*Review Articles***On Insulin Secretion***

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Summary. Aspects of insulin secretory mechanisms and models of diabetogenic B cell damage are discussed. Measurements of fluxes of ^3H -labelled triphenylmethylphosphonium ion, $^{86}\text{Rb}^+$, $^{42}\text{K}^+$, $^{22}\text{Na}^+$, and $^{45}\text{Ca}^{2+}$ in isolated islets indicate that the triggering of insulin release depends on alterations in the interaction of ions with the B cells. One difficulty in the detailed analysis of these alterations are uncertainties which arise when macroscopic concepts for homogenous phases are applied to microscopic and heterogenous compartments, as exemplified by the meaning of pH in insulin secretory granules and of membrane electric potential. Nonetheless, the importance of an apparent decreased K^+ permeability in mediating the insulin-releasing action of glucose, and of an apparent increased Na^+ permeability in mediating the potentiating action of acetylcholine is emphasized. Fluorescent probing of Ca^{2+} by chlorotetracycline revealed effects of glucose alone as well as glucose-dependent and atropine-sensitive effects of acetylcholine. Although acetylcholine, sulfonyleureas, and certain thiol-blocking agents may stimulate insulin release by direct effects on the B cell plasma membrane, a high capacity for D-glucose transmembrane transport has probably evolved in order that the interior of the B cells can always sense the circulating glucose concentration. A signal to secretion is thought to be transmitted from glucose metabolism to altered ion fluxes by intervention of reduced pyridine nucleotides and a hypothetical redox protein for which thioredoxin may be a model. The insulin secretory defect in hereditary diabetic C57BL/KsJ-*db/db*-mice is apparently linked to a decreased basal permeability for K^+ and a failure of the B cells to decrease further this permeability in response to glucose. Functioning B cells are acutely

damaged when exposed to heterologous serum or alloxan in vitro; cytotoxic activation of complement by the alternative pathway could perhaps occur during islet inflammation. Protection experiments with free-radical scavengers in vitro and in vivo support the theory that hydroxyl radicals are instrumental in the production of alloxan diabetes. Rapid reduction of alloxan by thioredoxin in the presence of molecular oxygen and NADPH leads to strong chemiluminescence from luminol indicative of an intense radical production. The sensitivity of B cells to alloxan may be due to physiological specializations of their plasma membranes, involving the highly effective glucose carrier or the hypothetical oxidation/reduction systems or both.

Key words: B cell, secretory mechanisms, cytotoxic mechanisms, diabetes.

Because diabetes may follow from disturbances of insulin secretion, I have wanted to understand normal insulin secretion and how it can be disturbed. This article is a solicited survey of some of my attempts. Perhaps it has relevance not only as such but also, to a certain extent, for the understanding of normal insulin secretion and how it can be disturbed.

Insulin Secretion Mechanisms*Insulin Storage and pH*

As much as 10–15% of a B cell's dry weight is insulin. Expressed as an average over the intracellular water, the concentration is about 100 million times that in serum. Considering the size of this gradient, and considering furthermore that only a few per cent

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Fig. 1. Poisson distributions illustrating the theoretical frequency of protons in insulin secretory granules of three different sizes assuming that the average proton concentration is 10^{-6} mol/l. The diagram illustrates the conceptual difficulties in applying the macroscopic pH concept to individual granules; it is meant neither to state the true number of protons in granules nor to imply that the activity coefficient should be taken as unity. Representative granule diameters were taken from Dean [130]

of the stored insulin is released in 1 h from islets stimulated with glucose, insulin storage appears an important aspect of the secretory mechanism.

In subcellular fractionation experiments with homogenized mouse islets, altogether 92–96% of the recovered insulin was sedimented by forces up to 110,000 *g* for 30 min [1–2]. Some of the supernatant insulin undoubtedly came from damaged secretory granules. Suppose, however, that a virtually perfect fractionation experiment could be done in which 99.99% of the insulin were accounted for and appeared in particulate fractions. In terms of the B cell's insulin store, the remaining one-tenth of an ml would still be equivalent to an apparent transmembrane gradient of 10,000:1. In the face of such numbers one can perhaps never know with certainty whether or not some insulin is secreted by diffusion. The success of the signal hypothesis for single-chain peptide transport across endoplasmic reticulum and prokaryotic plasma membranes [3] tends to restrain any inclination to viewing peptide transmembrane diffusion as an inherently absurd idea. However, concentrating on what we know [1, 2, 4–6], 60–80% of the B cell insulin sediments at fairly low centrifugal forces (6,000–28,000 *g*, 10–20 min). Most of that insulin is in the form of membrane-delimited granules that can function as vehicles for insulin secretion by exocytosis [5, 7, 8], the seemingly ubiquitous mode of peptide secretion in eukaryotic cells.

Coore et al. [4] observed that pH affects the stability of insulin secretory granules isolated from microdissected mouse-islets. As in most of my experiments to be described, we used non-inbred *ob/ob*-mice as experimental animals because their islets are

big and contain an unusually high proportion of functioning B cells. The B cell granules had optimum stability around pH 6. The reduced stability at pH 7.4 is probably a fundamental property of insulin secretory granules as similar observations have been made with granules isolated from rats [5], Swiss albino mice [9] and fish [6]. Coore et al. [4] also observed that the granules dissolved with increasing electrolyte strength in the suspending buffer; hence there is a simple physicochemical explanation of the rapid dissolution of insulin once the crystal gets out of the B cell and into the interstitial fluid which is probably pH 7.4 and relatively high in ionic strength.

But why are the granules stable *within* the B cell? Could it possibly be that the cell interior is moderately acid? We tried to answer this question by measuring the islet-cell uptake of dimethyl-oxazolidine-dione, DMO [10]. DMO is a weak acid whose ability to traverse cell membranes depends on the degree of dissociation and hence on the pH values on either side of the membrane. The uptake of DMO indicated that pH was 7.0 to 7.1 in the B cells, so those measurements could not explain the granule stability. Instead we must now ask: how can the granules be stable in the B cell when the pH of cytoplasm seems to favour their dissolution? We could perhaps postulate that the granule sac has the power to seclude insulin from the rest of the cytoplasm and that, therefore, pH within the granule sac is 6 although the rest of the cell is neutral; alternatively, we could distrust the DMO data and seek other reasons for believing that pH is not neutral but rather near to 6 in the B cell cytoplasm. Both alternatives are worth analyzing.

First we must consider what it means to say that pH could be 6 in the granules. Because for obvious reasons a pH meter electrode cannot be inserted into the individual granules, we must adopt the traditional view of pH as the negative logarithm of proton activity; in the first analysis one also naturally takes the activity coefficient to be unity. However, the granules are so small that 10^{-6} mol/l corresponds to no more than seven protons per granule of average size. This figure must be understood as the average number of protons in medium-sized granules. At any time-point, each medium-sized granule could contain more or less protons, and the probability that they do is likely to be governed by a Poisson distribution. Figure 1 illustrates what this means for granules of three different sizes. Even in the biggest granules the protons should be counted as individuals rather than as fractions of moles. A large proportion of the medium-sized granules is likely to contain as few as five, or fewer, protons. The smallest granules hardly ever contain more than five protons at pH 6. If the same calculations are performed for a macroscopic

pH of 7.05, the value actually measured for islet-cells by the DMO technique, one realizes that most of the granules for most of the time would not even contain a single proton; the average for medium-sized granules would be 0.6 protons per granule. This simple arithmetic suggests two principle conclusions. Firstly, if one tries to define pH for such a small compartment as a B granule, a stable pH value cannot be equated with a stable concentration of protons but rather with a certain average of protons in time. If the insulin crystal is sensitive to protons, as suggested by our granule stability data [4, 5], one may therefore expect certain fluctuations to constantly take place in the microscopic structure of the granules. I suggest that such fluctuations, whether due to protons or to any analogous stability factor, may be part of the explanation why B granules vary a great deal in outline and electron lucidity. The pleomorphology of granules is in itself an old observation and can have many causes. The point I want to make is mainly that random fluctuations should not really be ignored.

The second conclusion is simply a reservation against too deterministic models for the transport of protons, or of other ions for that matter. The ion-pump concept is by now well established in cell physiology. Yet, considering that in classical chemistry determinism and lawfulness arise out of mass action, it is unclear what should be meant by any pump operating on a compartment with singular protons.

Perhaps it is as simple to think that proton pumps are not needed in the granules because pH is 6 in the cytoplasm as well? Our actual data on DMO uptake are probably not to be distrusted as they have in principle been confirmed with rat islets [11], and as the calculated pH values are very similar to those for other cells on the basis of DMO uptake. What could perhaps be challenged is the basic assumption that DMO is not bound in the cells. Studies on muscle fibres have yielded occasional examples of discrepancies between the pH values recorded with DMO on the one hand and with intracellular electrodes on the other. Most electrode measurements have resulted in intracellular pH estimates around 7, but values as low as 6.0 have also been reported and, indeed, for cells which took up DMO as if the pH were 7.1 [12].

From the islet literature one can extract one positive reason for thinking that the cytoplasmic pH may be lower than is indicated by the DMO data, namely the measurements of membrane potential by high-resistance electrodes. The electromotive force recorded with such electrodes in non-stimulated B cells is about -60mV [13]. If protons distribute passively across the B cell membrane and, as is usually assumed in cell physiology, the equilibrium of such an ion can be validly described by the so-called

Fig. 2. Time course of triphenyl(^3H -methyl)phosphonium $^+$ (TPMP $^+$) uptake. *ob/ob*-Mouse islets were (●) or were not (○) pretreated with $50\text{ }\mu\text{mol/l}$ non-radioactive TPMP $^+$ for 90 min. They were then incubated with $5.6\text{ }\mu\text{mol/l}$ (^3H)TPMP $^+$ and 0.1 mmol/l L-(1- ^{14}C)glucose for the indicated periods of time. Uptake of labelled TPMP $^+$ in excess of the L-glucose space is shown as mean values $\pm$ SEM of five or six different experiments. The broken line shows the expected equilibrium uptake of TPMP $^+$ as predicted by the Nernst equation if uptake were solely due to an electric potential difference of -60 mV (inside negative) (from unpublished work together with J. Sehlin)

Nernst equation, then -60 mV predicts an intracellular pH of 6.4.

Membrane Potentials

On this point it is necessary to draw attention to a rarely discussed difficulty in the interpretation of membrane potentials. The driving force for moving a solute between two compartments is in general the electrochemical potential difference for that solute. If the two compartments have the same chemical composition, the electrochemical potential difference can be split into one chemical part, which is then zero, and one electrical part. In contrast, if the two compartments differ in composition, as is the case for cytoplasm and extracellular fluid, strictly speaking it is a rather arbitrary manoeuvre to split the electrochemical potential into an electrical and a chemical part [14, 15]. Under such conditions the electric potential may not have a defined value in volts independently of the measuring system. On the other hand, if different empirical approaches could be shown to yield the same value for the membrane

Fig. 3. Effects of various agents on the uptake of TPMP⁺. *ob/ob*-Mouse islets were incubated for 60 or 120 min with 5.6 μmol/l (³H)TPMP⁺ and 0.1 mmol/l L-(1-¹⁴C)glucose as well as D-glucose, D-mannoheptulose, or 2,4-dinitrophenol (DNP) in combination with 3 mmol/l D-glucose as indicated. Uptake of TPMP⁺ in excess of the L-glucose space is given as the mean values for 9–18 different experiments; thin bars denote SEM. Because there was no difference between 60 and 120 min, data for both incubation times have been pooled. The broken line, on which the bars stand, represents the uptake of (³H)TPMP⁺ by islets pretreated with 50 μmol/l non-radioactive TPMP⁺. *p* < 0.02 for nil effect of 20 mmol/l D-glucose (from unpublished data together with J. Sehlin)

electric potential, the lesser would be the metaphysical ambiguities surrounding the concept.

Triphenylmethylphosphonium cation (TPMP⁺) is a univalent organic cation that has been tried as a probe of electric potentials in other systems [16–18]. Because TPMP⁺ is lipid-soluble, it can move across plasma membranes with some ease. Using tritiated TPMP⁺, J. Sehlin and I have tested whether the ion is taken up by the B cells to the extent predicted by the Nernst equation in combination with an electric potential of –60 mV. The uptake predicted is a ten fold accumulation of the probe inside the cells.

Figure 2 shows the islet-cell uptake of TPMP⁺ with time, corrected for label in the extracellular space. The uptake more than reached the level predicted. Because the high uptake could in part be due to binding of the probe, some islets were pre-treated with an excessive concentration (50 μmol/l) of non-radioactive TPMP⁺. Such treatment reduced the subsequent uptake of (³H)TPMP⁺, but there was still more than enough. As another plausible way of correcting the TPMP⁺ uptake data, we analyzed the kinetics of label disappearance from islets that had been preloaded with (³H)TPMP⁺. The loss of label approached a non-zero asymptot which was tenta-

tively considered to represent high-affinity binding without relevance for the membrane potential. Correcting for this binding did not result in potential estimates smaller than those predicted. So, if it is at all meaningful to speak about a certain value for the membrane electric potential, for all we know it could be at least –60 mV.

Effects of Various Agents on Islet-Cell Uptake of TPMP⁺

Regardless of the exact objective value, if any, of the membrane electric potential, it seems important that the uptake of TPMP⁺ is no doubt influenced by D-glucose (Fig. 3); this effect of glucose has been reproduced with several concentrations of TPMP⁺ and with islets of normal lean mice as well. The metabolic poison, dinitrophenol, is also inhibitory. Mannoheptulose has no effect in itself but abolishes the effect of 20 mmol/l glucose. The effects of glucose and mannoheptulose on the TPMP⁺ uptake parallel those on insulin release. The results therefore suggest that specific changes in the interaction of one or more cations with the B cell is somehow coupled to the insulin-releasing action of glucose.

As TPMP⁺ is an artificial cation one naturally asks how its behaviour relates to the naturally occurring ions. One of the standard observations with intracellular electrodes is that the electromotive force diminishes when the extracellular concentration of K⁺ is increased. Figure 4 shows the effect of K⁺ on the islet-cell uptake of TPMP⁺. To facilitate comparison with electrode recordings [19, 20] the results are given in terms of electric potentials calculated by applying the Nernst equation to the uptake values. Because ⁴²K⁺ equilibration experiments have indicated that the present type of islet cells contain about 165 mmol/l K⁺ internally when incubated with 5.9 mmol/l K⁺ in the presence of 3 mmol/l D-glucose (Sehlin and Täljedal, unpublished data), one expects complete depolarization of the cells around 165 mmol/l K⁺ in the medium. Unadjusted TPMP⁺ uptake values were very far from meeting this expectation. However, both of the above methods for correcting the TPMP⁺ uptake data made the calculated potentials more sensitive to K⁺ within the range 5.9–130 mmol/l; one correction method even appeared compatible with complete depolarization around 165 mmol/l K⁺. Evidently, the TPMP⁺ uptake is influenced by K⁺ in qualitatively the same way, and quantitatively a similar way, as the electromotive force recorded by electrodes.

The membrane potential is classically envisaged as a diffusion potential whose magnitude depends on the gradients of free, dissociated ions and the permeabilities of the plasma membrane to such ions. If

Fig. 4. Effects of the extracellular K^+ concentration on apparent membrane potentials calculated by applying the Nernst equation to data for TPMP $^+$ uptake by *ob/ob*-mouse islets ($5.6 \mu\text{mol/l}$ TPMP $^+$ in the medium). All values are corrected for TPMP $^+$ in the extracellular (L-glucose) space. In the bottom curve ($\circ$) no correction has been made for any voltage-independent binding of TPMP $^+$. In the top curve ($\bullet$) potentials were calculated from the differences in (^3H)TPMP $^+$ uptake between islets not pretreated or pretreated with $50 \mu\text{mol/l}$ non-radioactive TPMP $^+$. In the middle curve ($\square$) the uptake values have first been reduced by $70 \mu\text{mol/kg}$ dry weight, representing a non-zero asymptote of TPMP $^+$ retained by islets in efflux experiments. The circle with the cross indicates the logarithm of 165, which in mmol/l is the intracellular concentration of K^+ in islets equilibrated with $^{42}\text{K}^+$ in salt-balanced medium containing 5.9 mmol/l K^+ and 3 mmol/l D-glucose (from unpublished work together with J. Sehlin)

this theory is essentially true, any factor that decreases the outward gradient of K^+ , or the membrane permeability to K^+ , should also diminish the electric potential. Hence the depolarizing action of high extracellular K^+ concentrations. The question is how does D-glucose produce this depolarizing action (Fig. 3), which was also observed by Dean and Matthew's measurements of electromotive forces as early as in 1968 [21]. A possible answer to this question has come from measurements of $^{86}\text{Rb}^+$ fluxes in isolated islets.

Fluxes of $^{86}\text{Rb}^+$, $^{42}\text{K}^+$, and $^{22}\text{Na}^+$

B Cells can accumulate $^{86}\text{Rb}^+$ ions avidly as a tracer for K^+ [22]. The accumulation is inhibited by ouabain or Na^+ deficiency and so resembles the cation pump that in most cells is held responsible for establishing opposing chemical gradients of K^+ and Na^+ across the plasma membrane. Measurements of $^{22}\text{Na}^+$ uptake by islets confirmed that the B cells have an inward Na^+ gradient, albeit rather small [23].

When $^{86}\text{Rb}^+$ -labelled islets were transferred to a non-radioactive buffer, they lost their radioactivity in a nearly exponential fashion [22]. In Figure 5 the radioactivity remaining in the islets is given as a function of washing time. An exponential curve has been fitted to a collection of data which comprise early experiments with $^{86}\text{Rb}^+$ [22] as well as more recent observations with $^{42}\text{K}^+$ (Sehlin and Täljedal, unpublished data). The approximately exponential behaviour of Rb^+ or K^+ retention suggests that the rate of isotope disappearance may reflect the permeability of the B cell plasma membrane. We were therefore interested in testing how D-glucose would influence the retention of isotope.

Figure 6 shows that the islets retain more Rb^+ if the D-glucose concentration during washing is 20 mmol/l compared with only 3 mmol/l [24]. In this capacity D-glucose cannot be replaced by L-glucose or methylated D-glucose. Because the specificity for D-glucose is the same as in the depolarizing and insulin-releasing action of the sugars, Sehlin and I proposed [24] that it is a decrease of the membrane permeability to K^+ that mediates the depolarizing and insulin-releasing action of D-glucose, or at least partially so. The correctness in accepting $^{86}\text{Rb}^+$ as a practical proxy for K^+ in those early experiments is evidenced by the more recent studies with $^{42}\text{K}^+$. The effect of D-glucose on $^{86}\text{Rb}^+$ retention is not only qualitatively reproduced with $^{42}\text{K}^+$ but is also proportionally the same with the two isotopes (Fig. 6).

It has been gratifying to see that this hypothesis of a glucose-sensitive electrodiffusion control mechanism, based on potassium, has been corroborated and extended in several other laboratories, both by measurements of isotopic fluxes and by recordings with micro-electrodes [25–27]. It must be emphasized, however, that we do not believe that K^+ electrodiffusion is necessarily involved in the action of all insulin secretagogues. Quite intuitively, it would seem purposeful if physiological potentiators of the glucose-initiated insulin release acted by mechanisms somewhat different from that of glucose itself. That this may be so can be illustrated by the actions of acetylcholine.

Cholinergic Effects on Insulin Release and Ions

Acetylcholine is the classical transmitter in the vagal nerves which may be important for controlling physiological insulin secretion in the intact body [28–30]. Atropine-injected mice had significantly smaller B cell nuclei than control mice, suggesting that the B cells may be subject to a long-term trophic influence of the cholinergic mechanisms [31]. Studies on isolated *ob/ob*-mouse islets showed that

Fig. 5. Approximately exponential efflux of $^{86}\text{Rb}^+$ and $^{42}\text{K}^+$ from *ob/ob*-mouse islets. After loading for 2 h with isotopes at the concentrations indicated, the islets were briefly (2 s) rinsed in non-radioactive medium and then incubated in non-radioactive medium (3 mmol/l D-glucose) for the indicated periods of time. The retention of isotope by the islets is given as percentages of the islet radioactivity after brief rinsing alone. Mean values $\pm$ SEM of 3–11 different experiments. The exponential curve is the explicit function of a straight line fitted to all points in a semi-logarithmic plot. From Sehlin and Täljedal [22; unpublished data]

acetylcholine has no effect in itself but collaborates with glucose as a typical potentiator of the glucose-initiated insulin secretory response [32]; species differences in this respect may exist, as cultured rat islets responded to acetylcholine at a non-stimulatory glucose concentration [33].

Quite unlike glucose, acetylcholine did not make the *ob/ob*-mouse islets retain more $^{86}\text{Rb}^+$ in efflux experiments [34]. Instead the drug influenced the rate of $^{22}\text{Na}^+$ influx; this effect was a proper cholinergic effect in being blocked by 5 $\mu\text{mol/l}$ atropine. In theory, an increased sodium permeability, as suggested by these data, would influence the membrane potential in the same direction as a decreased permeability to potassium. It seems plausible that the simultaneous occurrence of both changes, as in the presence of both a high glucose concentration and acetylcholine, would be especially effective. That glucose and acetylcholine have synergistic effects on the B cell's electrical activity has been demonstrated [32].

By analogy with other excitable cells it has been thought that depolarization of the B cell plasma membrane can open it to an inflow of Ca^{2+} ions [26]; alternatively or additionally, altered fluxes of K^+ or Na^+ could perhaps influence the intracellular distribution of Ca^{2+} . Whatever the mechanisms, it is

Fig. 6. Effects of sugars on the retention of $^{86}\text{Rb}^+$ and $^{42}\text{K}^+$ by *ob/ob*-mouse islets. Islets were loaded by incubation for 2 h in salt-balanced medium containing 3 mmol/l D-glucose and labelled with 70 $\mu\text{mol/l}$ $^{86}\text{Rb}^+$ or 5.9 mmol/l $^{42}\text{K}^+$. After brief (2 s) rinsing, the islets were incubated for 10 min in non-radioactive medium containing 3 or 20 mmol/l D-glucose alone or 3 mmol/l D-glucose in combination with other sugars as indicated. Mean values of 4–18 different experiments; thin bars denote SEM. The effects of 20 mmol/l D-glucose are significant with $p < 0.025$ (from Sehlin and Täljedal [24; unpublished data])

commonplace that several modifiers of insulin release affect the interaction of $^{45}\text{Ca}^{2+}$ with the islet cells. In Umeå we have also performed such experiments and among other things observed that glucose causes a real uptake of Ca^{2+} and not only alterations of isotopic flux rates [35], and that one can differentiate between more superficial and less superficial pools of glucose-sensitive calcium in the islet cells [36]. Yet, I shall be brief on $^{45}\text{Ca}^{2+}$ here and merely stress a rather important point that has emanated from many different experiments: there is no strict relationship between the total uptake of $^{45}\text{Ca}^{2+}$ by the B cells and insulin release.

For example, 11 mmol/l glucose stimulates the islet-cell uptake of $^{45}\text{Ca}^{2+}$. Acetylcholine at 3 mmol/l glucose also stimulates $^{45}\text{Ca}^{2+}$ uptake [34], although the drug has no effect on insulin release from these islets at low glucose concentration [32]. Moreover, at 11 mmol/l glucose acetylcholine had little or no effect on the $^{45}\text{Ca}^{2+}$ uptake despite its marked potentiation of insulin release at that glucose concentration [32]. Other subtle discrepancies of a similar nature suggest that calcium uptake is somehow related to insulin release but in its entirety is too crude a variable to be physiologically relevant [37, 38]. Against this background, other ways of studying Ca^{2+} seem worth considering.

Fluorescent Probing of Calcium in Islet Cells

I have studied whether the fluorescence of Ca^{2+} -chlorotetracycline chelates [39] may yield useful new information about the interaction of Ca^{2+} with islet cells [40–43]. Chlorotetracycline is a fairly flat molecule with 4 rings and altogether 8 oxygens. The molecule can bend somewhat to form a central crevice surrounded by the oxygens. Attracted by the electron excess in these oxygens, a Ca^{2+} ion can probably fit into the crevice. There the ion is likely to protrude on one side so as to allow interaction with negative charges in a cell at the same time. In an hydrophobic environment the Ca^{2+} -chlorotetracycline complex fluoresces with extreme intensity. Therefore tetracycline may preferentially probe Ca^{2+} ions located in membranes. At high concentrations the drug is also an ionophore [43] and stains calcium all over the cells [40, 44]. However, at sufficiently low concentrations there are reasons for believing that chlorotetracycline largely monitors Ca^{2+} in or near the plasma membranes [40, 43]. When working with the small amounts of islet cells available from mammalian pancreases, the light yield over background is too low for practical measurements to be performed in ordinary fluorometer cuvettes. A microscopic method was therefore developed [40].

The advantages and disadvantages of the new technique have not yet been fully analyzed, but its potential usefulness may be illustrated by some results concerning the effects of glucose and acetylcholine (Fig. 7). Dispersed islet-cells were incubated with Ca^{2+} and chlorotetracycline and then analyzed [34]. Acetylcholine had no effect in the absence of glucose, while, as found repeatedly [40, 41], glucose alone decreased the fluorescence. This decrease could mean that there was a loss of membrane-bound Ca^{2+} , or that the hydrophobicity of the micro-environment had diminished. Indeed, studies on the polarization of the fluorescent light suggest that glucose induced a change in the micro-environment of Ca^{2+} ions in the B cells [41].

In contrast to the failure of acetylcholine to influence fluorescence in glucose-free medium, at 11 mmol/l glucose acetylcholine enhanced the fluorescence (Fig. 7). This effect was blocked by atropine, as it should be if reflecting a true muscarinic receptor interaction. Thus, the fluorescent probing technique has revealed a cholinergic effect on calcium that may occur near the B cell's plasma membrane which is glucose-dependent, and which has an appropriate sensitivity to atropine. The only puzzling thing is that glucose and acetylcholine acted in opposite directions. Perhaps this paradox has something to do with the fact that the fluorescent signal responds to changes in the concentration of calcium as well as

Fig. 7. Fluorescence from dispersed *ob/ob*-mouse islet-cells that had been incubated for 1 h in salt-balanced medium containing 5 mmol/l Ca^{2+} and 10 $\mu\text{mol/l}$ atropine as indicated; acetylcholine (ACh) was added together with 5 $\mu\text{mol/l}$ eserine. Results are expressed as percentages of the light recorded with 10 $\mu\text{mol/l}$ chlorotetracycline and 5 mmol/l Ca^{2+} in the absence of glucose and drugs. The Ca^{2+} -independent chlorotetracycline fluorescence from the cells was about 50%; the background light recorded for cells in the absence of chlorotetracycline was about 25% and has not been subtracted from the data. Mean values are given for 5–11 different experiments; thin bars denote SEM. From [34]

to changes in the environment surrounding the ions; perhaps a solution should be sought in the postulated existence of both stimulatory and inhibitory functions of calcium in the B cells [45].

Interactions of Insulin Secretagogues with the B Cell Plasma Membrane

If insulin release is controlled by ionic events in or near the B-cell plasma membrane, the simplest way of action for any modifier of insulin release would be to bind directly to the plasma membrane. Given the above results for acetylcholine, one naturally assumes that the drug binds to traditional muscarinic receptors.

A direct effect on the B cell plasma membrane is also likely in the case of the blood-sugar-lowering sulphonylurea derivatives. The distribution volumes of such drugs in isolated islets suggest that they bind to the B cells without readily penetrating into them [46–50]. The magnitude of binding is clearly different for tolbutamide, glibornuride and glibenclamide, and this difference seems to match their relative efficiencies as insulin-releasing agents. Interactions between sulphonylurea and cations may be illustrated by the binding of glibenclamide to the islet cells, a process stimulated by Na^+ , Ca^{2+} , or

Mg²⁺ [51]. That sulfonylureas influence ionic fluxes in the islets has also been demonstrated [52–54].

A class of theoretically interesting stimuli of insulin release are thiols and thiol-blocking agents, some of whose effects are most likely exerted in the B cell plasma membrane [55–65]. Studies of their actions have led to a specific speculation on the role of membrane thiol groups in stimulus-secretion coupling [58, 66], a hypothesis considered in more detail below ("*Thioredoxin and Thioredoxin Reductase*", and "*Alloxan Toxicity*").

It has been suggested that amino acids may stimulate insulin release by virtue of their direct interaction with transport systems in the B cell plasma membrane [67]. Various systems for amino acid transport have been detected in the islet cells, and it is possible that the system for arginine is involved in the arginine-induced potentiation of glucose-initiated insulin release [68–70]. However, the ability of L-leucine to initiate insulin release in the absence of glucose is probably not linked to leucine transport. Although D-leucine is transported by the same system as L-leucine, D-leucine neither stimulated insulin release nor inhibited the release induced by L-leucine [71, 72]; similarly, L-isoleucine inhibited the transport of both L-leucine and D-leucine but had no effect on insulin release [71, 72]. That co-transport of an amino acid with a cation is in general not sufficient to elicit secretion is evidenced by the fact that alanine or α -aminoisobutyric acid does not stimulate insulin release from *ob/ob*-mouse islets [68] but is avidly transported into such islet cells by a Na⁺-dependent mechanism [69, 73].

With regard to the most important insulin secretagogue, D-glucose, there is very little positive evidence for membrane-located receptors other than the hexose transport system. Phlorizin and phloretin can stimulate insulin release, and the possibility has been discussed that they do so by interacting with receptors for the unmetabolized glucose molecule [74–76]. Analyses of chemical structures led Weaver et al. [77] to propose that D-glucose, D-mannose, alloxan, and ninhydrin share a common molecular architecture that could account for their recognition by a direct receptor to initiate insulin release. Although a role of direct glucose receptors, whether in the plasma membrane or elsewhere in the B cell, cannot be ruled out, there is comparatively much stronger evidence that at least an essential part of glucose recognition involves metabolism of the sugar. This evidence was elegantly reviewed by the 1979 Minkowski lecture [78]. To avoid repetition, and since I share Dr. Ashcroft's general views of the matter, I shall not dwell long on glucose metabolism but restrict myself to a few comments on glucose transport and glucose phosphorylation. First, it may be

noted that neither the inhibitory effect of neuraminidase treatment of isolated islets [79] nor the fact that glucose can raise the islet cyclic AMP level [80] is a valid argument for the direct receptor hypothesis.

Glucose Transport

Similar to the observations in fish islets [81], in mammalian islets rich in B cells the glucose transport system discriminates between D-glucose and L-glucose [82–85]. This property is most readily demonstrated by measuring the distribution volumes of ³H and ¹⁴C in islets incubated simultaneously with D-(6-³H)glucose and L-(1-¹⁴C)glucose. Double-labelling with D-(U-¹⁴C)glucose or L-(1-¹⁴C)glucose together with (6,6'-³H)sucrose indicated that L-glucose does not penetrate beyond the extracellular space. In contrast, D-glucose equilibrates across the B cell plasma membrane with a distribution ratio of about unity [82]. The capacity for D-glucose transport is so great as to bring about a nearly complete equilibration of the sugar within the first 1–2 min of incubation at 37 °C [83]. Therefore, the kinetics of transport are difficult to study accurately with isolated islets at a physiological temperature. However, by using suspensions of dispersed islet-cells and interrupting the incubation by centrifuging through oil, incubation periods as brief as 4 s can be employed [85]. Alternatively, the transmembrane transport rate in whole islets can be slowed down by lowering the temperature [82].

At any concentration of D-glucose in the medium, the rate of D-glucose entry (unidirectional) is several times higher than the rate of glucose oxidation or ³H₂O production from D-(5-³H)glucose [83, 84]. Thus, transport is not rate-limiting for glucose metabolism in the B cell. D-glucose transport is not normally rate-limiting for insulin release either, as phlorizin could inhibit the transport by 52–73% with only marginal, if any, effects on insulin release [74].

A consequence of the high transport capacity is that the interior of the B cells always senses the glucose concentration in the surrounding environment [86, 87]. As this is not typical of cells, a strong evolutionary argument can be made for linking the glucose transport system to the special task of the B cells, i. e. insulin secretion. However, the transport system as such cannot be the recognition system for glucose, at least not the complete system. 3-*O*-Methyl-D-glucose is transported like D-glucose [88] but does not affect insulin release [83]. 5-Thio-D-glucose is probably also a substrate for the transport system but markedly inhibits secretion [89]. Similarly, α -D-glucose and β -D-glucose appeared equally effective in inducing counter-transport of 3-*O*-methyl-D-glucose from the B cells, although the α anomer was a more potent insulin secretagogue than

the β anomer [90]. I am therefore convinced that evolution has endowed the B cells with their unusually effective glucose transport system in order that the *interior* of the cells can always know and keep track of the circulating glucose concentration. This brings us to metabolism, the first step of which is glucose phosphorylation.

Glucose Phosphorylation

Strong evidence has been gathered for the view that glucose phosphorylation is the rate-limiting step in the stimulation of insulin secretion by glucose [78]. In accordance with this hypothesis there is often a good correlation between the concentrations of D-glucose and glucose-6-phosphate in the islets, including the B-cell-rich islets of non-inbred *ob/ob*-mice [84]. Occasional deviations from the expected results suggest, however, that the complete metabolic recognition system for glucose may be somewhat more complicated. Thus, it is not understood why phlorizin at concentrations decreasing the islet content of fructose-1,6-diphosphate, glucose oxidation, and insulin release had no demonstrable effect on the glucose-6-phosphate level [74]. Similarly, β -D-glucose appeared at least as effective as α -D-glucose in raising the glucose-6-phosphate concentration in the islets, although α -D-glucose had a stronger effect than β -D-glucose on insulin release [90, 91]. Malaisse et al. [92] have presented evidence that the different insulin-releasing potencies of D-glucose anomers reflects a corresponding stereo-specificity of phosphoglucose isomerase; this explanation is considered insufficient by Niki and Niki [93], who reviewed the many complicated data on glucose anomers and judged them in favour of direct glucose receptors.

The concentration of glucose-6-phosphate in B cells is decreased by anoxia, a situation which simultaneously increases the concentration of fructose-1,6-diphosphate plus triose phosphates [84]. However, the islets did not exhibit a traditional Pasteur effect, as glycolysis ($^3\text{H}_2\text{O}$ production from glucose labelled in position 5) was not enhanced but inhibited. In contrast, when pieces of exocrine pancreas were examined by the same incubation techniques, they exhibited the expected stimulation of glycolysis [84]. Islet glycolysis may be especially sensitive to inhibition by anoxia because a comparatively low activity of lactate dehydrogenase limits re-oxidation of NADH for the phosphoglyceraldehyde dehydrogenase step. Under such conditions the availability of ATP is probably insufficient to maintain the concentration of glucose-6-phosphate.

Regardless of whether regulatory mechanisms other than glucose phosphorylation will in the end have to be incorporated into a comprehensive picture

of glucose recognition, it is a fact that glucose-6-phosphate co-varies with glucose within the physiological and diabetic glucose phosphorylation range. This phenomenon may be due to the existence in the B cells of a glucose-phosphorylating enzyme with especially high K_m for glucose; the kinetics of phosphorylation in whole islet extracts can be interpreted as reflecting a mixture of such an enzyme (resembling liver glucokinase), and hexokinases with low K_m for glucose [78, 87]. However, so far the hypothetical islet glucokinase has not been directly demonstrated by separating it from other enzymes. Attempts in that direction have been performed in our laboratory by subjecting tissue extracts to electrophoresis in polyacrylamide gel slabs (Söderberg and Täljedal, unpublished data). As shown in Fig. 8, at a high glucose concentration at least two major zones of enzyme activity could be demonstrated with extracts of exocrine pancreas. The fastest band was considerably less impressive when the gels were incubated with only 0.5 mmol/l D-glucose, while the more slowly migrating zone seemed to be comparatively little affected by lowering the glucose concentration. Surprisingly enough, the clearly glucose-sensitive fast band was hardly visible in extracts of isolated islets, although the more slowly-running zone was about as active as in the exocrine pancreas. Other separation techniques could perhaps be employed to resolve the major zones in Fig. 8 into finer iso-enzyme bands, and also for other reasons the results certainly do not disprove the existence of a high- K_m phosphorylating enzyme in the B cells. However, they seem to indicate that there is still more to be done in the area of pancreatic glucose phosphorylation.

Thioredoxin and Thioredoxin Reductase

The question as to how the insulin-releasing signal is transmitted onwards from an enhanced rate of glucose metabolism to the triggering of exocytosis is among the most unclear ones in the whole area of insulin secretory control. To fill in a gap in the envisaged signal chain we have considered the possibility that membrane-located thiol groups play a role in the coupling of glucose metabolism to ionic events. The hypothesis arose out of studies on thiols and thiol-blocking agents as stimuli of insulin release [55–66]. The existence in the B cells of a membrane protein was proposed [58, 66], whose conformation depends on whether a couple of sulphurs are oxidized to an S-S bridge or reduced to thiol groups. Only the reduced form would allow the ionic fluxes eliciting secretion. Glucose would shift the protein to the reduced form through intervention of reduced pyridine nucleotides and perhaps glutathione. This was rather a venturesome speculation, not the least because the

Fig. 8. Polyacrylamide gel electrophoresis of glucose-phosphorylating enzymes in mouse islets and exocrine pancreas. Tissues were homogenized in 50 mmol/l Tris-HCl buffer (pH 7.4) containing 10 mmol/l D-glucose, 150 mmol/l KCl, 2.5 mmol/l EDTA, 5 mmol/l $MgCl_2$, and 0.5% Triton X-100. Samples of homogenate (20 μ l) were applied to gel slabs containing glucose-6-phosphate dehydrogenase and 0.5 or 100 mmol/l D-glucose as required. After running the gels at 150 V for about 3 h, they were exposed to ATP and 0.5 or 100 mmol/l D-glucose in a histochemical staining solution catalyzing the oxidation of glucose-6-phosphate and a corresponding colour-forming reduction of tetrazolium salt. The protein concentration (by native fluorescence and human serum albumin as standard) of the undiluted homogenates was estimated to be 1.8 mg/ml (0.5 mmol/l D-glucose) or 2.8 mg/ml (100 mmol/l D-glucose) for exocrine pancreas, and 0.6 mg/ml (both glucose concentrations) for the islets. From left to right the eight lanes represent: exocrine pancreas undiluted, exocrine pancreas diluted 1:1, exocrine pancreas diluted 1:3, islets undiluted, islets undiluted, exocrine pancreas diluted 1:3, exocrine pancreas diluted 1:1, exocrine pancreas undiluted. The four lanes to the left were developed at 0.5 mmol/l D-glucose, while the four lanes to the right were developed at 100 mmol/l D-glucose (unpublished experiments together with M. Söderberg)

Fig. 9. Scheme illustrating the function of the thioredoxin/thioredoxin reductase system [94, 95]

Fig. 10. Demonstration of thioredoxin in *ob/ob*-mouse islets. In the assay employed, exogenous insulin was first reduced by islet homogenate or purified calf thymus thioredoxin (standard) in the presence of purified calf thymus reductase and NADPH. The amount of insulin reduced was then photometrically determined by 5,5'-dithiobis(2-nitrobenzoic acid) (from unpublished data together with Holmgren and Luthman)

strategic thiol protein was pure imagination. The hypothesis remains speculative, but we can definitely state that a protein with some of the desired properties exists in the B cells.

Thioredoxin is a 11,700 molecular weight protein functioning as the electron acceptor in the transfer of electrons from NADPH as catalyzed by the enzyme thioredoxin reductase [94, 95]. Thioredoxin and its reductase are rapidly becoming recognised as a reduction/oxidation system of very wide significance in micro-organisms as well as mammalian cells. Originally discovered in connection with studies on ribose reduction and DNA synthesis, its range of possible substrates today include a variety of biologically important factors; the reactivity with insulin is exceptionally high, suggesting that thioredoxin among

other things may play a physiological role in insulin action and degradation [95]. As schematically outlined in Fig. 9, thioredoxin may be a model for the type of protein envisaged in our regulatory hypothesis for B cells [58, 66]. Alternatively, its ability to react with a whole range of thiol/disulphides makes it interesting as a conceivable member of any

Fig. 11. Individual weight curves for 16 male C57BL/KsJ-*db/db* mice which from 10 weeks of age were fed a diet rich in rye bran (solid lines) or an iso-caloric bran-free diet instead containing refined rye flour (broken lines). The bran diet is in many ways comparable to standard laboratory mouse food, whereas the bran-free diet is very poor in fibre. Spontaneous deaths are indicated by crosses (from unpublished data together with Berglund, Hallmans and Nygren)

longer electron transport chain between glucose metabolism and the B cell plasma membrane.

Figure 10 shows that thioredoxin exists in mouse B cells and can be assayed with the aid of calf thymus thioredoxin reductase. In three batches of islets from different *ob/ob*-mice, 0.05% of the protein content was found to be thioredoxin, a significant figure in consideration of the protein's catalytic properties [94]. When assayed with calf thymus thioredoxin as substrate and with calf thymus reductase as standard, the islet homogenates contained reductase in amounts corresponding to $0.04 \pm 0.01\%$ (mean $\pm$ SEM of three homogenates) of the islet protein (Holmgren, Luthman and Täljedal, unpublished data). That these regulatory proteins occur in the islet cells is not only interesting from the point of view of physiological secretory control but may also be essential for the understanding of B cell damage (see below, "Alloxan Toxicity").

Damage to B Cells and Disturbances of Insulin Secretion

Experimental Models

With the above aspects of the normal secretory mechanisms as a background, it has been natural to examine various models of B cell damage. C57BL/KsJ-*db/db*-mice have been used as a model of genetically determined and progressive B cell deterioration leading to diabetes. Healthy B cells isolated and exposed to serum or alloxan *in vitro* have been investigated as examples of acutely inflicted cytotoxicity. Although the results obtained with these experimental models cannot be immediately extrapolated to

diabetes in man, they might provide some insight into mechanisms that in principle could also apply to the human situation.

Genetically Diabetic Mice

C57BL/KsJ-*db/db*-mice are overweight early in life but then start to lose weight abruptly and die prematurely [96, 97]. Terminal mice have a severe form of diabetes with markedly defective insulin secretory responses *in vitro* [98–100]. The fact that this pathological development is not only genetically determined but is also influenced by such a simple environmental factor as dietary fibre enhances the similarity with certain types of human diabetes. The effect of fibre is illustrated by the weight curves in Fig. 11. Mice fed a diet rich in bran exhibited a weight development similar to that of mice on regular laboratory diet; both of those diets are rich in fibre. In contrast, when the KsJ-*db/db*-mice were given a bran-free diet extremely poor in fibre, they quickly lost weight and their life-spans became very short. Control experiments with healthy KsJ-*+/+*-mice showed no reduction of body weight or life span in response to the diet poor in bran (Berglund, Hallmans, Nygren and Täljedal, unpublished data).

The islets of KsJ-*+/+*-mice respond to 20 mmol/l D-glucose with an inhibition of $^{86}\text{Rb}^+$ efflux in much the same way as in Fig. 6 for the B cell-rich islets of non-inbred *ob/ob*-mice. In contrast, no such effect of a high glucose concentration was seen in KsJ-*db/db*-mouse islets [101]. Instead, the basal K^+ permeability appeared to be decreased in the diabetic mouse islet-cells, a finding that could explain their persistent depolarization at low glucose concentration [102]. The satisfying thing with this

Fig. 12. Schematic illustration of B cell damage by immune mechanisms (for explanation see text)

Fig. 13. Inhibition of $^{86}\text{Rb}^+$ accumulation in dispersed mouse islet cells exposed to 25% human serum. To differentiate between the classical and alternative ways of complement activation, the serum was tested without pretreatment, after addition of 10 mmol/l EGTA in combination with 10 mmol/l MgCl_2 , and after heating at 50 °C for 30 min. Control cells were not exposed to serum at all. All cells were incubated for 2 h in salt-balanced medium labelled with 28 $\mu\text{mol/l}$ $^{86}\text{Rb}^+$. Results are expressed as percentages of the Rb^+ uptake by control cells. Mean values $\pm$ SEM of four different experiments are given; thin bars denote SEM (from [110])

result is that it connects our potassium electrodiffusion hypothesis for normal insulin secretion (see above, “Fluxes of $^{86}\text{Rb}^+$, $^{42}\text{K}^+$, and $^{22}\text{Na}^+$ ”) with a defined disturbance of insulin secretion in at least one form of diabetes. This conclusion does not mean that altered K^+ permeability is the only defect in the regulation of ion fluxes and membrane potential in the KsJ-*db/db*-mouse islet cells. The B cell membrane potential may also depend on the Cl^- permea-

bility, which is increased in these diabetic mice [103, 104]; a defective efflux of $^{45}\text{Ca}^{2+}$ has also been reported [105].

Another interesting defect in the KsJ-*db/db*-mouse islets is their failure to respond to methylxanthine with an increased concentration of cyclic AMP [100]. Although the initiation of insulin release by glucose is probably not mediated by cyclic AMP, the nucleotide undoubtedly plays an important role in modulating the secretory response to glucose [80, 106]. That disturbances of both ion fluxes and cyclic AMP occur in the islets of KsJ-*db/db*-mice supports the idea of close interrelationships between ionic events and/or the membrane potential on the one hand and the metabolism of cyclic AMP on the other [106–108].

Serum Cytotoxicity

In an attempt to search for cytotoxic factors in the blood of human diabetic patients, it was accidentally observed that normal human serum or plasma markedly inhibits the accumulation of $^{86}\text{Rb}^+$ by mouse islet-cells [109]. Subsequent studies clarified that this effect follows from a cytotoxic activation of the serum complement system [109, 110]. What relevance, if any, could these findings have for diabetes?

Figure 12 is a schematic summary of the mechanisms by which B cells could conceivably be attacked by the immune system. First there is the direct killer-cell attack proposed by Huang and McLaren [111]. Secondly, there is the hypothetical involvement of humoral antibodies against the B-cells. Lernmark et al. [112] have shown that some of them bind to the surface of whole B-cells. Their pathogenetic role is not clear, but they may well be toxic by activating complement along the classical pathway. Thirdly, there is the often neglected possibility of eliciting the toxicity of complement even in the absence of antibody, the so-called alternative pathway. It is well known from experiments with other cell types that this pathway can be activated by polysaccharides and heterologous cells, and probably also by viral transformation of the cells [113–115]. As diagnostic criteria, the classical pathway requires calcium ions, whereas the alternative pathway is inhibited by destroying factor B by heating at 50 °C [116].

Figure 13 shows that the inhibitory effect of human serum on rubidium accumulation by mouse islet-cells is not blocked by calcium deficiency but is entirely prevented by heating at 50 °C. Together with several other related experiments, the results strongly suggest that mouse B cells can activate the alternative complement pathway in human serum [109, 110]. From other experiments it is also clear that in such a heterologous system, cells from the

spleen, the liver and the exocrine pancreas are also attacked. However, in a homologous system represented by normal mouse serum and normal mouse cells, neither spleen nor liver cells were affected whereas islet-cells were, provided that they came from other individuals than those donating the serum. In a fully autologous system no significant effect was observed. It therefore appears that the effect of serum is determined by at least three different classes of identifiers on the cells, namely for species, organ, and self. Against this background, I think that when one discusses the possibility that infectious or inflammatory alterations of the B cell surface can entail sufficient deviations from the self-configuration to elicit killer cell or immunoglobulin responses, one could as well consider the possibility of an early activation of the alternative complement pathway.

Modern considerations of possible B cytotoxic mechanisms probably should not neglect the role of free radicals produced by the non-lymphocyte inflammatory cells [117, 118]. Interestingly enough, free radicals have also been proposed to mediate the classical diabetogenic action of alloxan, the third model of B cell damage now to be discussed.

Alloxan Toxicity

The free-radical hypothesis of alloxan action was originally based on protection experiments in whole animals [119]. In Umeå we believe that we have confirmed this hypothesis by measuring $^{86}\text{Rb}^+$ accumulation and Trypan Blue exclusion as viability criteria in isolated *ob/ob*-mouse islets and islet cells in vitro [120, 121]; similar conclusions have been reached in independent studies of the alloxan-induced inhibition of insulin release from isolated rat islets [122, 123].

According to this hypothesis, the double-bonded oxygens of alloxan can be reduced by suitable groups in the islet tissue to yield dialuric acid. This reduction is not necessarily harmful in itself. However, dialuric acid spontaneously oxidizes back to alloxan [124], and in this process a number of reactive compounds can be formed, such as hydrogen peroxide, superoxide anion radicals, and most notoriously, hydroxyl radicals. The generation of hydroxyl radicals from superoxide radicals and hydrogen peroxide ("Haber-Weiss reaction") in biological systems appears to require catalysis by traces of transitional metals. It can be seen as the summation of the reduction of ferric ion by superoxide ($\text{Fe}^{3+} + \text{O}_2^- \rightarrow \text{Fe}^{2+} + \text{O}_2$) and the Fenton reaction ($\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{OH}^\cdot + \text{OH}^- + \text{Fe}^{3+}$). Against this background we have tested the free-radical hypothesis by studying whether scavengers of peroxide, free radicals or iron ions influence the effects of alloxan in vitro.

Table 1. Thioredoxin-catalyzed light production from luminol in the presence of alloxan and molecular oxygen

Additions to basal reaction mixture	Luminescence (thousands of counts)
NADPH	3.9 ± 1.0
NADPH + thioredoxin	2.9 ± 0.6
NADPH + thioredoxin reductase	5.5 ± 1.1
NADPH + thioredoxin + thioredoxin reductase	26.4 ± 3.0

The basal reaction mixture consisted of 100 $\mu\text{mol/l}$ alloxan, 10 $\mu\text{mol/l}$ FeSO_4 , and 1 $\mu\text{mol/l}$ luminol in a salt-balanced buffer of essentially the same formula as Krebs-Ringer bicarbonate, except that the bicarbonate was replaced by 20 mmol/l Hepes. These basal conditions were chosen in the light of extensive methodological tests aimed at defining optimum conditions for using luminol to monitor the production of radicals in the alloxan-dialuric acid cycle (Grankvist, unpublished data). Luminescence was measured in a liquid-scintillator, and the figures denote thousands of counts recorded within 30 s after the addition of 100 $\mu\text{mol/l}$ NADPH, or 3.4 $\mu\text{mol/l}$ purified thioredoxin from *E. coli*, or 0.2 $\mu\text{mol/l}$ purified thioredoxin reductase from *E. coli*, or the combination of these compounds. All values are corrected for the background in the presence of buffer alone (2.1), and they represent mean values $\pm$ SEM of 6 observations (from unpublished data together with Grankvist, Holmgren and Luthman)

Fig. 14. Scheme of conceivable interrelationships between various normal and cytotoxic processes in the B cell plasma membrane (for explanation see text)

When the ability of butanol, dimethylsulfoxide, benzoate, histidine, urea, theophylline, caffeine, and pyridine nucleotides to protect the $^{86}\text{Rb}^+$ -accumulating capacity was plotted against the concentration of protector multiplied by its known reactivity with free hydroxyl radicals, there was a marked correlation, except that the methylxanthines were better protectors than indicated by their hydroxyl-scavenging capacity [120]. By the same methods it was also found that alloxan cytotoxicity is inhibited by chelating any catalytic traces of iron in the islets [121]. In the same way, the enzymes catalase and superoxide

dismutase, in a dose-dependent manner, protected the ability of islet cells to exclude Trypan Blue [120].

Cooperstein, Lazarow, Watkins and collaborators have long argued that alloxan acts on thiol groups in the B cell plasma membrane [125, 126]. This view is in part supported by our protection experiments, as neither the enzymes nor some of the hydroxyl-scavenging compounds are likely to penetrate easily into the cells. The extraordinary efficiency of glucose and methylxanthines as protectors [120, 127, 128] suggests that steric hindrance in the hexose transport system may contribute to the protection, and so the primary site of action, i. e. the reduction to dialuric acid, may well be embedded within the membrane.

An important role in insulin secretion has hypothetically been attributed to thiol/S-S redox reactions in the B cell plasma membrane and their dependence on reduced pyridine nucleotides (see above, "*Thioredoxin and Thioredoxin Reductase*"). It is therefore noteworthy that alloxan is reduced by NADH and NADPH [120]. As thioredoxin may be a model for the hypothetical signal protein, it is striking that alloxan reacts extremely rapidly with purified thioredoxin [129]. Finally, as the toxicity of alloxan is probably mediated by hydroxyl radicals [120, 121], we have used a chemiluminescence method to test directly whether thioredoxin can catalyze the production of free radicals from alloxan. As shown in Table 1, 3.4 $\mu\text{mol/l}$ thioredoxin from *E. coli* markedly stimulated the light yield from luminol. The specificity of the reaction and the efficiency of the complete reductive system (NADPH/thioredoxin/reductase) are evident from the low light yield with as much as 100 $\mu\text{mol/l}$ NADPH alone, or with NADPH in combination with only thioredoxin (Grankvist, Holmgren, Luthman and Täljedal, unpublished data). Based on these considerations, an integrated picture of several interrelated events in the B cell plasma membrane could perhaps be sketched as in Fig. 14.

Measurements of the activities of endogenous superoxide dismutases, catalase, and glutathione peroxidase in mouse islets showed that the islets have modest activities of these important protectors against toxic oxygen reduction products (Grankvist, Marklund and Täljedal, unpublished data). Even if the deviation from other tissues was not large enough to explain fully the great sensitivity of B cells to alloxan, it seems logical to assume that injections of exogenous superoxide dismutase could improve the protection of the B cells against free superoxide and hydroxyl radicals under conditions when such agents are generated. In support of this assumption, in my laboratory Grankvist and Marklund have found that mice are protected against alloxan diabetes if injected with superoxide dismutase several hours beforehand, provided the enzyme has been linked to

polyethylene glycol in order to prevent its elimination through the kidneys. How far this protective effect can be generalized to other forms of diabetes is entirely unknown but seems suitable topic for research.

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