis before proceeding to debunk his main effort to resurrect the sodium pump hypothesis.

5.1 Rebuttal of Miller's three minor criticisms

5.1.1 Passive component ignored (p. 26 of Thesis)

In order to reduce the energy need to below what I calculated, Miller suggested that a part of the Na^+ efflux measured might not need energy-consuming pumping. This part is what he called the passively diffusing fraction of Na^+ efflux and he suggested that I did not take this into consideration—which is not true. I have been fully aware of this problem all along. In 1965 and thus eight years before Miler wrote his Ph.D. Thesis, I published in a footnote that this passive component of the Na^+ efflux is below the experimental error (i.e., 0.1%) and thus negligible (Ling, 1965, p. S-105, footnote).

5.1.2 Part of the sodium ion is adsorbed, and this reduces the energy need of the pump (p. 28)

This is the only valid minor criticism Miller cited in his Thesis—a testimonial that he had not "forgotten" everything he had once learnt from his former Counselor and Professor. Confirming my own theory, then known as Ling's Fixed Charge Hypothesis—which was to evolve into the AI Hypothesis—I have shown that in the presence of an external Na^+ concentration of 100 mM and 2 mM of K^+, about half of the total intracellular Na^+ concentration in a normal frog muscle is adsorbed on the β- and γ-carboxyl groups of cell proteins most prominently myosin. This reduction of free intracellular Na^+ concentration, established in my laboratory long *after* the 1950s during which the energy balance study was made (Ling, 1966, p. 853, Figure 15; Ling, 1969, p. 24, Figure 15), would reduce the minimum energy need of the sodium pump, but this reduction is partly compensated by the increase in the Na^+-concentration gradient across the cell surface which in turn *increases* the minimum energy need.

However, even if we totally disregard this compensatory effect, the energy discrepancy from the recognition of the adsorption of a part of the intracellular Na^+ would lower the minimum energy need of the sodium pump from between 600 times to 1200 times of the maximally available energy to between 300 times to 600 times.

From the viewpoint of resurrecting the Na^+ pump hypothesis, this two-fold reduction of the pumping rate really makes no difference whatsoever, just like shooting a horse 1000 times in the head or only 500 times makes no real difference. You get a dead horse in one case, and a dead membrane-pump theory in the other.

5.1.3 In a poisoned muscle, part of the effluxing Na^+ may be running down *the gradient and thus requires no pumping (p. 34)*

This comment also has no validity.

The intracellular Na^+ concentration in the poisoned muscle at 0°C remained essentially unchanging from its normal initial value of healthy muscles till at least the 6th hour after poisoning began, hovering meanwhile between 20 to 30 micromoles per gram of muscle (while the external sodium ion concentration was at a steady 107.6 mM). From the 6th hour to the 10th hour, the Na^+ concentration in the cells rose to about 42 micromoles per gram, a

concentration still way below the external concentration of 100 mM. Of the three sets of data presented, two sets lasted only 4 hours during which time there was no gradient degradation whatsoever. (The intracellular Na^+ concentrations were not explicitly given but can be easily calculated from the data of E_{na}/F shown in Figure 8.7 in Appendix 1 and the formula given in the footnote at the bottom of the legend of Table 8.6. Thus at E_{na}/F equal to 40 mV, 35 mV and 22 mV, the intracellular Na^+ concentrations are respectively 19.5 mM, 24.6 mM and 41.7 mM respectively).

Only the set of experiments carried out on 9/12/1956 lasted till the 10th hour. But even at the 10th hour the overall electrochemical gradient shown as the top curve of Figure 8.7 was still 90 mV outside positive (compared to between 120–130 mV outside positive when the muscles were still normal). It is true that in this particular third set of experiments, there was such a decline of some 28% of the electrochemical gradient and the energy need accordingly reduced by that percentage—as it had already been taken into account in my original calculations. And it was *after* this correction had been taken into account, that the minimum need of energy still exceeds the maximum available energy by a ratio of 3060%.

The basic law of physics is incompatible with the idea that a positively charged ion like Na^+ could run *"down"* a 100 mV *outside-positive* electrochemical gradient as Miller suggested, no more so than water can flow "down" from the bottom to the top of a hill.

5.1.4 Two other comments

There remain two other comments made by Miller in his Ph.D. Thesis. One was taken from work from another pair of my fleeing students, Palmer and Gulati, which is briefly described and fully rebutted in *Science* (Ling, 1977) and briefly rebutted again in a linked page to my web site (Ling, 1997a under section (6)). The other concerning the electrical field effect at the cut end of muscles on K^+ diffusion from cut muscle cells was fully rebutted in TIBS (Ling, 1979) and also in the same linked page (Ling, 1997a under section (1)). Since these comments are not directly concerned with the resurrection of the sodium pump hypothesis, I have decided not to include them here.

5.2 Debunking Miller's main efforts to resurrect the Na^+ hypothesis

Miller's main effort to resurrect the Na^+ pump hypothesis has three components: (1) the sequestration of a large portion of cell in the sarcoplasmic reticulum or SR; (2) Ling's exploitation of an abnormal behavior or anomaly to obtain (3) an abnormally high "pumping rate." I shall deal with each of these accusations one by one next.

5.2.1 "Compartmentation" of labeled Na^+ in the sarcoplasmic reticulum (SR) (p. 28 of Thesis)

This is one of the major thrusts in Miller's effort to resurrect the Na^+ pump hypothesis. The idea that a part of the fast-effluxing Na^+ coming from a separate compartment inside the muscle cell but separate from the muscle cytoplasm (or sarcoplasm) began with Levi and Ussing (1948). Keynes and Steinhardt (1968) pondered over a "multicompartment system" with the sarcoplasmic reticulum (SR) arranged in parallel to the sarcoplasm and open to the outside, but noted the difficulty posed by Peachey's electron-microscopic demonstration that the "reticulum is located entirely *within* the sarcoplasm (italics mine)" (Peachey, 1965). Ignoring this serious difficulty, a whole bunch of workers has subsequently adopted the idea

(Birks and Davey, 1969; Zierler, 1972; Kulczcky and Mainwood, 1972; Rogus and Zierler, 1973) that the fast fraction of effluxing Na^+ comes from the Na^+ sequestered in the SR.

Now the energy need of the (hypothetical) Na^+ pump depends on the free Na^+ concentration within the cell (and other factors including the Na^+-concentration gradients across the cell surface). Trying to reduce the energy need of the pump, Miller adopted the idea of Na^+ sequestration in the SR of the above-cited workers, and claimed that a large (fast exchanging) fraction of intracellular Na^+ is not actively pumped because it is located at high concentration inside the SR (p. 28). This SR sequestration or compartmentation idea is also what he referred to in his June 28, 1996 letter to me cited above as "compartmentation effects" and "multicompartment membrane theory."

However, there was abundant evidence showing that the idea of Na^+ compartmentation in the SR is wrong. For brevity, I shall cite just two here. A third one will be brought up below.

(1) Returning to Figure 2A and Figure 2B, I would like to remind the reader that the size of the fast fraction of Na^+ efflux grows steadily in the poisoned muscles until at the time of death, Na^+ in the fast fraction takes up the whole volume of the muscle cells. Now if the fast fraction is truly Na^+ sequestered in the SR, then one is forced to conclude that as the muscle cell deteriorates, its SR expands in volume at the expense of the rest of the muscle cell until, at the time when the muscle cell dies, the entire muscle cell becomes a gigantic SR and all the thin filaments and thick filaments etc. of the contractile apparatus simply vanish. This is absurd.

(2) The most straightforward disproof of this SR compartmentation idea came from electron probe microanalysis of Somlyo and coworkers who concluded from their study of muscle cells "that there is no compartmentalization of chlorine and sodium in the sarcoplasmic reticulum, or in other cellular organelles . . . " (See Maugh, 1977, p. 358, column 1, 4th paragraph; Somlyo *et al.*, 1977).

5.2.2. The so-called "anomalous influx" (p. 32)

The reader recalls that earlier in this section, Miller has accused me of concocting an unacceptably high Na^+ pumping rate by exploiting an abnormal phenomenon he called anomalous efflux. This is yet another major pillar of Miller's argument intended to resurrect the membrane-pump hypothesis. Before showing what Miller called an anomaly really amounts to, let us examine a broader issue of scientific anomalies.

In his "The Structure of Scientific Revolutions," science historian, Thomas Kuhn (1962) alluded to two divergent responses to a scientific anomaly. They are respectively to suppress or to discover:

> "Normal science, . . . often suppresses fundamental novelties because they are necessarily subversive of its basic commitments" (p. 5)
>
> "Discovery commences with the awareness of anomaly (p. 52).

In my view, the pivotal point between these two divergent responses to an anomaly lies in what Albert Einstein called "inward freedom" of a scientist—a quality Einstein found quite rare (Einstein, 1956, p. 10), but I suspect may be "educated" out of many—as

witnessed on the one hand, by the often noted lack of formal education among scientists like Michael Faraday and Benjamin Franklin and innovators like Thomas Edison, and on the other hand, by the proverbial "village child" or even "idiot" who sees what scholars with advanced degrees do not.

Miller apparently wanted to suppress the anomaly, rather than to question the validity of the "basic commitment" of the majority of workers in this field, i.e., that the slow fraction truly represents the membrane-limited Na^+ efflux. Miller also made a highly reproducible phenomenon seemingly erratic and beyond comprehension—by befuddling it with claims based apparently on some casual unpublished observations made by another graduate student as a favor (page 34): "(the anomaly occurs) only when measured on small fiber bundles at 0°. The anomaly does not show up on whole muscles or at 20°C." None of these claims can be confirmed.

As shown in Table IVA taken from Ling (1981), the small fiber bundles as well as whole sartorius muscle show the same "inconsistency" between the efflux rate obtained from the intercept of the slow fraction and from the slope of the slow fraction—all carried out at room temperature just like the earlier ones described in Appendix 1 carried out at 0°C.

The same Table IVA also shows how with the new "basic commitment," it is the fast fraction of the Na^+ efflux of the centrifuged fiber bundle after CTE correction which represents the surface-, or, membrane-limited Na^+ efflux; the efflux rate determined by the intercept and by the slope of the plot of the fast fraction now agree with each other nearly perfectly. This experience tells us that Nature is never anomalous. However, through a pair of distorting eye glasses of faulty "basic commitment," Nature might appear anomalous.

5.2.3. Truth behind the alleged over-estimated pumping rate once more

Under Section 4, we have already shown how the claim that I overestimated the Na^+ efflux rate was totally wrong. Instead of my overestimating the Na^+ efflux rate 20 times, it was the conventional method which has underestimated the Na^+ by a factor of 10 at least. However, to keep my promise of answering in detail all the specific criticisms Miller posed, I now return to the problem again. To begin, let us examine exactly what Kolata proclaimed in her *Science* article and what Miller wrote in his Ph.D. thesis:

Miller:

> "The extremely large Na^+ pump rate used by Ling is about 20 times higher than the flux measured . . . by Keynes and Steinhardt" (p. 31 of Thesis).

Kolata:

> "They (Miller and Freedman) report that Ling's analysis of his data led him to assume that the sodium was being transported out of the muscle cells at least 20 times faster than the rate accepted by muscle physiologists" (*Science*, vol. 192, p. 1222, column 1).

Note that "about 20 times higher" from Miller is not the same as "at least 20 times faster" from Kolata. Nor is the "flux measured by Keynes and Steinhardt" the same as "(flux) rate accepted by muscle physiologists". But spending more time on these offenses would detract from far more serious offenses below.

Nor do I want to do more than just asking: "Why did Miller choose to select from my data

the flux rate of the order of 100 M/kg.hr or μmoles/gm.hr (cited at the beginning of section 5) and compare them with Keynes and Steinhardts's data from normal muscle?" A look at the Table 8.7 in Appendix 1 reveals that all the values around this 100 M/kg.hr figure were taken from muscles fiber bundles that had been exposed to the metabolic poison IAA. This is all the more difficult to understand when immediately beneath these data is that of a normal control muscle—with a considerably slower rate of Na^+ flux.

The gross offense is that Miller compares my correctly but deliberately under-estimated Na^+ pumping rate described in Appendix 1 (and in Section 4.3) with an erroneously determined flux rate obtained from the slope of the slow fraction without CTE correction by Keynes and Steinhardt. Finding them different, he proclaims that my pumping rate is 20 times too high. As will be made crystal clear below, Miller reached this spurious conclusion by repeating what he has been doing all along: *deliberate withholding of decisive key information.*

For the reader to understand without ambiguity what are withheld, it would be worthwhile to review the contents of Sections 4.1.1, 4.2.1 to 4.2.4 and 5.2.1 to show how new findings presented in these sections permitted our step-by-step logical progress toward the conclusion that it is from the fast fraction (of centrifuged muscle fiber bundles) and not from the slope of the slow fraction, one obtains the true membrane-limited Na^+ efflux rate. These steps are summarized next:

(1) Section 4.1.1 demonstrates an extracellular space of only around 10%, thus precluding the much larger fast fraction to be entirely due to Na^+ in the extracellular space.
(2) Section 4.2.3 shows that single and multiple fiber bundles studies revealed that a part of the fast fraction come from within the muscle cells.
(3) Section 5.2.1 shows that the fast fraction from single muscle fiber or multiple fiber bundles free from contamination of the extracelluar space or CTE, does not come from Na^+ in the sarcoplasmic reticulum (SR).
(4) Section 4.2.1 shows that from the studies of IAA-poisoned muscles, the fast fraction coming from within the muscle cells must come from the entire cytoplasm of the muscle cell interior or sarcoplasm.

When considered together, these findings have established that the conventional assignment of the slow fraction as representing membrane-permeation limited Na^+ efflux to be wrong. And that it is the fast fraction from the muscle (after removal of extracellular space and CTE contaminations) which measures the true surface, or membrane-limited Na^+ efflux rate, which in the context of the membrane-pump theory, is virtually identical to the pumping rate.

With this information in mind, let us examine the following replay of the same step-by-step logical sequence and Miller's active participation in it.

(1) On page 16 of the review coauthored by Ling, Miller and Ochsenfeld (1973), 3 out of the 5 new methods for determining the extracellular space size given in Section 4.1.1 are described, in addition to the conclusion that the newly estimated extracellular space volume is less than 10%. This recognition precludes the fast fraction as coming exclusively from the extracellular space and demands an additional source of Na^+ in the fast fraction.
(2) On pages 16 and 17 of this same review co-authored by Ling, Miller and Ochsenfeld, the single and multiple fiber studies described above in detail under Section 4.2.3 were already included. The study shows clearly that not all the fast fraction comes from the

extracellular space or the connective tissue elements. A fast fraction of the effluxing Na^+ must come from within the muscle cells.

(3) On pages 17 to 18 of this review co-authored by Ling, Miller and Ochsenfeld, the evidence was described—including the figure reproduced here as Figure 6—why this fast effluxing Na^+ from within the muscle cells cannot originate from the sarcoplasmic reticulum (SR). To prevent misunderstanding, I have reproduced verbatim here this section by Ling, Miller and Ochsenfeld—offering yet another set of evidence against the SR compartmentation interpretation in addition to the two later ones already described under section 5.2.1:

> "that only the T-tubules are open to the outside, (Porter and Bonneville, 1964), that the T-tubule space, at some 0.2–0.4% in volume (Peachey, 1965; Hill, 1964; Huxley, 1964) can be reached by the extracellular space probes, and therefore is already included in the below-10% figure (for the extracellular space) cited. However, even if we assume that this membrane separating the sarcoplasmic reticulum (from the muscle cytoplasm, or sarcoplasm—added by GL for clarity) to have specific high permeability toward small molecules and ions, there are still ample reasons to reject Keynes and Steinhardt's suggestion (that the SR contains most of the intracellular Na^+): (1) No such fast fraction is observable in the (small, added by GL) Br^- ion exchange (Ling, 1972). (2) When sartorius muscles are exposed briefly to Ringer solution containing 100 mm K^+ or Rb^+ ion (in which the K^+ and Rb^+ concentrations were made equal to the Na^+ concentration in a normal Ringer solution), the efflux curves can be resolved into two single fractions—a very fast fraction corresponding to an extracellular space of below 10% and another slow fraction from the cell. No second fast fraction exists (Figure 5)." (Figure 5 in the original is reproduced here as Figure 7).

(4) On pages 20 to 21 of the same review co-authored by Ling, Miller and Ochsenfeld, the Na^+ effluxes from IAA poisoned dying muscles described in detail under section 4.2.1 were reviewed, leading to the conclusion that the fast fraction of Na^+ efflux coming from within the cells must come from all parts of the muscle cell interior or cytoplasm.

(5) All told, the 4 quotes above have already disproved that the slope of the slow fraction measures the pumping rate. In affirmation, on page 21 of the same review co-authored by Ling, Miller and Ochsenfeld, they wrote: "the true intracellular-extracellular exchange rate . . . has been shown to be approximately eight times faster than the exchange rate of the γ-fraction (both at 25°C). The γ-fraction referred to in this 1973 article is what we call the "slow fraction" here, the slope of which has been used in virtually all conventional studies to yield the erroneous membrane-limited extra-, intra-cellular exchange rate of Na^+, including those of Keynes and Steinhardt.

In summary, Miller's claim that Ling's pumping rate is 20 times too fast is entirely upside down. It is the conventionally accepted figure that is 10 times too low. There is no labeled Na^+ concentrated inside the SR. Nor is there anything anomalous except from a wrong viewpoint. Miller's central claim that I overestimated the Na^+ pumping rate is an illusion created entirely by withholding key information pointing to the opposite in each and every turn of the case. So is Miller's conclusion that "Ling's energy calculation for the Na^+ pump . . . is equivocal enough that it should not be taken as disproof of the membrane theory"

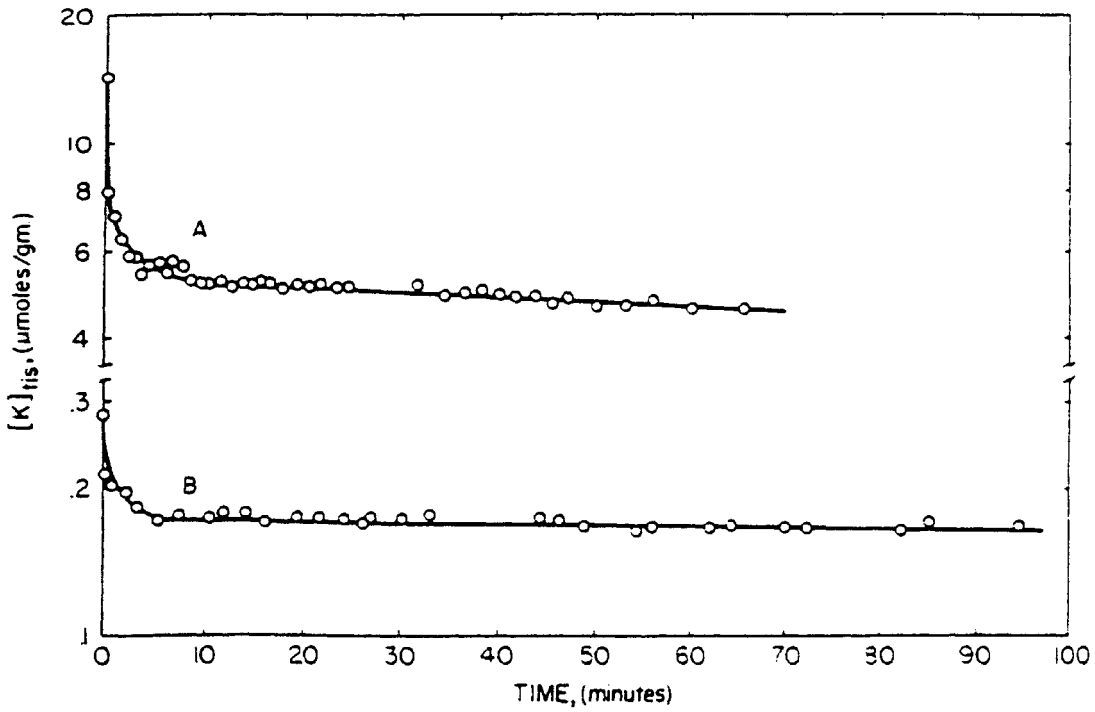

FIGURE 7. K^+-ion-efflux curves of frog sartorius muscles briefly exposed to ^{42}K-labeled Ringer's solution. **A:** 7-min exposure in 0.118 M KCl with enough glucose to prevent swelling. **B:** 7.9-min exposure to normal Ringer's containing 2.5 mM labeled K^+-ion. All incubation and washout carried out at 0°C. Fast fraction was $\frac{100}{14.3-5.4} = 8.9\%$ in A and $\frac{0.282-0.174}{2.5} = 4.3\%$.
(From Ling *et al.*, 1973 by permission of New York Academy of Sciences.)

(Ph.D. thesis, p. 36). Miller was not being falsely modest when he himself described the materials he once circulated around and set Kolata on her misbegotten mission, as "useless junk."

The fact that a reporter like Gina Kolata should fall for something Miller and Freedman circulated around gave me one reason that I have to put the record straight. And that is what I have done here—in part also fulfilling a personal belief that I answer every printed criticism of my work no matter how trivial it may appear to me—as I have been doing all my life.

There is little question in my mind that the mass exodus of my former students per se, aided by the kind of unfounded claims put out by Miller, Freedman (and my other fleeing students) in addition to Gina Kolata's cavalier endorsement have all contributed to the plight of legitimate cell physiology today. Nevertheless, there is also little doubt in my mind that Miller and all my other graduate and postdoctoral students would have behaved altogether differently if they did not see a total hopelessness in front of them following what they once started to do: to lead the life of a real scientist. Read my home page (Ling, 1997); you should have a good idea from where the evilness really came.

Finally a reader may ask: "Have you spent too much time dwelling on something which

took place so long ago?" My answer is No. To convince yourself of what I think, you can go to the library and borrow a copy of any major newspaper published on October 16 this year (1997). There you will discover an announcement from the Royal Swedish Academy of Sciences which stated that

> One half of the 1997 Nobel prize for Chemistry is awarded to Jens Skou from Denmark for his work on the sodium pump.

This announcement tells you volumes on how far the practice of the game of illusion has infested the world of cell physiological science in the closing years of the 20th century. And a great opportunity for rapid progress in this basic science and for healing the sick have been squandered in the obscenity of glorifying the Holy Cow named the Sodium Pump. And generations upon generations of students from high schools to graduate schools are still routinely spoon-fed the same useless junk.

Is the future of our last major domain of unexplored relevant basic science entirely hopeless then? To answer, I shall tell you a story. (However, for a more direct and specific answer, read the concluding sections of my home-page, Ling, 1997).

The story I tell is that of a frail Japanese historian, Saburo Ienaga, now 83 years old. Thirty-two years ago Ienega went to court to contest the right of the Government to delete parts of the history textbook he wrote, describing the atrocities committed by the Japanese army during the Second World War, including the holocaust of Nanking (see Chang, 1997), and the use of Chinese prisoners for conducting medical experiments, killing thousands. After an initial victory at a lower court for Ienaga, the Supreme Court in 1993 ruled that the Government was well within its right to delete whatever it sees as inappropriate (Sanger, 1993).

This total defeat, however, did not faze Ienaga. Instead, he told a reporter: "I did not start this thinking I could win. In the end almost no one wins a lawsuit in Japan against the Government. But for 20 years I think I have proved a great deal. Even if I couldn't win in court, in the court of history I think I have been victorious." "I think I have been motivated by guilt, nothing else," Ienaga added. In spite of his outward cynicism, he continued to battle in court even after the 1993 defeat.

Then in 1997 the Supreme Court in a 3–2 split decision, reversed its earlier verdict. In pronouncing the court's verdict, Presiding Justice Maso Ono quoted from one of Japan's most famous novelists, Ryotaro Shiba: "A country whose textbooks lie . . . will inevitably collapse." Ono then pronounced that the Education Ministry had illegally forced the deletion of accurate description of Japanese atrocities during World War II (Efron, 1997).

Ienaga at long last won his court battle. And truths, harsh savage and mortifying truths though they are, have replaced the soporific fabrications taught in Japanese schools.

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APPENDIX 1

The Role of Metabolism in Biological Functions

(Reproduced from Chapter 8 of Ling, 1962)

ALTHOUGH ISOLATED nonliving systems approach lower states of energy and higher degrees of randomness with the passage of time, living systems appear to counter this universal trend in two ways. They perform work reversibly, returning to their initial state after each cyclic event. They also maintain—for a considerable length of time—a labile but constant state, which invariably disintegrates after death. To reconcile these phenomena with physical laws, biologists have contended that reversible work performance, as illustrated by mechanical work in muscular contraction and electrical work in nerve action potentials, is coupled with energy-yielding reactions. Many biologists maintain that the sustenance of the labile living system, even in the resting condition, requires a continuous supply of energy; for example, maintenance of the high level of K^+-ion selectively accumulated in most living cells is thought to require a machinelike pump operation. This type of work is referred to as osmotic work and is conceived as the continuous use of metabolic energy to pump out undesirable elements (the Na^+ ion, for instance) and to maintain a steady living state.

While they were developing these ideas, physiologists and biochemists added a comprehensive store of exact information about the complex sequence of chemical processes that constitute metabolism. Of central interest is the oxidative and glycolytic conversion of food matter into those usable packets of metabolic energy, which we now call high-energy phosphate bonds, residing in compounds such as phosphoenolpyruvate, acetyl phosphate, creatine phosphate (CrP), and particularly ATP. This process is outlined in Figure 8.1 (see Baldwin, 1957).

8.1 The Role of High-Energy Phosphate-Bond Compounds in the Maintenance of Biological Functions

To point out the significance of the high-energy phosphate bond in the present theory, first, we shall show that oxidation, glycolytic reactions, and the stores of high-energy phosphate-bond compounds such as CrP and ATP are the only significant sources of metabolic energy that maintain biological function; then we shall demonstrate that normal functions are maintained when both oxidative and glycolytic activities are entirely blocked, leaving high-energy phosphate bonds in the form of CrP and ATP as the only source of energy. Finally we shall ask: If ATP and CrP can maintain functions, do they accomplish this by

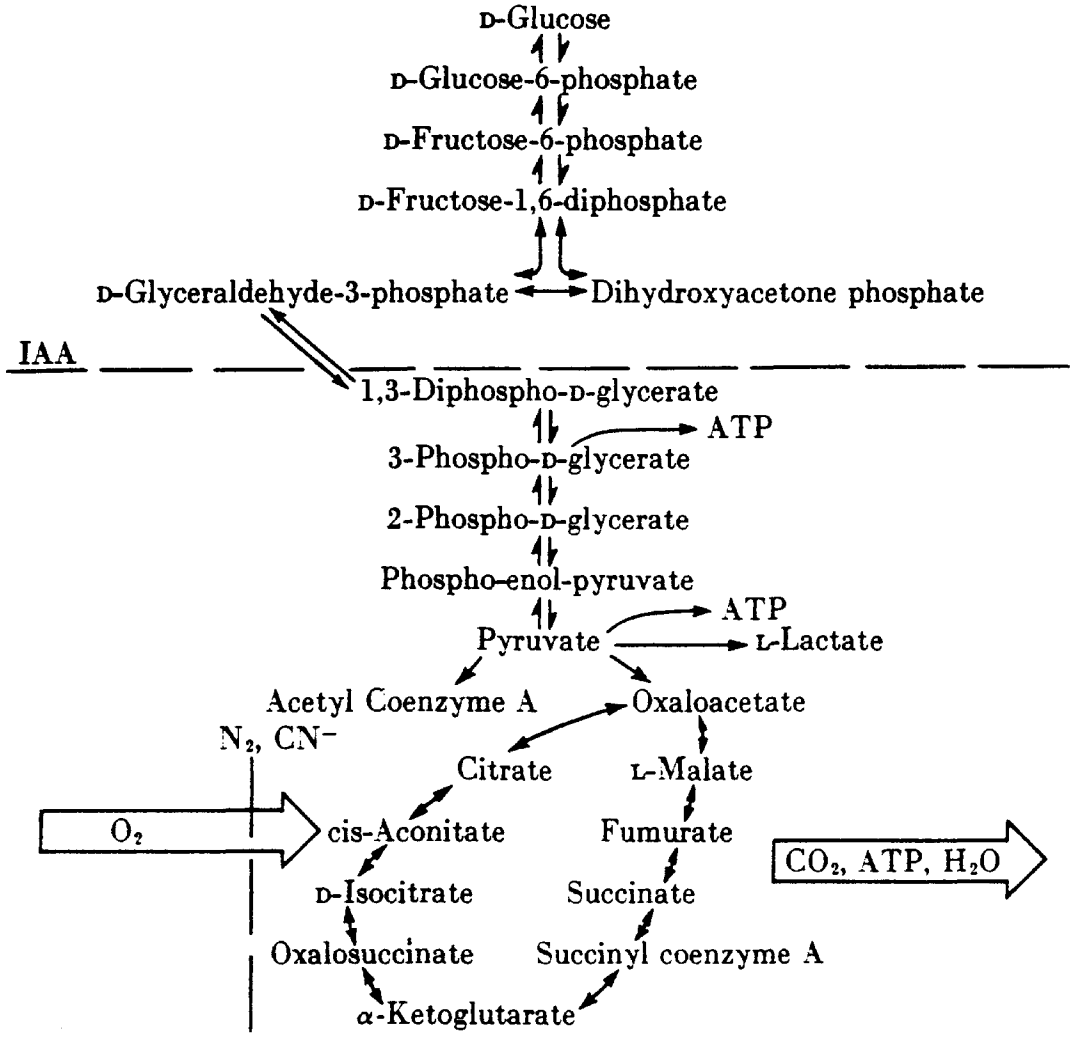


FIGURE 8.1. The main pathways of glycolytic and oxidative metabolism. The main products of metabolism are ATP, lactate, CO_2, and H_2O. The points of action of the inhibitors, iodoacetate (IAA), cyanide (CN^-), and nitrogen (N_2), are indicated by broken lines.

continually *hydrolyzing* and directly delivering their high-energy phosphate-bond energy or do they act through another mechanism?

Figure 8.2 presents the results of an experiment in which a frog *sartorius* muscle, poisoned with iodoacetate (which completely blocks glycolysis) and nitrogen (which completely inhibits respiration), was stimulated electrically. Exhaustive stimulation under such conditions removed the ATP and CrP content of the muscle. As the figure shows, following such stimulation, the muscle promptly began to lose its selectively accumulated K^+ ions. On the other hand, any one of the factors, pure nitrogen (Table 8.1), glycolytic blockage (Table 8.4), or *sciatic* stimulation (Figure 8.2, control muscle), by itself, produced little effect (pure

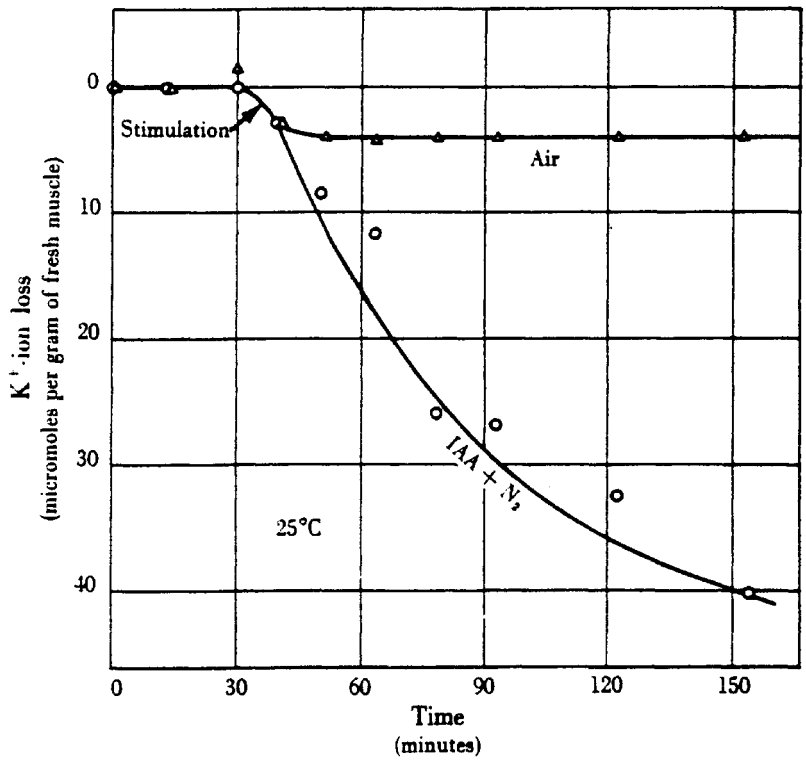


FIGURE 8.2. The loss of K^+ ion from muscle stimulated in the presence and in the absence of iodoacetate (IAA) plus nitrogen (25°C). Paired muscles from the same frog were placed in tandem in separate watch glasses electrically connected through a moist cotton wick. At the time indicated by the arrow, tetanic stimulation from an inductorium was applied directly to both muscles for two minutes. Successive aliquots of the bathing solution were then analyzed for K^+ ion. Points marked with triangles indicate the control muscle in normal Ringer's solution and air; points marked with circles indicate the experimental muscle in Ringer's solution containing 0.005*M* IAA and N_2. Other experiments showed that the loss of K^+ ions from the experimental muscles continued until external and internal K^+-ion concentrations were equal—about four and a half hours.

nitrogen, electrical stimuli), or a very slow one (0.005*M* iodoacetate alone, Ling, unpublished; see Dean, 1940). Table 8.2 shows the analogous behavior of the resting potential of muscle fibers; Figure 8.3, Table 8.3, Figure 8.4, and Figure 8.5 illustrate the companion observations on contractile function, action potentials, and the rate of Na^+-ion exchange in frog muscle. All of these functions can be performed normally in the absence of active oxidative and glycolytic activity as long as the normal CrP and ATP contents are present. But, as soon as these compounds are removed from the poisoned cells, all functions cease. We must conclude that *respiration, glycolysis, and high-energy phosphate-bond compounds in the form of* CrP *and* ATP *are the only energy sources which effectively maintain cellular excitability, contractility, and ability to accumulate ions selectively. Other sources of energy, if they exist, are so trivial in effect that they can be ignored.*

Date	Frog No.	Environment	Muscle types	Muscle wt, mg	Temp., °C	Duration, hr	K, μM/g
3-3-55	1	Air	1, 2, 3	287.6	25	1	77.6
	1	N_2		293.4		1	81.7
	2	Air		280.6		2	72.6
	2	N_2		281.0		2	77.0
	3	Air		325.6		3	70.0
	3	N_2		318.2		3	74.9
	4	Air		265.2		4	65.9
	4	N_2		240.0		4	68.5
	5	Air		221.4		5	65.1
	5	N_2		222.4		5	65.4
2-2-55	6	Air	1, 2, 3, 4	368.2	20	4	87.4
	6	N_2		376.6		4	92.8
	7	Air		318.6		4	87.4
	7	N_2		317.0		4	83.5
	8	Air		270.6		4	91.7
	8	N_2		260.8		4	96.1
	9	Air		210.6		4	95.4
	9	N_2		201.2		4	81.2
	10	Air		392.8		4	95.2
	10	N_2		408.6		4	83.8
	11	Air		316.6		4	95.6
	11	N_2		314.8		4	82.7

TABLE 8.1 The effect of anoxia upon the K^+-ion content of frog muscles. The K^+-ion content of paired *sartorius* (1), *semitendinosus* (2), *tibialis anticus longus* (3), and *iliofibularis* (4) muscles from frogs was determined by means of flame photometry after the muscles had been soaked for one to five hours in Ringer's solution bubbled with either air or N_2 purified by passage through a column of activated copper. The data indicate no significant loss of K^+ ions after complete blockage of oxidative metabolism for up to five hours at room temperature.

Next, experiments were designed to assure complete suppression of respiration (oxidative metabolism) and glycolysis in frog muscle. Under these conditions and at a temperature of 0°C, the tissues maintained their excitability, contractility, and normal selective ionic accumulation pattern for at least eight hours (Tables 8.4 and 8.5). We concluded that, in adequate quantities, the high-energy phosphate-bond compounds, ATP and CrP, are capable, by themselves, of maintaining the tissue's capacity for normal performance of mechanical and electrical work as well as the normal asymmetric ionic distribution. This conclusion serves as the foundation for our discussion on the mechanisms of functions such as contraction and nerve activity.

8.2 Evidence that the Maintenance of Selective Ionic Accumulation in the Resting Cell Represents a Metastable Equilibrium State Rather than a Steady State

We have shown that respiration, glycolysis, and the store of high-energy phosphate-bond compounds are the only significant sources of energy in the cell. Yet a normal ionic

Muscle condition	Potential[b]	
	Average, mv	Maximum fiber
In 0.005*M* IAA for 1 hr at 0°C, then at 22.5°C	85.2 ± 2.9	
After 100 shocks to muscle, 2/sec[a]	82.1 ± 3.8	
After 100 more shocks to muscle	76.0 ± 3.8	
After 100 more shocks to muscle	80.3 ± 3.0	
After 100 more shocks to muscle	84.9 ± 5.1	
20 min after stimulation	84.1 ± 2.0	
40 min after stimulation	64.4 irreg.	
60 min after stimulation	15.0 irreg.	
In 0.01*M* NaCN for 3 hr at 20°C	80.0 ± 2.2	
After 90 sec tetanization of muscle, 100/sec	47.7 ± 5.5	
45 min after tetanization	62.8 ± 9.5	
120 min after tetanization	75.4 ± 4.8	
In 0.005*M* IAA for 15 min at 22.5°C	77.6	
After 3 min tetanization of nerve, 100/sec	55.7	56.0
30 min after tetanization	55.7	56.0
60 min after tetanization	13.0	47.5
90 min after tetanization	12.0	45.6
120 min after tetanization	8.5	43.0

TABLE 8.2 Resting potentials of single muscle fibers in iodoacetate or cyanide before and after stimulation. These experiments demonstrate that the normal resting potential (84.5 ± 3.2mv *in vivo*, Ling and Gerard, 1949a) can be maintained in the absence of active glycolytic and oxidative metabolism. The potentials of such poisoned muscle cells degenerate after electrical stimulation which removes CrP and ATP from them, see Table 8.3, also Ling, unpublished. (Data *from* Ling and Gerard, 1949b.)

[a] The interval between successive periods of stimulation is 3 to 5 minutes during which readings are made.

[b] Each figure is obtained on 4 to 12 fibers. The probable error of most figures given is between 2 and 5 mv.

accumulation pattern is maintained when both respiration and glycolysis are blocked. Thus, *if the maintenance of the ionic accumulation pattern depends upon a continuous energy expenditure, this energy must be that liberated in the degradation of the ATP and CrP originally present in the system.* Thus we can estimate the *maximum energy-delivery rate* by observing the difference between the amount of ATP and CrP in a muscle at the beginning of an experiment and the amount present at the end (in paired muscles).

We can also estimate the *minimum energy-delivery rate* required by the membrane-pump theory to maintain the normal asymmetric ionic distribution through the operation of a metabolic pump. If Na^+ ions are to be pumped out of a cell, work must be done against both a concentration gradient (the intracellular concentration $[Na]_{in}$ is much lower than the extracellular concentration $[Na]_{ex}$) and an electrical potential gradient (the resting potential is positive outside the cell). The work done against the concentration gradient, per mole of

Date	Environment	Creatine phosphate in milligrams P_2O_5 per gram of *sartorius* muscle
5-17-30	Muscle in N_2	0.20
	Muscle in O_2	1.32
5-19-30	Muscle in N_2	0.15
	Muscle in O_2	1.23
5-19-30	Muscle in N_2	0.17
	Muscle in O_2	0.89
5-20-30	Muscle in N_2	0.09
	Muscle in O_2	0.73
5-23-30	Muscle in N_2	0.11
	Muscle in O_2	1.25
5-23-30	Muscle in N_2	0.27
	Muscle in O_2	0.97
5-25-30	Muscle in N_2	0.16
	Muscle in O_2	0.93
Average	Muscles in N_2	0.164
	Muscles in O_2	1.046

TABLE 8.3 Creatine phosphate content of iodoacetate-poisoned *sartorius* muscles after stimulation in oxygen and nitrogen. Paired muscles were used as for Figure 8.3. The early exhaustion of the ability to perform mechanical work in IAA plus nitrogen was accompanied by an early exhaustion of CrP content as compared with the paired muscle contracting in IAA plus oxygen. (Data *from* Lundsgaard, 1930.)

Na^+ transported, is given by

$$E_{Na}(t)\, RT \ln \left(\frac{[Na]_{ex}}{[Na]_{in}} \right)_i \tag{8-1}$$

From microelectrode measurements, we obtained the electrical potential gradient $\psi(t)$ at various times during the experiment (Table 8.6). The work per mole done against the potential gradient is equal to $\mathcal{F}\psi(t)$, where $\mathcal{F}$ is the Faraday constant. The minimum energy required by the Na pump to sustain selective ionic accumulation for a length of time $t_f - t_o$ is given by the total work ΔW done in this time:

$$\Delta W = \int_{t_o}^{t_f} [\mathcal{F}\Psi(t) + E_{Na}(t)] J_{Na}^{in \rightarrow ex}(t)\, dt \tag{8-2}$$

where $J_{Na}^{in \rightarrow ex}(t)$ is the rate of pumping in moles of Na^+ ion exchanged per kilogram of fresh muscle per hour; this measurement was also made from time to time on the experimental muscles using the radioactive tracer Na^{22} (Table 8.7); these measurements enable us to

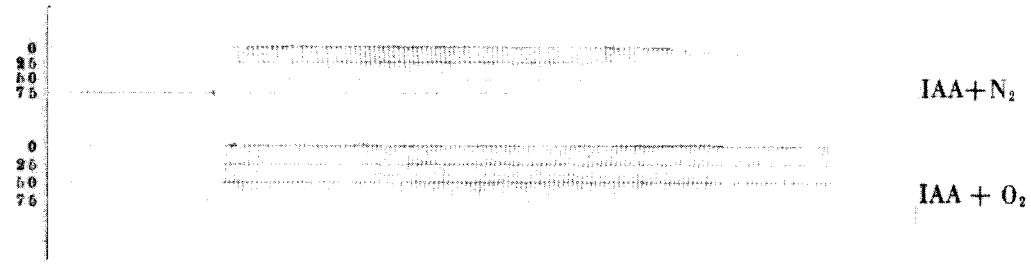


Figure 8.3 Successive muscle twitches from paired frog *sartorius* muscles poisoned with iodoacetate. Stimulation was carried out in nitrogen (upper record) and in oxygen (lower record). These experiments establish that normal contractile function can be totally independent of active glycolytic and active respiratory metabolism. The ability to perform mechanical work ceases with the exhaustion of the CrP in the muscles, see Table 8.3. (Figure *from* Lundsgaard, 1930.)

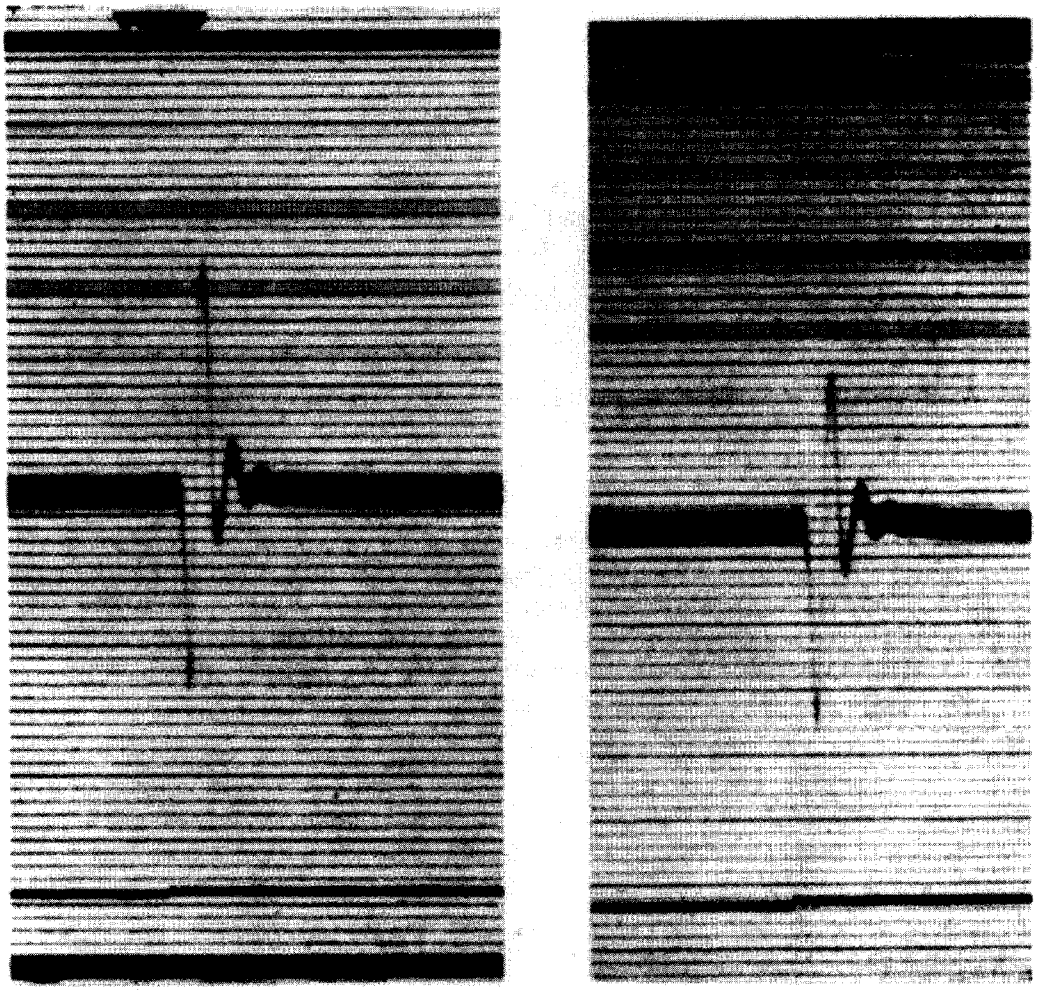
estimate the minimum energy necessary for the effective operation of a hypothetical Na pump for the duration of the experiment; we made the unlikely assumption that all energy transfers and utilization processes are 100 per cent efficient.

Representative data accumulated from more than five years of work (1950–1956) are presented in Tables 8.5, 8.6, 8.7, 8.8, and 8.9, and in Figures 8.6 and 8.7. We were forced to conclude that the mean maximum energy liberated per kilogram of muscle per hour cannot exceed 25 calories (Table 8.9); the mean minimum energy needed to perform the postulated pumping operation is more than ten times greater (343 cal).

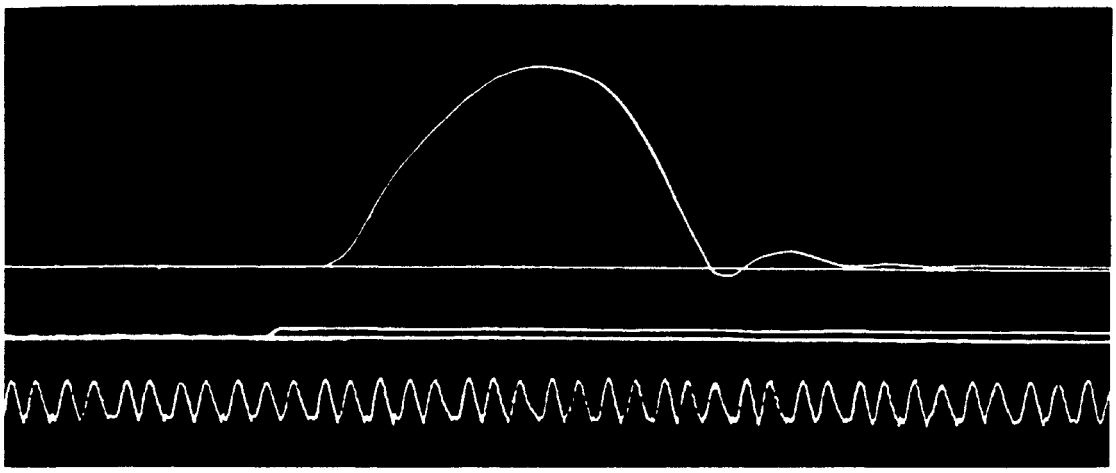
Experiments of this type necessitate the conclusion that the energy available to the cell is not sufficient to operate the Na pump—even if the cell performs no energy-requiring function but the maintenance of its asymmetrical Na^+-ion distribution. Thus the phenomenon of selective ionic accumulation, *per se*, does not belong in the same category as the performance of mechanical work (in contraction) or electrical work (in the action potential). It cannot depend upon a continuous expenditure of energy, and, therefore, cannot be said to involve the performance of osmotic work.

Although the above experiments indicate that selective ionic accumulation cannot depend on a continuous expenditure of energy, they do not suggest that metabolism is not essential. Metabolism is essential; even though muscle deprived of its ability to carry on either oxidative or glycolytic metabolism can maintain its functions at 0°C for eight hours or longer, the tissue eventually does degenerate. It loses its excitability and contractility, and liberates all of its selectively accumulated K^+ ions. It has been noted that there is a simultaneous exhaustion of the CrP and ATP contents.

We conclude that the maintenance of physiological function depends crucially upon the presence of ATP and other metabolic products within the protein fixed-charge system. This dependence rests on the role of ATP and CrP as cardinal adsorbents onto the fixed-charge system rather than on the continuous liberation of energy from these compounds through their hydrolytic degradation. This conclusion follows naturally from our hypothesis which



A B



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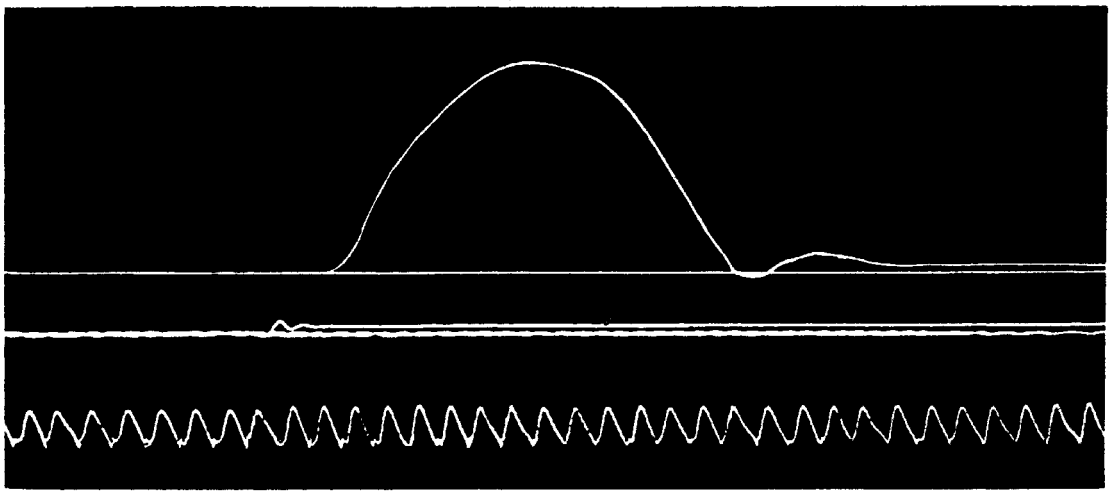

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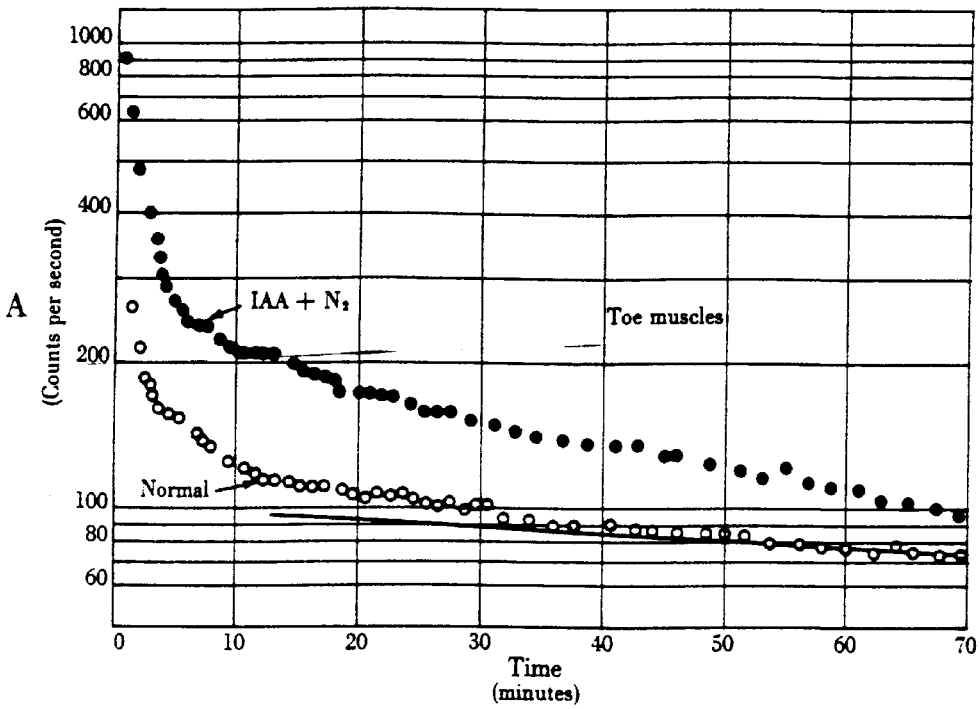
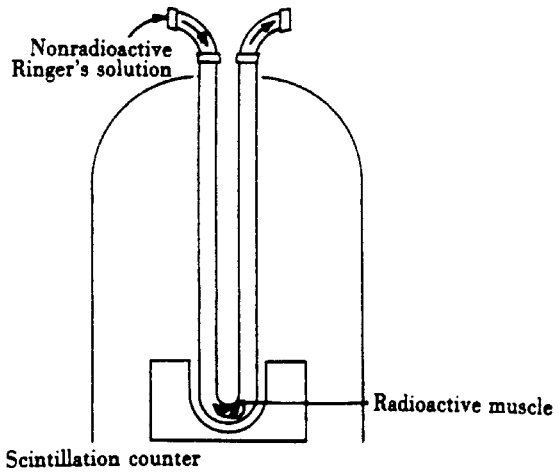
FIGURE 8.4 Electrical and mechanical activity of iodoacetate-poisoned frog *gastrocnemius* muscles. (Room temperature.) A and B are electromyograms from indirectly stimulated *gastrocnemii;* C and D are isometric contraction tracings. Muscles A and D have been poisoned with IAA; muscles B and C are normal controls. The figure shows that normal electrical and mechanical behavior can be maintained in muscles whose glycolytic metabolism is blocked. (Figures *from* Henriques and Lundsgaard, 1931.)


A
1000
800
600
400
200
100
80
60
(Counts per second)
IAA + N₂
Toe muscles
Normal
0
10
20
30
40
50
60
70
Time
(minutes)


Nonradioactive
Ringer's solution
Radioactive muscle
Scintillation counter
B

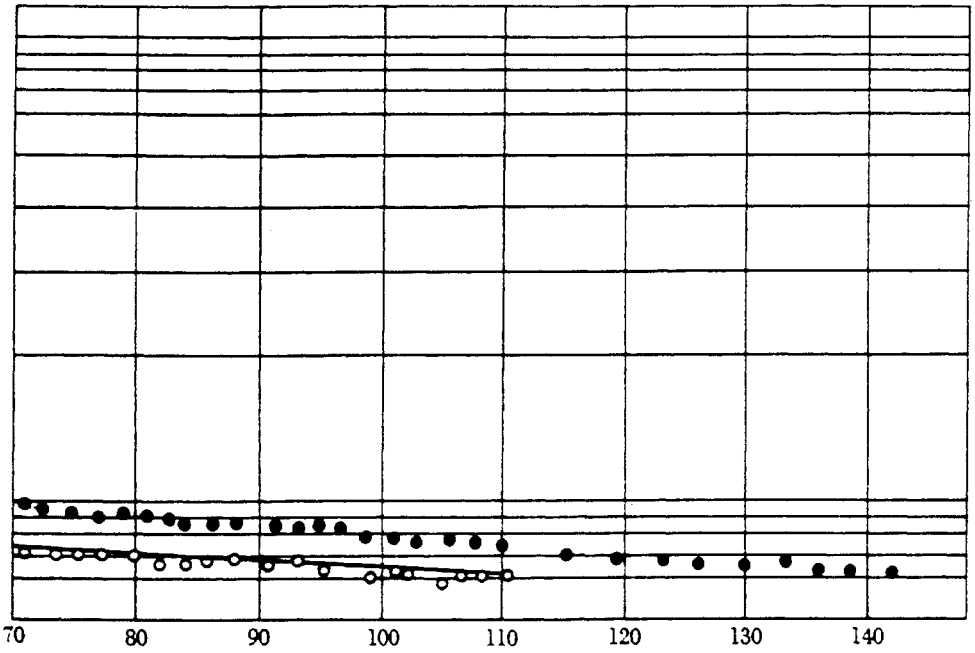


FIGURE 8.5 Washout of Na^{22} from frog toe muscle. A, paired *extensor digitorium* IV muscles were equilibrated overnight at 0°C in Ringer's solution containing Na^{22} and washed in Ringer's solution at 0°C. The washing solution used to produce the upper curve contained 0.001*M* IAA and was bubbled with nitrogen; the lower curve was obtained with normal Ringer's solution bubbled with oxygen. These curves are similar in nature; the differences in their absolute magnitudes are largely results of the initial quantities of tracer present in and weight difference among the muscles. B, the washing-out apparatus used in this experiment consists of a U-shaped glass tube that can be placed in the well of a scintillation counter. A radioactive muscle is held at the bottom of the tube by pieces of thread tied to either end of the muscle and affixed to the inlet and outlet of the glass tube. A continuous stream of nonradioactive Ringer's solution is drawn through the tube by negative pressure. Since small changes of position of the specimen do not affect the radioactive count (because of the 4π geometry of the well), several samples can be studied simultaneously if the U-tubes containing them are placed in the well alternately. To assure a constant temperature for the experiments the entire apparatus was kept in a constant-temperature room.

Frog No.	Tissue	Control or experiment	Tissue wt, mg	K, μM/g	Na, μM/g
1	muscle 2[a]	control	96.8	78.6	25.8
	muscle 2	experiment	96.8	78.6	28.5
	muscle 3[a]	control	94.6	82.8	23.0
	muscle 3	experiment	93.6	81.1	24.6
2	muscle 2	control	109.6	70.2	18.0
	muscle 2	experiment	109.6	73.0	18.0
	muscle 3	control	105.6	67.6	20.8
	muscle 3	experiment	103.6	70.5	20.5
3	muscle 2	control	85.4	74.8	25.2
	muscle 2	experiment	84.6	74.4	30.9
	muscle 3	control	100.0	64.6	30.5
	muscle 3	experiment	101.4	75.6	23.2
4	muscle 2	control	86.4	55.5[b]	41.6[b]
	muscle 2	experiment	86.4	79.5	18.5
	muscle 3	control	88.8	45.1[b]	57.7[b]
	muscle 3	experiment	91.3	77.5	24.9
	nerve	control	33.0	34.8	73.0
	nerve	experiment	31.4	35.0	69.8
5	muscle 2	control	100.2	71.8	29.9
	muscle 2	experiment	101.0	71.0	29.9
	muscle 3	control	83.2	71.0	24.5
	muscle 3	experiment	84.3	62.0	34.4
	testis	control	26.8	56.0	35.8
	testis	experiment	23.0	56.7	41.3
	kidney	control	66.4	30.0	51.4
	kidney	experiment	64.0	35.2	51.9
	nerve	control	32.8	38.7	62.8
	nerve	experiment	28.0	35.8	51.1
6	muscle 2	control	100.0	70.5	36.7
	muscle 2	experiment	102.6	75.5	26.3
	muscle 3	control	97.0	71.4	31.0
	muscle 3	experiment	97.0	65.6	52.0
	testis	control	26.4	43.5	52.3
	testis	experiment	17.4	39.1	47.1
	kidney	control	74.2	40.4	60.7
	kidney	experiment	69.4	45.9	54.8
	nerve	control	30.2	37.1	79.5
	nerve	experiment	28.6	40.6	84.0
7	muscle 2	control	102.2	75.2	38.6
	muscle 2	experiment	103.6	86.7	27.5
	muscle 3	control	97.0	83.8	29.5
	muscle 3	experiment	97.0	82.6	28.1

TABLE 8.4 (*Continued on facing page*)

Frog No.	Tissue	Control or experiment	Tissue wt, mg	K, $\mu M/g$	Na, $\mu M/g$
8	muscle 2	control	88.0	83.0	27.6
	muscle 2	experiment	82.0	86.2	31.7
	muscle 3	control	80.4	78.4	33.7
	muscle 3	experiment	83.4	84.4	27.6
9	muscle 2	control	98.2	80.0	28.6
	muscle 2	experiment	94.8	84.3	27.4
	muscle 3	control	92.6	78.9	30.4
	muscle 3	experiment	92.8	77.4	35.9
10	muscle 2	control	90.8	71.5	29.6
	muscle 2	experiment	90.8	72.7	32.8
	muscle 3	control	98.2	73.5	28.1
	muscle 3	experiment	100.3	72.8	26.1
9	heart	experiment	97.6	29.8	52.5
5	heart	experiment	104.0	21.4	38.7
6	heart	experiment	88.6	33.9	48.6
7	heart	experiment	90.4	22.2	63.0
8	heart	control	83.4	36.3	56.3
4	heart	control	80.0	45.0	49.0
10	heart	control	83.4	40.2	47.2

TABLE 8.4 The effect of iodoacetate and pure nitrogen on the K^+- and Na^+-ion contents of frog tissues. Isolated frog tissues were placed in a Ringer's solution (0°C) containing 0.005*M* sodium iodoacetate (Eastman). The solution had been equilibrated with 99.99% pure nitrogen (Linde Air Corp.). At the end of the experiment (after 7 hours and 45 minutes), the individual tissues were boiled in 3ml of distilled water to extract their K^+- and Na^+-ion contents. (For experiments after 1953, a different procedure was adopted; extraction was accomplished by overnight emersion of the tissues, in 3 ml of 0.1*N* HCl without heating.) The aliquot for the K^+-ion determination was diluted to contain a final concentration of 0.1*M* NaCl; aliquots for the Na^+-ion determination contained 0.1*M* KCl. These radiation buffers in the samples, as well as in all standards, eliminate both self-interference and radiation interference which can be considerable for direct flame photometry. A Beckman DU spectrophotometer with a flame attachment was employed. The results presented are those of an experiment performed on February 27, 1953; this experiment is one of more than 20 similar series performed since 1950; all yielded similar results. (For example see Table 8.5; here the control of respiration and glycolysis was more rigorous.)

[a] Muscles 2 and 3 represent the *semitendinosus* and *tibialis anticus longus*, respectively; "nerve" refers to *sciatic* nerve.

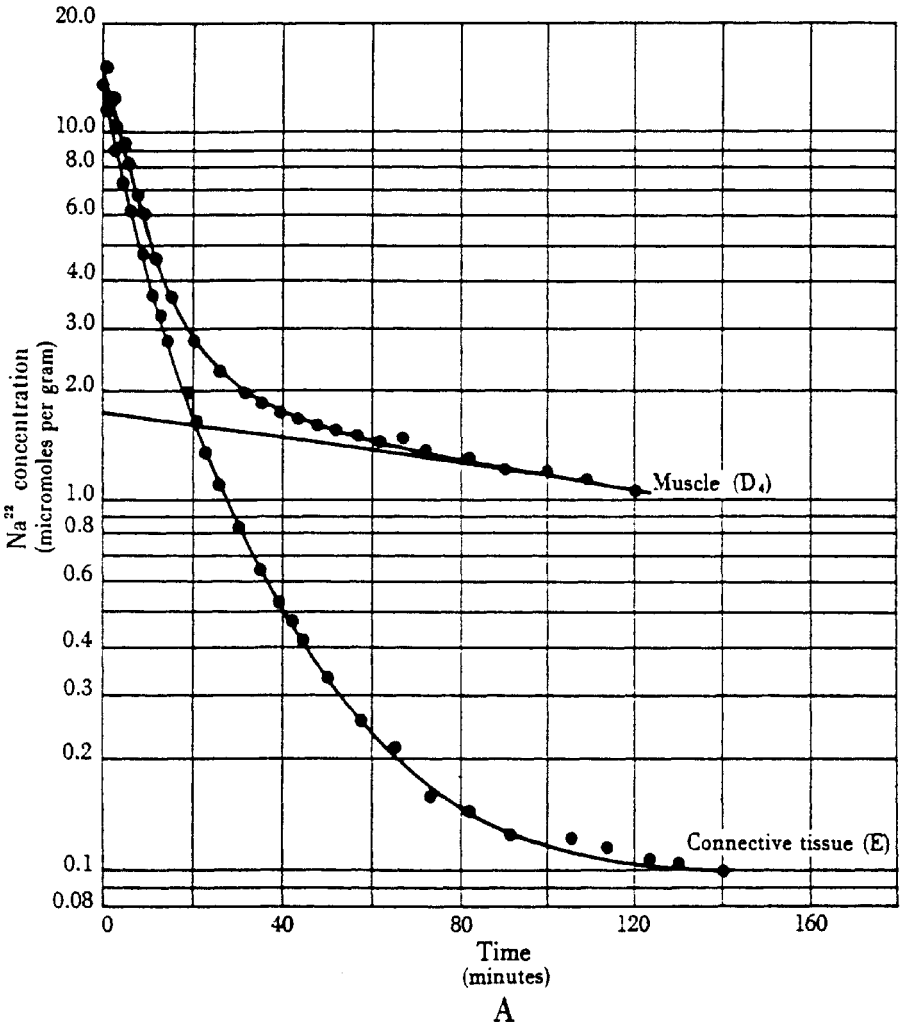
[b] Low K^+, high Na^+ found in these control muscles were probably from the same abnormal leg.

Frog No. and sex	Control or experiment	Muscle wt, mg	Duration of soaking in IAA-CN-N_2, hr	K, μM/g	CrP, μM/g	ΔCrP, μM/g	ΔF (CrP), cal/kg/hr	ATP, μM/g	ΔATP, μM/g
1 ♀	Control	418.1	0	72.5	10.5	−3.7	−11.8	7.31	−0.83
	Experiment	421.5	4	61.7	6.8			6.48	
2 ♀	Control	421.3	0	72.5	17.7	−7.4	−23.7	7.00	−0.34
	Experiment	441.1	4	67.8	10.3			6.66	
3 ♀	Control	373.3	0	77.7	11.3	−5.8	−18.6	6.78	+0.27
	Experiment	350.5	4	68.3	5.5			7.05	
4 ♂	Control	367.9	0	73.5	17.2	−8.5	−27.2	7.46	−0.65
	Experiment	374.9	4	72.4	8.7			6.81	
5 ♂	Control	407.3	0	69.5	24.2	−7.1	−22.8	6.49	+0.53
	Experiment	412.7	4	76.3	17.1			7.02	
6 ♂	Control	307.1	0	66.4	19.2	−7.9	−25.3	6.15	+0.63
	Experiment	313.8	4	74.7	11.3			6.78	
Average							−21.57		

Frog No. and sex	ΔF (ATP), cal/kg/hr	ADP, μM/g	ΔADP, μM/g	ΔF (ADP), cal/kg/hr	Lactate, μM/g	Δ Lactate, μM[a]	ΔF (Lactate),[a] cal/kg/hr	ΔF (Total), cal/kg/hr
1 ♀	−6.10	0.09	+0.36	+1.35	0.972	−0.235	Total loss from muscles, 0.744μM. Bathing solution contained 0.0196μM/ml before experiment; 0.0298μM/ml after experiment. Total gain in solution, 0.929μM. Net production, 0.185μM.	−17.20
		0.45			0.415			
2 ♀	−2.49	0.0	+0.42	+1.58	0.848	−0.219		−25.18
		0.42			0.352			
3 ♀	+1.00	0.10	+0.11	+0.45	0.719	−0.117		−17.71
		0.21			0.387			
4 ♂	−4.75	0.93	−0.32	−1.20	0.623	−0.055		−33.71
		0.61			0.478			
5 ♂	+3.87	0.38	−0.38	−1.35	0.511	−0.065		−20.84
		0.0			0.364			
6 ♂	+4.63	0.50	−0.50	−1.95	0.540	−0.053		−23.18
		0.0			0.369			
Average	−0.64			−0.18			−0.56	−22.97

TABLE 8.5 Maximum rate of energy delivery in muscles poisoned with iodoacetate, cyanide, and pure nitrogen. *Sartorius, semitendinosus, tibialis anticus longus,* and *iliofibularis* muscles from one frog were used in each experimental group; the paired muscles served as controls. First, both control and experimental muscle groups were equilibrated with 0.001*M* IAA at 0°C for one hour; this ensures complete penetration of IAA and inhibition of glycolysis. The control muscles were then homogenized in 10 per cent perchloric acid and the extract was immediately neutralized. The experimental muscles were transferred to a special flask with 100ml of Ringer's solution (*p*H 7.4) containing 0.001*M* IAA and 0.001*M* NaCN (also at 0°C); the solution had been equilibrated with a 5% CO_2 + 95% N_2 mixture. The oxygen tension of this mixture was no higher than 4×10^{-5} per cent; to assure this, it was passed first through heated copper turnings and then through a tower of activated copper. After the solution containing the experimental muscles had been bubbled for two hours with the CO_2–N_2 mixture, both inlet and outlet tubes were closed and the entire flask was immersed in the ice water bathing the flask. At the end of four hours in IAA-CN-N_2, the experimental muscles were also homogenized. Aliquots of the perchloric acid extract from both controls and experimentals were analyzed for K^+ ion, CrP, ATP, ADP and lactate. The lactate contents of the bathing solution were determined at the beginning and end of the experiments. K^+-ion concentrations were determined by flame photometry, CrP by Gomori's modification (1942) of the method of Fiske and Subbarow (1929), ATP and ADP by a modification of the method of Kalckar (1947) using 5-adenylic-acid deaminase, myokinase, and potato apyrase. (Appendix D, reproduced as Appendix 2 below, outlines an easy method for preparation of a large quantity of stable adenylic-acid deaminase that can be used for the accurate and simultaneous determination of the ATP, ADP, and AMP contents of a large number of samples.) Average lactate production (under IAA) was determined by the method of Barker and Summerson (1941), taking into account the changes both in the muscles and in the bathing solution. The calculation is shown in the column under ΔF (lactate); the average shown is the total change in free energy, taking both the muscle and the bathing-medium lactate into account. For calculation of the maximum rate of free energy delivery, we used the relatively high values of Burton and Krebs (1953; see also Levintow and Meister, 1954; Vladimorov *et al.,* 1957; George and Rutman, 1960): ΔF (ATP $\rightarrow$ ADP + P) between –12.5 and –16.0, averaging –14.3 kcal/mole; ΔF (ADP $\rightarrow$ AMP + P) –15.0 kcal/mole; ΔF (CrP $\rightarrow$ Cr + P) –12.8 kcal/mole and ΔF (glucose $\rightarrow$ 2 lactate) –28 kcal/mole of lactate. These ΔF values are free energy changes for the conditions presumed to exist in living cells and are not standard free energy changes.

[a] Total amount of lactate change by muscle was obtained by multiplying the difference between experimental and control lactate concentrations by the weight of the experimental muscle.


20.0
10.0
8.0
6.0
4.0
3.0
2.0
1.0
0.8
0.6
0.4
0.3
0.2
0.1
0.08
Na^{22} concentration (micromoles per gram)
Muscle (D_4)
Connective tissue (E)
0
40
80
120
160
Time (minutes)

A

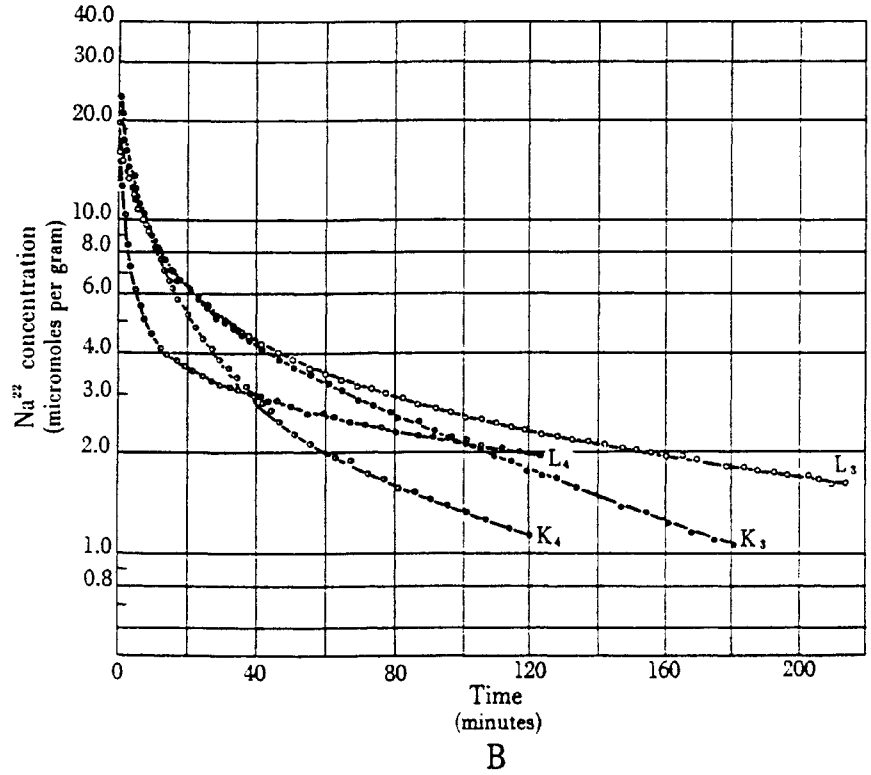


FIGURE 8.6 The rate of Na^+-ion exchange in normal connective tissues and muscle-fiber bundles poisoned with IAA-CN-N_2. A, connective-tissue elements weighing 5.35 mg were soaked in Na^{22} Ringer's solution for 4 minutes and 24 seconds. For data on normal muscle-fiber bundle D_4, see Table 8.7. For details of the experimental procedure, see legends of Table 8.7 and Figure 8.5. The straight line through the last points on curve D_4 gives the extrapolated initial concentration of Na^{22} ion. B, similar to A except that all muscle-fiber bundles were treated with IAA-CN-N_2. Details are given in Table 8.7. From these curves, the extrapolated initial concentrations of Na_{22} ion were obtained.

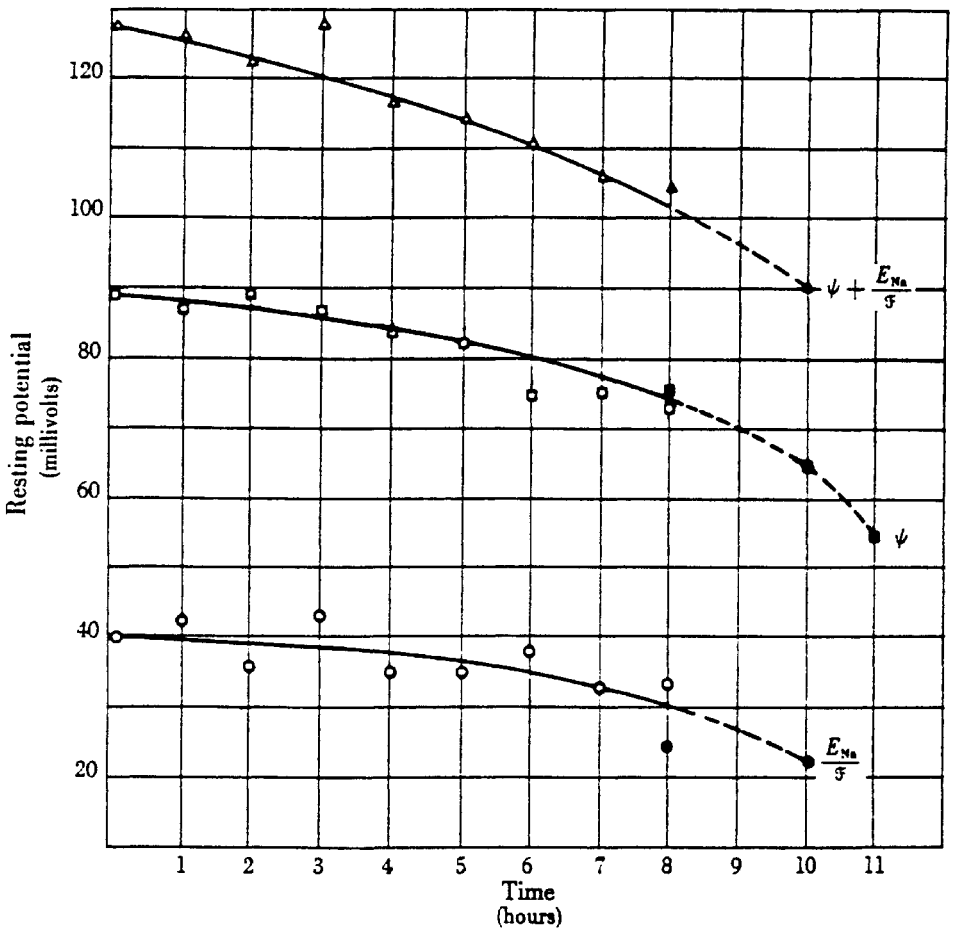


FIGURE 8.7 Time course of change of the resting potential ψ and the intracellular Na^+-ion concentration in iodoacetate-, cyanide-, and nitrogen-poisoned frog *sartorius* muscles (0°C). The Na^+-ion concentration is expressed as $E_{Na}/\mathcal{F}$, a potential gradient derived from the Nernst equation, $E_{Na} = RT$ in $([Na]_{ex}/[Na]_{in})$ for 0°C. Empty triangles, squares, and circles are taken from a single experiment (Table 8.5); solid points are averages of other experimental values.

implies that the major properties of normal resting cells can be explained best if the cell is described, not as a system in an energy-consuming steady state, but rather, if it is described as a system in a metastable equilibrium state. Like any equilibrium state, this equilibrium state requires no expenditure of energy for its maintenance. How ATP functions as a cardinal adsorbent to maintain this metastable equilibrium state and the role of ATP in the establishment of new metastable states form a central theme of later chapters.

Frog No.	Muscle No.	Muscle wt, mg	Duration in CN-N_2-IAA, hr	No. of muscle fibers measured	Resting potential ψ, mv: Mean ± standard deviation	Resting potential ψ, mv: Average of means	$E_{Na}/\mathcal{F}$[a], mv: Individual	$E_{Na}/\mathcal{F}$[a], mv: Mean	$\psi + E_{Na}/\mathcal{F}$, mv
1	1	58.0	0	8	99.5 ± 4.0	97.0	40.5	39.1	128.0
	2	62.5	0	8	94.5 ± 2.9		37.7		
2	1	84.0	1	8	91.5 ± 5.5	93.3	37.0	41.4	126.8
	2	86.6	1	8	94.8 ± 2.9		45.8		
3	1	62.4	2	8	97.5 ± 4.9	96.7	31.7	33.8	122.3
	2	62.3	2	8	96.0 ± 1.1		35.8		
4	1	62.2	3	10	94.8 ± 3.5	94.0	43.2	42.6	128.6
	2	60.2	3	10	93.0 ± 3.7		41.9		
5	1	41.3	4	8	90.4 ± 3.1	89.0	34.6	34.0	115.5
	2	38.6	4	8	97.5 ± 6.6		33.3		
6	1	44.9	5	8	84.9 ± 2.5	88.0	34.3	33.6	114.1
	2	42.6	5	8	90.8 ± 2.0		32.8		
7	1	63.7	6	8	79.4 ± 4.6	80.0	33.3	37.7	111.0
	2	60.5	6	8	80.6 ± 4.8		42.0		
8	1	61.5	7	8	79.4 ± 4.9	80.5	39.0	32.5	106.2
	2	62.9	7	8	81.7 ± 3.8		26.0		
9	1	57.2	8	10	74.2 ± 7.8	78.5	36.6	34.6	106.5
	2	51.6	8	10	82.6 ± 5.0		32.6		

TABLE 8.6 The average resting potential and intracellular Na^+-ion concentration of frog *sartorius* muscles poisoned with nitrogen, cyanide, and iodoacetate (0°C). We determined the intracellular Na^+ ion by flame photometry and measured resting potentials of individual fibers with Gerard-Graham-Ling microelectrodes. The intracellular-extracellular Na^+-ion concentration gradient is expressed as a potential gradient derived with the Nernst equation for 0°C. The last column gives the minimum energy, on the basis of a membrane-pump hypothesis, for the extrusion of one mole of Na^+ ions against the measured electrical and concentration gradients. Experimental conditions were similar to those described under Table 8.4 except that only *sartorius* muscles were used.

[a] $E_{Na}/\mathcal{F}$ values are given by the relation $E_{Na}/\mathcal{F} = 54.3 \log \frac{[Na]_o}{[Na]_i}$, where $[Na]_o = 107.6$ millimoles per liter.

	Muscle No.	Muscle wt, mg	Muscle-fiber length, cm	Total No. of muscle fibers in bundle	Fiber diameter: No. of fibers counted	Fiber diameter: Average, μ	$\frac{V}{A}$, μ	$\frac{A}{W}$, cm^2/g	Duration in poison, hr
A	1	6.82	1.8	59	9	71	18	530	0.33
	2	2.07	1.8	17	9	73	20	477	0.33
	3	2.26	1.2	24	10	63	16	596	0.83
	4	5.68	1.3	70	11	63	16	596	1.50
	5	5.18	1.5	53	9	63	16	596	1.50
	6	8.60	1.4	80	10	82	20	477	2.33
	7	2.76	1.5	20	10	71	18	530	2.33
	8	7.45	1.4	73	10	75	19	500	4.33
	9	3.49	1.5	32	11	67	17	560	4.33
	10	9.84	1.7	67	10	69	17	569	8.00
	11	4.99	1.5	39	10	65	16	596	8.00
	Average							550	
B	D_4	17.03							0.00
	L_4	6.00		76					1.00
	L_3	17.45		204					2.50
	K_3	3.50		45					4.50
	K_4	8.60		101					8.00

	Muscle No.	Duration in Na^{22} Ringer's solution, min	Rate of Na^+ flux: M/kg/hr, Uncorrected	M/kg/hr, Corrected	M/kg/hr, Average	M/cm^2/sec, Uncorrected	M/cm^2/sec, Corrected
A	1	3.0	0.133	0.121	0.118	6.97×10^{-11}	6.33×10^{-11}
	2	3.0	0.125	0.114		7.28×10^{-11}	6.62×10^{-11}
	3	3.0	0.073	0.066	0.066	3.40×10^{-11}	3.09×10^{-11}
	4	3.0	0.114	0.104	0.107	5.31×10^{-11}	4.83×10^{-11}
	5	3.0	0.121	0.110		5.64×10^{-11}	5.13×10^{-11}
	6	3.0	0.071	0.064	0.081	4.13×10^{-11}	3.75×10^{-11}
	7	3.0	0.108	0.098		5.66×10^{-11}	5.14×10^{-11}
	8	3.0	0.173	0.157	0.128	9.61×10^{-11}	8.73×10^{-11}
	9	3.0	0.109	0.099		5.41×10^{-11}	4.92×10^{-11}
	10	3.0	0.169	0.154	0.152	8.25×10^{-11}	7.50×10^{-11}
	11	3.0	0.165	0.150		7.69×10^{-11}	6.99×10^{-11}
B	D_4	3.1	0.035	0.035			1.76×10^{-11}
	L_4	2.8	0.074	0.074			3.74×10^{-11}
	L_3	2.7	0.162	0.162			8.18×10^{-11}
	K_3	2.5	0.197	0.197			9.95×10^{-11}
	K_4	2.3	0.113	0.113			5.71×10^{-11}

TABLE 8.7 The rate of Na^+-ion exchange in muscle-fiber bundles poisoned with IAA and pure nitrogen. Muscle-fiber bundles were isolated from the parts of frog *semitendinosus* muscles that are practically free of small-fiber-inervated muscle fibers (Kuffler and Vaughan-Williams, 1953). Series-A bundles were soaked in Ringer's solution containing IAA (0.001*M*) and bubbled with pure N_2 for up to eight hours at 0°C. The container of Ringer's solution was in a large covered glass jar containing ice water and continuously flushed with pure nitrogen; the glass jar was kept in a constant-temperature room maintained at 0° to 2°C. After the specified time, the muscle bundle was transferred by means of a remote-handling device to a smaller vessel of Ringer-IAA mixture containing Na^{22}. After soaking for 3 minutes in this solution, the muscle bundle was placed in a large beaker and washed there for 1.5 minutes in nonradioactive Ringer's solution vigorously bubbled with nitrogen. The radioactivity of the tissue was then assayed in the well of a scintillation counter. Diffusion through the extracellular space can be determined from the following equation (Jost, 1952):

$$\frac{\bar{C}(t) - C(\infty)}{C(0) - C(\infty)} = \frac{8}{\pi^2} \sum_{v=0}^{\infty} \frac{1}{(2v+1)^2} \exp\left[-\pi^2 \left(\frac{2v+1}{h} \right)^2 Dt \right].$$

Here $\bar{C}(t)$ is the concentration at time t; $C(\infty)$ is the final concentration, and $C(0)$ is the initial concentration; v is an integer equal to 0, 1, 2 . . . ; h is the height of the capillaries, and D is the self-diffusion constant which, for the Na^+ ion, is equal to 1.30×10^{-5} cm^2/sec (Mills, 1955). From this calculation, we find the washing for 1.5 minutes removes virtually all Na^{22} contained in the extracellular space. A more significant error arises from the connective-tissue elements which, in whole *sartorius* muscle, constitute about 9.1 per cent of the wet weight (see Table 8.8). Such connective-tissue elements take up less Na^{22} than whole muscle does (see Figure 8.6) and release it more rapidly. A deliberately over-estimated correction for connective tissue was made by the subtraction of 9.1 per cent of the assayed Na^{22} content from this latter figure. For series B, a totally different method of estimating the Na^+ ion exchange rate was adopted. The muscle-fiber bundles were kept in IAA-CN-N_2 Ringer's solution for the specified length of time. Then they were soaked in a Ringer's solution containing Na^{22} for a few minutes, mounted in the washing apparatus (see Figure 8.5), and washed continuously with nonradioactive Ringer's solution containing IAA and cyanide. In this case, the muscle-fiber bundle was tied to a small lucite rod to prevent tearing of the tissue. A straight line was fitted to the last part of the plot of Na^{22} content against time (see Figure 8.6). Extrapolation of this line to zero time gives another deliberately underestimated initial Na^{22} content for the muscle fibers. Dividing this by the time the muscle was actually soaked in the Na^{22} Ringer's solution, we obtained a minimum value of the rate of Na^{22} exchange in such poisoned muscles. The last column of values for series B gives the rate of flux in moles per square centimeter per second; we obtained this by using the average value for area/weight of 550 cm^2/g obtained for series A.

Source	No.	Tissue wt, mg	Collagen wt, mg	Collagen content, %	Average percentage of connective tissue in muscle, fresh wt/fresh wt
	1	496.9	1.2	0.24	
Muscle	2	550.4	1.3	0.23	
	3	546.6	1.6	0.29	
	4	547.3	1.5	0.27	
Average				0.26	9.09
	1	51.9	1.7	3.28	
Connective tissue	2	49.1	1.5	3.05	
	3	56.4	1.3	2.30	
	4	42.6	1.2	2.81	
Average				2.86	

TABLE 8.8 The percentage in weight of connective tissues in frog *sartorius* muscles. To estimate this, we used the fact that pure collagen can be isolated from tissues by alkaline digestion (Lowry *et al.*, 1941) and the reasonable assumption that the collagen content of the connective-tissue elements is closely approximated by the similar connective-tissue elements immediately surrounding the muscle. From the percentage of pure collagen found in these "pure" connective tissues and the content of pure collagen found in intact muscles, the average percentage by weight of fresh connective tissues in fresh muscle was calculated.

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Date	Duration, hr	Rate of Na exchange, integrated average, M/kg/hr	$\psi + E_{Na}/\mathfrak{F}$ integrated average, mv	Minimum rate of energy required for Na pump, cal/kg/hr	Maximum rate of energy delivery, cal/kg/hr	Minimum required energy / Maximum available energy
9-12-56	10	0.138	111	353	11.57 (highest value, 22.19)	3060%
9-20-56	4	0.121	123	343	22.25 (highest value, 33.71)	1542%
9-26-56	4.5	0.131	122	368	20.47 (highest value, 26.10)	1800%

TABLE 8.9 Energy balance sheet for the Na pump in frog *sartorius* muscles (0°C). The minimum rate of energy delivery required to operate a Na pump according to the membrane-pump theory was calculated from integrated values of the measured rates of Na^+-ion exchange (Table 8.7) and the energy needed to pump each mole of Na^+ ion out against the measured electrical and concentration gradients (Table 8.5; Figure 8.7). The maximum energy-delivery rate was calculated from the measured hydrolysis of CrP, ATP, and ADP, the only effective energy sources available to the muscles which were poisoned with IAA and N^2. Total inhibition of respiration and of glycolysis was assured by the simultaneous presence of 0.001*M* NaCN (in addition to N_2) and verified by the actual measurement of residual lactate production (in addition to IAA). The ratios between the required and available energy are underestimations. There is no significant difference if the data on flux rate for series A in Table 8.7 are used rather than those for series B. Details of one of the three complete sets of data obtained in September of 1956 are given in Table 8.4. It should be pointed out that six more series of similar experiments were completed (3-20-53, 4-12-54, 1-13-55, 5-20-55, 5-30-55, and 8-9-55); the duration of soaking in the poison gave a mean maximum rate of energy delivery even lower than the data from the three series used in these calculations. Since the development of the procedure for ATP-ADP determinations (Appendix D, reproduced as Appendix 2 below) was not completed until the end of 1955, the earlier data have not been included.

APPENDIX 2

The Procedure for the Determination of ATP, ADP, and AMP Described in Appendix 1

THE FOLLOWING PROCEDURE, which has been modified from Kalckar (1947), has been used successfully in the author's laboratory for the past seven years. It is presented for two reasons. First, several important arguments depend upon the accurate determination of the ATP and ADP contents of tissues. Second, there is a real need for an easy, time-saving, and accurate procedure for the assay of the AMP, ADP, and ATP contents of a large number of tissue samples. The following procedure requires neither special apparatus nor the experience of an enzymologist.

The basis of this procedure is the fact that adenosinemonophosphate (AMP, or 5-adenylic acid) has an absorption peak at 265mμ, whereas deaminated AMP (inosinic acid) has no absorption peak at this wavelength. The transformation from adenosinemonophosphate (AMP) to inosinic acid is brought about by the action of the enzyme, 5-adenylic acid deaminase, which is specific for this reaction. The determination of ATP and ADP utilizes enzymes specific for the transformation of these compounds into AMP. In the determination of ATP, the enzyme used is potato apyrase which also converts ATP into AMP. In the determination of ADP, the enzyme, myokinase, which is specific for the reaction ADP→AMP, is used; one-half of the ADP present in the sample is converted to AMP.

(1) Purification of adenylic acid deaminase (method modified after Nikiforuk and Colowick,[1] 1955)

A rabbit is killed by a blow on the head and skinned. The muscles are removed as quickly as possible and placed in a beaker which is sitting in an ice bath. The muscles are weighed and ground in a meat grinder in a cold room (4°C). An equal part of 0.9 per cent NaCl is added to the ground muscle and the mixture is shaken or stirred vigorously in the cold for 15 minutes. It is then filtered through two layers of cheesecloth. The filtrate is put aside in the cold. The ground muscle is again shaken for 15 minutes in the cold with an equal volume of 0.9 per cent NaCl and again filtered through cheesecloth. The second filtrate is combined with the first and saved for the later preparation of myokinase [see Section (2)]. The ground muscle is washed twice more using the same procedure; the third and fourth filtrates are discarded.

After the fourth washing, the sediment is shaken for 90 minutes at room temperature with two volumes of 2 per cent $NaHCO_3$. The resultant sticky mixture is filtered through three layers of cheesecloth; the filtrate volume is made as large as possible by squeezing. The sediment is again shaken for 15 minutes with two volumes of 2 per cent $NaHCO_3$ (at room

[1] The author is indebted to Dr. S. Colowick for making available to him in 1952 the then unpublished manuscript of Nikiforuk and Colowick (1956). The present modified procedure is based on this, as well as on the original work of G. Schmidt (1928).

temperature). After filtering through cheesecloth, the second filtrate is added to the first and both are measured in a large graduate. Celite, a diatomaceous filtering agent (Johns Manville), is added to a concentration of 4 per cent (weight/volume) and the mixture is filtered through large Buchner funnels employing Whatman No. 1 paper. Since this procedure may be very slow, it is advisable to add only a very thin layer of the extract to the funnel at a time. If it is necessary to complete the filtering overnight, the funnel and flask must be placed in the cold; a frequent change of filter paper may hasten the procedure somewhat. A translucent filtrate is obtained.

The filtrate is adjusted to *p*H 7.0 with a 0.3*M* acetic-acid-sodium-acetate buffer at *p*H 5.0. It can now be frozen in a deep freeze; in this condition it will remain active for several months. When purified adenylic acid deaminase is needed, part of the filtrate may be thawed and further purified to isolate the enzyme as follows: The thawed Celite filtrate is adjusted to *p*H 6.0 by drop-by-drop addition of the 0.3*M* acetate buffer at *p*H 5.0. To one volume of this adjusted Celite filtrate, 0.3 volumes of saturated ammonium sulfate, also adjusted to *p*H 6.0, are added. This should be added drop by drop in the cold, using extreme care to mix well during the addition. The mixture is then allowed to stand for 15 minutes in the cold and spun for 10 minutes at high speed (approximately $12{,}000 \times g$). The precipitate is discarded and twice the original volume of ammonium sulfate is added to the clear filtrate in the manner described above. The resultant mixture is again allowed to stand in the cold for 10 minutes and again spun at high speed. Following this, the supernatant is discarded and the precipitate treated in the following way.

Into each centrifuge tube, one or two drops of 2 per cent $NaHCO_3$ are pipetted and the precipitate is then taken up in twice the minimum amount of a mixture of one part 0.3*M* sodium-acetate-acetic-acid buffer at *p*H 6.0 and two parts of a 0.75*M* NaCl solution. The resultant solution should be clear and the *p*H should be 6.0. Otherwise, the *p*H should be adjusted with the sodium-acetate-acetic-acid buffer and spun once more to get rid of particulate matter. The completely soluble enzyme is now ready for use and is free from ATPase or myokinase activity.

(2) Myokinase and potato apyrase preparation

A satisfactory myokinase preparation may be obtained from the combined NaCl washings of the rabbit muscle discussed in Section (1), using the method of Colowick-Kalckar (1943):

The muscle extract is acidified with 0.05 volume of 1*N* HCl and heated to 90°C for three minutes. It is then cooled rapidly in ice bath, neutralized with 2*N* NaOH to *p*H 6.5, filtered, and the precipitate is discarded. Ammonium sulfate is added to the supernatant to about 80 per cent of saturation at room temperature and the precipitate spun down at the same temperature. The resultant precipitate is dissolved in 15 to 20 milliliters of H_2O. The preparation is then frozen in the deep freeze; a small amount may be thawed and used as needed.

Potato apyrase may be satisfactorily prepared by the method of Krishnan (1949). One kilogram of peeled potatoes is ground in a Waring blender with an equal volume of 0.01*M* NaCN solution and the supernatant centrifuged in the cold to remove particulate matter. After returning to room temperature solid $(NH_4)_2SO_4$ is added to the supernatant with stirring to a concentration of 45 grams per 100 milliliters of extract. The mixture is filtered through Whatman No. 1 filter paper at 4°C. The "precipitate" is then taken up in water, dialyzed against distilled water (changed frequently) in the cold for 24 hours. The dialysate

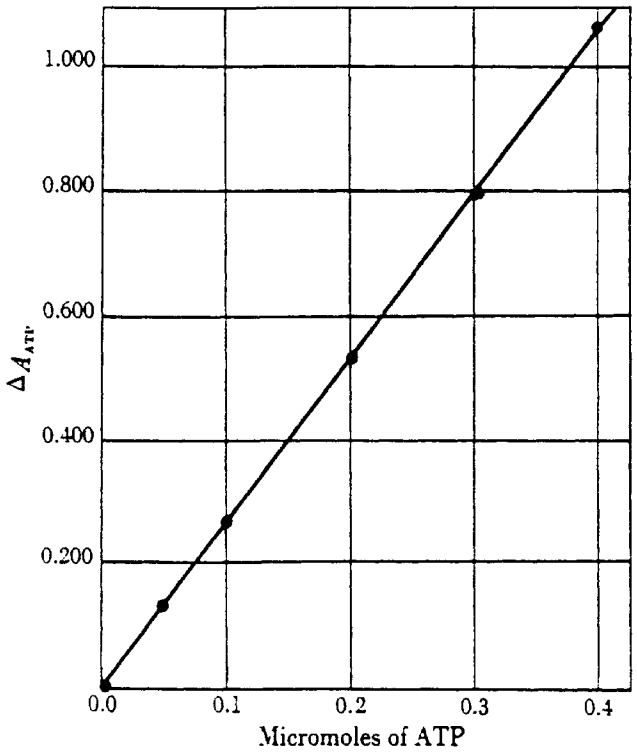

FIGURE 1. The relationship between ΔA_{ATP} and the amount of ATP in sample. A fresh preparation of crystalline Na_2·ATP·$3H_2O$ (Sigma Chemical Co., St. Louis) was employed.

is then lyophilized. The powdery preparation, if kept in the deep freeze, will remain active at least for several months. For use, the powder is dissolved in a small amount of distilled water.

(3) Determination of ATP, ADP, and AMP

The tissue, in cold 2 per cent perchloric acid, is homogenized at low temperature and centrifuged. To the clear supernatant is added one drop of 0.1 per cent phenolphthalein in alcohol. The *p*H is then adjusted to near neutrality with NaOH. A final *p*H of 7.0 is achieved by further adjustment with the aid of brom-thymol-blue on a porcelain spot test plate. The neutralized samples can be frozen and preserved overnight or even somewhat longer. Due to the ease of ATP hydrolysis in basic or acidic medium, extreme caution must be exercised that a neutralized sample does not become contaminated by droplets of acid or base on the side of the centrifuge tubes. For the determination of AMP, ADP, and ATP, three aliquots, each 0.5 milliliters in volume, are needed for each piece of tissue.

(a) Reagents. The reagents used are (1) succinic acid buffer containing 15 parts 0.1*M* succinic acid adjusted to *p*H 5.9 with NaOH, 1 part 0.3M $MgCl_2$, and 9 parts 1.5*M* NaCl; (2) 2 per cent perchloric acid neutralized to *p*H 7.0 with NaOH; (3) samples prepared by the cold perchloric-acid extraction method given above; and (4) solutions of adenylic acid

deaminase, myokinase, and potato apyrase as prepared above, kept in small test tubes immersed in an ice-water mixture.

(b) Determinations. The succinic acid buffer (2.5ml) and 0.5ml of sample (well shaken before pipetting) are pipetted into a silica cuvette. The blank contains 0.5 ml of neutralized 2 per cent perchloric acid instead of a sample. The solutions are stirred with small polyethylene stirring rods and the absorption is read on a Beckman DU spectrophotometer at 265mμ. This initial reading is only tentative and aids in the rapid reading of the following samples.

(i) AMP determination. Add 0.05 ml of 5-adenylic acid deaminase to the samples and to the blank prepared as above. The contents of the cuvettes are rapidly stirred and the absorption at 265mμ read three times in rapid succession. The points so obtained are plotted on semilogarithmic graph paper; the initial absorption is obtained by extrapolation to zero time. The samples and blank are then poured into small stoppered tubes and kept in the refrigerator overnight or longer. After that the absorption at 265mμ is determined to give the final reading. The difference between the initial and final readings will be referred to as ΔA_1.

(ii) ADP determination. The procedure is exactly the same as that for AMP, except that 0.05ml of myokinase is added to the samples and to the blank when the 5-adenylic deaminase is added. The difference between the initial and final readings at 265mμ will be called ΔA_2.

(iii) ATP determination. The procedure is exactly the same as that for AMP, except that 0.05ml of potato apyrase is added to the samples and to the blank when the 5-adenylic deaminase is added. The difference between the initial and final readings at 265mμ will be referred to as ΔA_3.

(c) Calculations. The relationships between the ΔA values obtained above and the concentrations of ATP, ADP, and AMP in the sample expressed in terms of the changes of absorption at 265mμ are given by the equations,

$$\Delta A_{AMP} = \Delta A_1$$

$$\Delta A_{ADP} = 2(\Delta A_2 - \Delta A_1)$$

$$\Delta A_{ATP} = \Delta A_3 - 2\Delta A_2 + \Delta A_1.$$

If the procedure is followed *exactly* as outlined a ΔA value of 0.260 corresponds to 0.1 micromole of adenosine compound in the sample. The relationship between ΔA_{ATP} and the micromoles of ATP in the sample as determined for the purest preparation of ATP available as a standard is shown in Figure 1. Note that the relationship is a straight line to at least 0.4 micromoles of ATP. The same relationship holds true for both ΔA_{AMP} and ΔA_{ADP}.

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APPENDIX 3

The Characterization of a Protein by Its *c*- and *c′*-Value Ensemble; The Protein as a Functional Unit

(Reproduced from Chapter 7 of Ling, 1962)

FIGURE 7.1 SHOWS the conspicuously different plots of *p*H versus apparent heat of ionization $\Delta H'$ for four different proteins: myosin, wool, lysozyme, and oxyhemoglobin.[1] Figure 7.2 shows the *p*H-versus-$\Delta H'$ plots of serum albumins from three different species. With respect to polar amino-acid composition, myosin and human serum albumin differ about as much as human serum albumin and bovine serum albumin (Table 7.1). The functional characteristics of these proteins indicate that there is no distinct difference between human and bovine serum albumin (for ion adsorption data; see Carr, 1952). But the tremendous functional difference between serum albumin and the contractile protein, myosin, needs no emphasis. We may thus draw the important conclusion that a *p*H-versus-$\Delta H'$ plot characterizes the properties of protein in its interactions better than a description of the amino-acid composition does.

A. The Heterogeneity of Nominally Identical Polar Groups on Proteins

The work of Derick, Ingold, and others demonstrates that the affinity of a carboxylic group for a proton depends on the nature of the remainder of the molecule.[2] It is well known that the acid-dissociation constants or *p*K values of the same α-carboxyl groups on different amino acids differ from each other. The *p*K value of the carboxyl group of an amino acid is also changed its amino group is linked to another amino acid to form a dipeptide (see Section 5.2 in Ling, 1962; also Cohn and Edsall, 1943, p. 84). Similarly, the *p*K value of the side-chain polar group in a trifunctional amino acid (glutamic acid or lysine, for example) alters when the amino acid participates in the formation of a protein molecule. Such changes have usually been considered small (scarcely over 2 *p*H units). Many studies made in this field have emphasized uniformity in the characteristics of different proteins. However, a number of exceptions exist, notably, the work of Crammer and Neuberger (1943), who studied the phenolic group of the tyrosine residue; and the work of Karush and Sonenberg (1949), Teresi and Luck (1948), and Tanford and Wagner (1954), who studied β- and γ-carboxyl groups. All concluded that individual groups may have very different *p*K values.

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[1] The apparent heat of ionization is defined as

$$\Delta H' = -2.303RT^2\left(\frac{\partial pH}{\partial T}\right)_{\bar{x}}$$

where $(\partial pH/\partial T)\bar{x}$ refers to the change of the pH of a solution with temperature when the amount of base or acid (represented by $\bar{x}$) is kept constant.

[2] Derick, 1911; G. N. Lewis, 1923; Bjerrum, 1923b; Kirkwood and Westheimer, 1938a,b; Ingold, 1953; Branch and Calvin, 1941.

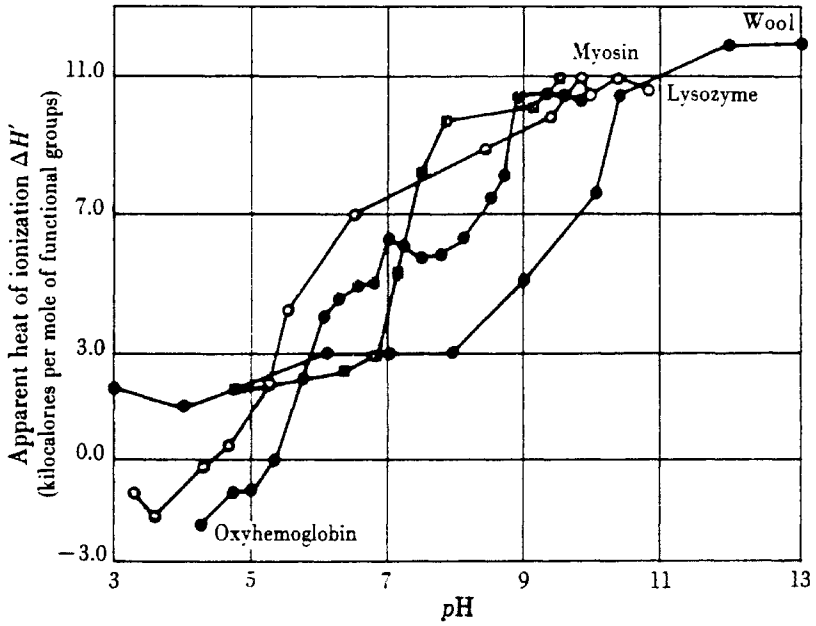

FIGURE 7.1. The apparent heat or ionization ΔH′ of ionizing groups of various proteins plotted against the *p*H. Note the great diversity of the ΔH′ values in functionally different proteins. [Data *from* Mihályi (1950): myosin (25°–38°C); *from* Wyman (1939): oxyhemoglobin (25°–37°C); *from* Tanford and Wagner (1954): lysozyme (4°–25°C); *from* Steinhardt *et al.* (1941): wool (0°–25°C).]

For the present, we emphasize the heterogeneity manifested by the nominally identical carboxyl, amino, and other functional groups. We shall consider both their *p*K values (which are directly related to the free energies of dissociation of the protons ΔF), and their apparent heats of dissociation $\Delta H'$, which are closely related to their true heats of dissociation ΔH.

(1) Carboxyl groups

The $\Delta H'$ values usually given for carboxyl groups on various organic molecules vary between +1.5 and −1.5 kcal/mole (Cohn and Edsall, 1943). This variation represents true variation resulting from changes in the nature of the rest of the molecule. Thus, although the α-carboxyl group of oxyvaline has a $\Delta H'$ value of −1.30 kcal/mole, the $\Delta H'$ value for the β-carboxyl group of aspartic acid is +2.10 kcal/mole and the $\Delta H'$ value of the γ-carboxyl group of glutamic acid is +1.04 kcal/mole (Cohn and Edsall, 1943, Chapter 4, Table 6). Wyman (1939) has shown that within a given *p*H range the $\Delta H'$ of protein molecules seems to depend solely upon the relative abundance of the various groups which ionize in that range.

In most large protein molecules, all the side-chain carboxyl groups belong to either glutamic- or aspartic-acid residues. If the *p*K and ΔH values of the carboxyl residues in protein are only slightly different from the *p*K and ΔH values of the corresponding groups in the free amino acid, the $\Delta H'$ of all proteins of large molecular weight should be about +1.0 to +2.0 kcal/mole of carboxyl groups at a *p*H near 4. However, as we have indicated, the

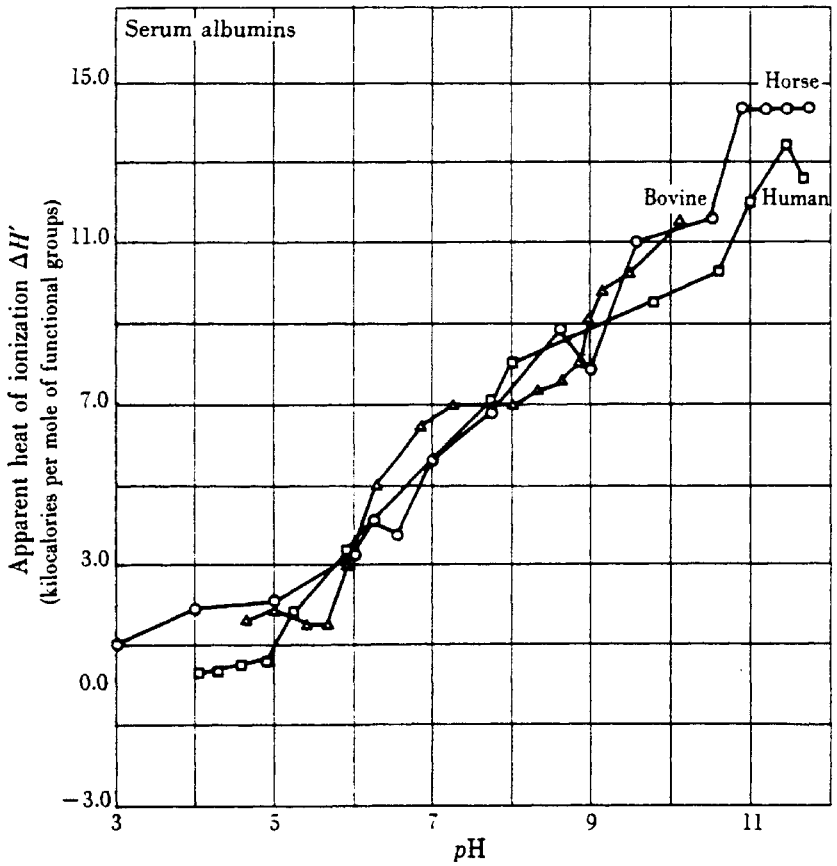

FIGURE 7.2. The apparent heat of ionization ΔH′ of ionizing groups of three serum albumins plotted against *p*H. Compare the plots of these functionally similar proteins with those of the functionally different proteins shown in Figure 7.1. [Data *from* Tanford (1950): human serum albumin (0°–25°C); *from* Tanford *et al.* (1955): bovine serum albumin (5°–25°C); *from* E.J. Cohn *et al.* (1943): horse serum albumin (5°–25°C).]

Protein	Amino-acid content							
	Tyr	Try	CySH	Arg	His	Lys	Asp	Glu
Wool	4.65	1.8	—	10.4	1.1	2.76	7.2	14.1
Myosin (rabbit)	3.4	0.8	—	7.36	2.41	11.92	8.9	22.1
Hemoglobin (horse)	3.03	1.7	0.56	3.65	8.71	8.51	10.60	8.50
Lysozyme	3.6	10.6	0	12.7	1.04	5.7	18.2	4.32
Bovine serum albumin	5.06	0.68	0.3	5.90	4.0	12.82	10.91	16.5
Human serum albumin	4.70	0.2	0.7	6.20	3.50	12.30	8.95	17.0

TABLE 7.1. The trifunctional amino-acid composition of several proteins. [Data (given in grams of amino acid per 100 grams of protein) *from* Tristram (1953).]

usual $\Delta H'$ values assigned to protein carboxyl groups vary from −1.5 to +1.5 kcal/mole. In fact, the *p*H-versus-$\Delta H'$ plots of wool protein, myosin, and lysozyme, as well as Wyman's original plot for oxyhemoglobin, demonstrate an even wider variation (Figure 7.1). At *p*H 4, the $\Delta H'$ value of the carboxyl group can be as high as +2.0 kcal/mole or lower than −2.0 kcal/mole. In proteins such as β-lactoglobulin, wool keratin, and myosin, plateaus in the curves indicate a uniformity in the *p*K values of the carboxyl groups; in proteins like oxyhemoglobin and lysozyme, the steepness of the slope indicates multiple *p*K and $\Delta H'$ values. The possibility of multiple *p*K values for protein carboxyl groups has often been suggested recently. In the case of some small protein molecule, a high proportion of C-terminal residues (α-carboxyl groups) may account for very low $\Delta H'$ values; this appears to be the case with insulin (Tanford and Epstein, 1954). The shape of the *p*H-versus-$\Delta H'$ curve in Figure 7.1 for lysozyme (molecular weight, 14,700), which has one C-terminal residue and 10.5 dissociable carboxyl groups, indicates that the $\Delta H'$ values for the β- and γ-carboxyl groups vary considerably.

(2) Amino groups

It is probable that in the *p*H range 7.0 to 9.5, only the imidazole group of histidine and the ε-amino groups of lysine significantly determine the measured $\Delta H'$ of proteins. If these groups, as present in protein, possessed uniform *p*K′ values, one would expect the *p*H-versus-$\Delta H'$ plots of lysozyme, myosin, and wool keratin to show that the $\Delta H'$ values at the *p*H range follow the relative abundance of histidine as compared with lysine (for lysozyme, the histidine content is 18.2 per cent of the lysine content, Fromageot and deGarilhe, 1950; for myosin, it is 20.2 per cent; and for wool keratin, 39.8 per cent; see Table 7.1). However, the $\Delta H'$-versus-*p*H plot of these proteins (Figure 7.1) shows wide variation in this range; and the variation is not at all predictable on this simple basis. Bailey (1951), using the fluorodinitrobenzene method of Sanger, showed an absence of α-amino groups in myosin; this indicates that the high $\Delta H'$ value for myosin at *p*H 8 cannot be due to terminal α-amino groups. A possible explanation is that the *p*K values of the ε-amino groups in myosin are much lower than the *p*K values of 9.4 to 10.6 usually given for ε-amino groups in protein[3]

[3] If a lower *p*K value for the ε-amino groups of myosin is accepted, at least two interesting paradoxes can be resolved. (1) Dubuisson (1941) and Dubuisson and Hamoir (1943) compared the actual titration curve of myosin with a theoretical one constructed according to its known constituent amino acids, assigning the usual *p*K values to each of these groups. They found that there is an amino-acid residue which inonizes in the *p*H range from 7 to 8; but this is unknown from the chemical analysis. A reduction of the *p*K value of the ε-amino-acid residue would fill in this gap. (2) A great discrepancy exists between the value of the heat of dephosphorylation of ATP given by Meyerhof and Lohmann (1932) as −12.0 kcal/mole and the much smaller figure of −4.7 kcal/mole determined by Podolsky and Kitzinger (1955) and Podolsky and Morales (1956). Podolsky and Morales suggested, in a footnote, that the difference originated from the heat of neutralization (equal to minus the heat of dissociation) of the imidazole group of histidine residues on muscle proteins present in the *Muskelsaft* used by Meyerhof and Lohmann. Bernhardt (1956), discussing the same general topic, mentioned that there is insufficient histidine residue in the intracellular fluid of muscle to make a significant contribution to the total heat change measured during ATP cleavage. Meyerhof and Lohmann's *Muskelsaft* could not contain a higher concentration of histidine than muscle itself; consequently, we suspect that histidine residues cannot account for all the extra heat. However, if we assume a low *p*K value for the ε-amino group of myosin, the participation of these groups in the buffering capacity of muscle proteins near *p*H 8 should be expected. The concentration of ε-amino groups is much higher than that of the histidine groups, and the ε-amino groups also have a much higher heat of neutralization (−10 to −12 kcal/mole); thus they could easily compensate for the difference between Podolsky and Morales' figure and the larger one found by Meyerhof and Lohmann.

(Cohn and Edsall, 1943). This is certainly an admissible possibility since amino groups in simpler molecules are known to have pK values as low as 7.60 (hexaglycine, see Section 5.2 in Ling, 1962), and 7.17 (α-hydroxyasparagine, Cohn and Edsall, 1943, p. 84). Conversely, in wool protein, the pK of the ϵ-amino groups of the lysine residue may be much higher than that usually given. We conclude that the pK and $\Delta H'$ values of ϵ-amino groups as well as those of carboxyl groups vary significantly when these groups are part of a protein molecule.

B. The c-Value Ensemble as the Determinant of the Functional Characteristics of Proteins

The apparent heat of ionization $\Delta H'$ is usually considered virtually equal to the true heat of ionization ΔH. In turn, ΔH is related to the dissociation energy $-\Delta F$ by the relation

$$\Delta H = -\Delta E + p\Delta V \tag{7–1}$$

where $p\Delta V$ represents the compressional work done on nearby water molecules during ionization. An estimate of this value, based on the treatment of Webb (1926), shows that $p\Delta V$ contributes much less than the electrical work ΔE does. Thus, $\Delta H \approx -\Delta E$.[4] The energy of dissociation of a proton at a definite temperature, pressure, and concentration is substantially determined by the c-value. Thus the pH-versus-$\Delta H'$ plot in the acidic range may be visualized as a "profile" of the c-value ensemble of a protein.[5]

We have mentioned that the variation in the pK value of a specific polar group in a protein (which is related to the c-value and $\Delta H'$ variation) is not large, and usually does not exceed two pH units. If each fixed carboxyl group could combine with a proton and nothing else, a pK shift of this magnitude would not be important. However, a pK shift of 2 units will result in an estimated difference of more than 2.8 kcal/mole in the free energy of dissociation of H^+. A change of dissociation energy of this order of magnitude will involve significant shifts of c-value. Consider, for example, the carboxyl groups: we may expect such a c-value shift to be very important when these anionic groups have cations of several species as possible counterions. Since all physiologically active proteins have both cationic and anionic side-chains, even in a salt-fee solution, the carboxyl groups have the choice of associating with H^+ ions from water or with the fixed positive groups on the protein molecule itself (ϵ-amino group or guanidyl group, for example). In living cells, ions of the alkali metals, the alkaline

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[4] Actually, the $\Delta H'$ values measured from the change of pH with variation of the temperature are related to the ΔE values but not quite as simply as equation (7–1) predicts. This is because the acid dissociation phenomenon is not really

$$H^+ + A^- \leftrightarrow HA$$

but rather

$$H^+(H_2O)_m + A^-(H_2O)_n = HA(H_2O)_l$$

and m, n, and l vary with temperature. This involvement of varying numbers of water molecules also accounts for the seemingly unreasonable negative values of $\Delta H'$ found for some protein carboxyl groups.

[5] This statement is usually true. The $\Delta H'$–versus–pH plots in the basic range are related to, but are not equivalent to, the c-value profile.

earths, and others are always present. Thus in general, the fixed anionic groups of proteins may combine with a variety of counterions. According to the present theory, small *p*K differences are sufficient to alter the preference for these counterions. This alteration will bring about profound changes in the physicochemical nature of the protein.

It should now be clear why the *p*H-versus-$\Delta H'$ plot of a protein characterizes its functional aspects better than its amino-acid composition does; this plot is a reflection of the *c*-value ensemble of the polar groups of the protein. The *c*-value ensemble is of basic importance in the interaction of a protein with its surroundings, (ions, water, and other proteins) and these interactions determine its functional characteristics. Let us now discuss the mechanism that produces the marked heterogeneity in the *c*-value, and therefore, in the $\Delta H'$ values and the *p*K values of the polar groups on a protein.

C. The Origin of the Heterogeneity of Functional Groups

The electrostatic effect of the ionization of charged groups on the dissociation of other side-chain polar groups is generally recognized. The Debye-Hückel theory of ionic solution was applied to proteins by Linderstrøm-Lang in his well-known electrostatic theory. In this theory, the protein molecule is treated as a sphere with its net electric charge uniformly smeared over its surface. The effect of the removal or addition of charges from or to such a body is a uniform one; it affects all charged groups in the molecule equally. This theory of proteins attributes no significance to the exact location of the ionizing or the affected groups or to the sequential order of arrangement of amino-acid residues on the protein polypeptide chain.

In theory, a direct electrostatic effect transmitted through space along a path of minimum length always exists.[6] The weight of evidence to be presented shows that in molecules of high polarizability, like proteins, particularly in the form of true fixed-charge systems, neither the macroscopic nor the microscopic electrostatic effect plays as important a role as do the inductive effects. The combined *F*-effects acting through the resonating, highly polarizable polypeptide chain, produce changes on groups all along the molecule; these changes depend upon the relative position of the interacting groups and the molecular structure that intervenes between the affecting and the affected groups. An example of the variety thus produced in the polar groups is the amino-acid sequence of a particular protein. The structural analysis of β-corticotropin (Shepherd *et al.*, 1956) led to the complete identification of its amino-acid sequence. Part of the molecule may be represented as:

Try	Gly	Lys	Pro	Val	Gly	Lys	Lys	Arg	Arg	Pro
9	10	11	12	13	14	15	16	17	18	19

where the numbers designate the sequential position of the amino-acid residues (Table 0.1 in Ling, 1962). The present theory emphasizes the fact that the *p*K value of the 11-lysine determined by, say, electrometric titration would be considerably different from that of the 16-lysine, which is flanked by three other positively charged side chains. Further, the ionization of the 16-lysine would have a very different effect on the ionization of the 15-lysine than it would on the ionization of the 11-lysine. When we consider the β-cortico-

[6] This microscopic electrostatic effect is not quite the same as the macroscopic electrostatic effect in Linderstrøm-Lang's theory.

tropin molecule as a whole, it is evident that the pK value of the lysine residues as well as those of many other polar side chains must be heterogeneous. Only in exceptional cases will they be the same.

Although we have focused our attention on the pK and ΔH value of the fixed ionic groups, these are by no means the only groups whose interactions are affected by their neighboring amino-acid residues.

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