It is a great privilege to be asked for a "Reflections" essay; I admire those prepared by my predecessors. My teachers were less prestigious than Arthur Kornberg's (1), and there was no single major theme in my research as was the case with several previous contributors to this series. Instead we studied a wide variety of metabolic phenomena that I have described in a summary of my first 50 years of biochemical research (2).

Our findings included a treatment for selenium poisoning in livestock (undergraduate thesis; selenium-containing mercapturic acids are excreted in the urine) that was applied successfully to a human case; our studies of spermatozoa will be described in a following section. We elucidated the mechanism by which L-glyceraldehyde inhibits glycolysis (3). That disproved Needham's non-phosphorylating glycolysis in embryos and tumors. Could that have encouraged him to drop experiments and to devote his talents to prepare his magnificent history of Chinese science instead? We found that the function of biotin was to fix CO₂ in heterotrophic organisms (4); cellular respiration rates varied with the availability of inorganic P and phosphate acceptor (5, 6); propionate was metabolized by CO₂ addition to ultimately yield succinate (7, 8). My students purified and crystallized some 10 phosphate-transferring enzymes, and we demonstrated that most of them required MgATP as substrate and were inhibited by free ATP; we found 16 different antibiotics that affected oxidative phosphorylation (9, 10) and a dozen that acted as ionophores (11), some of which are still being used in experiments. We also found that caffeine increased respiration and dramatically induced whiplash-type motility in sperm by increasing cyclic AMP (12, 13); the respiratory response was dependent on the utilization of acetylcarnitine (14). Thyroid hormone and also dehydroepiandrosterone induced the synthesis of mitochondrial glycerol-3-phosphate dehydrogenase to as much as 20 times the normal concentration (15–17) and formed part of a thermogenic system (17, 18). The path of carbon in gluconeogenesis was found to involve carboxylation of pyruvate (Utter reaction) in mitochondria, reduction of oxalacetate to malate, malate transport to cytosol in exchange for pyruvate, oxidation of malate to oxalacetate (the precursor of phosphopyruvate) together with the generation of the NADH required to reduce 3-phosphoglycerate to triose phosphate (19, 20); serine was found to be converted to glucose by an entirely different pathway, probably the reverse of its synthesis from hydroxypyruvate (21). We also found that levels of liver cytosolic phosphoenolpyruvate carboxykinase (PEPCK) are regulated by the need for gluconeogenesis; they are increased by fasting and decreased in well fed animals; PEPCK is activated by ferrous ion, and in liver free calcium activates PEPCK by releasing Fe²⁺ from mitochondria to the cytosol (22); feeding tryptophan inhibits gluconeogenesis because its metabolite, quinolinate, forms a complex with ferrous ion that blocks PEPCK (23, 24). The widely reported enhancement of liver mitochondrial respiration following exercise or the administration of glucagon or adrenaline to rats was found to be mediated by elevated malate concentration in the liver (25, 26). Malate is known to facilitate mitochondrial uptake of substrates by exchange across the mitochondrial membranes.
Naturally there was also an abundance of studies that yielded useful facts but not new concepts and many experiments undertaken to test hypotheses that turned out to be without merit! One reason for the diversity of research is that we wanted graduate students to have their own thesis research problems. Sixty-four candidates earned their Ph.D. degree in our group between 1945 and 1989 and more than 100 postdoctorate fellows conducted their research in our laboratories at the Institute for Enzyme Research. Relationships with these scholars and friends have always meant a great deal to me.

At the time my research history was written (2), we were studying an intriguing class of Janus-like proteins, caltrins, that function in fertilization. Because the work was in progress it was not described in that essay. The caltrins have not been widely publicized and therefore are probably not familiar to most biochemists. The caltrins of different species have widely different structures and their multiple functions are achieved by disparate mechanisms.

The Caltrin Story

No aspect of living processes is more awe-inspiring than the union of a microscopic spermatozoon with an egg of the same species to initiate a new life. In this process the contribution of the male is to present a set of haploid chromosomes to join those of the egg. However, this presentation is a complex ceremony involving “capacitation,” i.e., alteration of sperm plasma membranes to permit penetration by Ca\(^{2+}\). Calcium uptake is followed by disruption of the acrosome, a sac containing hyaluronidase and proproteinases that autocatalytically are converted to active acrosins. The sperm attach to the protective layer of the egg, a glycoprotein matrix (zona pellucida), and the calcium-activated acrosomal enzymes attack the zona to provide a path for sperm entry. Calcium uptake by the contractile components in the sperm tail facilitates the acquisition of “hyperactivated” motility characterized by rapid lashing and wider excursion of the sperm tail. This causes the sperm to swim in tight arcs to drive through the zona and then penetrate the egg. The role of caltrins in regulating each of these processes is complex and fascinating.

Our work with spermatozoa had been continuous since 1939 when my professor, Paul Phillips, and I developed a medium for the preservation of animal sperm (27). It permitted the retention of motility and fertility for 8–12 days and launched the artificial insemination industry in livestock. Because we had solved the practical problem, I was free to study basic aspects of sperm metabolism and the regulation of energy capture for motility. The findings during that period included the first clear statement concerning the mechanism by which 2,4-dinitrophenol functions: “the fact that DNP decreased the motility of the spermatozoa, while the processes of glycolysis and oxidation are increased, indicates an interference of the energy-coupling mechanism with the result that oxidation and glycolysis run rampant, while the energy is lost as heat rather than as work” (28).

During that same period we discovered that, unlike most substrates that increased both respiration and motility (29, 30), “β-hydroxybutyrate was unusual in that it depressed endogenous respiration slightly, but supported an excellent degree of motility. It is possible that the oxidation of this metabolite, in spermatozoa, is more efficiently coupled with phosphorylation than is the oxidation of the endogenous lipid reserve” (29). This explanation seems also to apply to the working heart (31) and may have therapeutic implications (32).

Nearly a century ago the eminent physiologist Jacques Loeb demonstrated that fertilization of sea urchin eggs does not occur in the absence of Ca\(^{2+}\) (33, 90). This failure is based on the need for Ca\(^{2+}\) to promote the lysis of the acrosomal membranes on the sperm head (acrosomal reaction) of both invertebrates (34) and vertebrates (35, 36).

We had been investigating the role of calcium transport in the regulation of sperm behavior, including the acrosome reaction and enhancement of motility for some years (35, 37, 91) when Donner Babcock found that the rapid uptake of calcium by bovine epididymal sperm did not occur in sperm separated from ejaculates (38). Epididymal sperm contain 6 ± 1 nmol of calcium/10^8 cells and will accumulate up to 50 nmol/10^8 sperm when incubated in a medium containing 0.2 mM calcium and an oxidizable energy source such as β-hydroxybutyrate (37, 38, 91). Ejaculated bovine sperm have the same low calcium content despite being bathed in 9 mM calcium in seminal fluid. When washed free of seminal fluid and suspended in media containing calcium, ejaculated sperm still do not take up this divalent cation. The obvious next experiment was to add seminal fluid to epididymal sperm, which demonstrated the presence of a calcium transport inhibitor that we termed caltrin. The inhibitor was purified to homo-
geneity (39) and the amino acid sequence was determined (Fig. 1) (40). A protein with the properties of caltrin was demonstrated to be bound to plasma membranes of ejaculated sperm and was not detected on the membranes of epididymal sperm (41). The sequence also disclosed that a similar protein, termed bovine seminal plasmin, had been isolated from bovine semen and was described as having antimicrobial activity (42). Errors in the sequencing of seminal plasmin (43) were later corrected (44), thus confirming our structure and the identity of seminal plasmin and caltrin. Analyses for bovine caltrin based on inhibition of calcium uptake by epididymal bull sperm indicated that bull seminal fluid contained about twice as much caltrin as was required to inhibit calcium uptake 90% by the sperm present in an ejaculate. On storage, caltrin lost activity as a blocker of calcium transport and became an enhancer of calcium uptake (45). This transformation could be accomplished rapidly by binding the fresh inhibitory caltrin to a cation exchanger. The recovered caltrin increased both the rate and extent of calcium uptake. The acidic components of the eluate contained ether-extractable phospholipids that restored calcium transport inhibition to the enhancer caltrin protein. Among the pure phospholipids tested only phosphatidylserine converted enhancer caltrin to a calcium transport inhibitor. Phosphatidylcholine, phosphatidylinositol, and citrate abolished the stimulation of calcium uptake but did not change the enhancer to an inhibitor (46).

With the aid of anti-bovine caltrin antiserum, two caltrin proteins were detected and isolated from guinea pig seminal vesicle secretions (47, 92). There were no common amino acid sequences in these peptides designated G.P. caltrins I and II, and the only identity with bovine caltrin was a group of four (Gly-Asn-Arg-Ser) near the carboxyl terminus of bovine caltrin and G.P. caltrin I (Fig. 1); nonetheless, the anti-bovine caltrin antiserum recognizes these guinea pig proteins. Both G.P. caltrins contain carbohydrate residues as detected with concanavalin A (47, 92); bovine caltrin does not (39). The molecular weights of the peptide portion of G.P. caltrins I and II are 5082 and 6255, respectively. The maximal inhibition of calcium uptake by each of the G.P. caltrins approached 50%. Deglycosylation of G.P. caltrins using trifluoromethanesulfonic acid caused both I and II to enhance the rate of calcium uptake by guinea pig epididymal sperm, i.e. they become enhancer caltrins (48).
The seminal vesicles of rats and mice contain caltrins that have been purified and sequenced (Fig. 1) (49). Their calculated molecular weights are 6217 and 8476, respectively. Rat caltrin is derived from a 54-kDa inactive precursor produced in the seminal vesicles (50); the biosynthesis of rat caltrin and its precursor is androgen-dependent (51). The active rat protein has a sequence of 13 amino acids nearly identical with a segment of G.P. caltrin I. Neither rat nor mouse caltrins have any significant sequence similarity to G.P. caltrin II or bovine caltrin. Each of the caltrins, with the exception of the bovine, contains cysteine residues that are not reactive with thiol reagents until the protein has been treated with reducing agents such as dithiothreitol. Reducing the cystine disulfide bonds of rat caltrin and carboxymethylating the protein diminishes, but does not eliminate, the effect on calcium transport. The locations of the disulfide bonds are known (52). In the case of mouse caltrin, which contains 7 cysteine residues, the protein appears to be a disulfide dimer formed between the odd cysteines. Reduction converts the 17-kDa native mouse caltrin to 8.5 kDa. Bovine caltrin also behaves as a dimer of $M_r$ 9600–10,500 by gel permeation and gel electrophoresis, but from its amino acid content we found a $M_r$ of 5411 and no cysteine.

FIG. 2. Bull sperm bind caltrin to the tail and over the acrosome. a, epididymal sperm treated with 0.40 mg of caltrin/10⁶ cells in 1 ml and washed. c, ejaculated sperm washed free of seminal fluid and not exposed to purified caltrin. Sperm were spread and dried on glass slides, treated in succession with rabbit monospecific caltrin antiserum and goat anti-rabbit IgG that had been labeled with fluorescein isothiocyanate. Slides were washed to remove excess protein and viewed with a Zeiss fluorescence microscope (a and c); b and d are corresponding phase contrast photomicrographs. Epididymal sperm not exposed to caltrin did not bind the fluorescent-labeled antibody.

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A caltrin protein designated SVS VII has been purified from mouse seminal vesicles by Yee-Hsiung Chen and co-workers (53). It has 76 amino acids, 71 of which are identical with our sequence for mouse caltrin (49). Luo et al. (53) determined the sequence of their protein from the corresponding cDNA and the first 18 amino acids by automated Edman degradation; they ascribe the differences to errors in our structure. Our sequence was determined by the Edman procedure applied to five different peptides isolated from caltrin subjected to partial proteolysis. Four of the peptides contained the amino acids in question; each of these fragments contained the same sequence. Therefore we are confident of our structural assignment and assume that there are genetic differences between CD-1 and Swiss white mice. Three of the differences can be explained by single base changes. The Chen group (54) reported another caltrin-like protein, P12, from mouse seminal vesicles with no appreciable sequence similarity to our mouse caltrin.

Bovine caltrin binds over the acrosome and the entire tail of bull sperm but does not bind to the posterior part of the head nor to the midpiece, which contains the mitochondria (45). Washed sperm separated from bull semen show the same fluorescence staining pattern as epididymal sperm treated with caltrin (Fig. 2), but epididymal sperm not exposed to caltrin do not bind the fluorescence-labeled antibody (45). Caltrin binding at these two sites was assumed to regulate the acrosome reaction and the hyperactivation of motility, respectively. These separate functions were clearly defined in the case of guinea pig sperm (see below).

The immunofluorescence test showed G.P. caltrin I binding to the G.P. sperm over the acrosome but not to other parts of the sperm (Fig. 3). Caltrin I (0.5 mg/10^8 sperm) nearly completely inhibited hyaluronidase release from the acrosome during 30 min of incubation in the presence of 1 mM calcium; many of these sperm were hyperactive but had intact acrosomes (48). Guinea pig epididymal sperm not treated with caltrins released hyaluronidase and were
hyperactive, indicating calcium access to both the acrosome and the tail. Guinea pig caltrin II (0.5 mg/10⁸ sperm) bound to the sperm tail and very sparingly to the head (Fig. 4). It depressed hyaluronidase release from the acrosome only partially, and during 2 h of incubation the sperm maintained linear motility because calcium access to the contractile mechanism was blocked (47, 92). These separate sites of binding and function account for the fact that each of the GP caltrins inhibits about 50% of the calcium uptake that occurs in the absence of these seminal vesicle proteins (48).

The “Eureka!” announcement (55) that “observations of differences in the effect of seminal plasma contamination on hyperactivation and capacitation made in the present study provide further evidence for the existence of functionally separated, distinct regions in the spermatozoon” in effect confirmed for the human sperm what had been demonstrated much earlier in guinea pig sperm (48).

Some years ago Tschesche et al. (56) reported the presence of proteolytic inhibitory proteins in mammalian seminal plasma, and their observation has been confirmed by several laboratory groups. Rat caltrin and guinea pig caltrin I are also trypsin inhibitors (52), and the former is identical with the inhibitors from the pancreas isolated by Uda et al. (57) and from liver by Kido et al. (58). There is extensive homology between rat caltrin and trypsin inhibitors isolated from pancreatic secretions of many different mammalian species. Guinea pig caltrin II, bovine, and mouse caltrins are not trypsin inhibitors (50). The gene for caltrin (seminal plasmin) has been characterized (59) and, remarkably, has been shown to be a member of the extensive neuropeptide Y gene family (60). Seminal plasmin/caltrin was the subject of an excellent review (61).

In summary, the role of caltrins in fertilization can be postulated from their known functions. On ejaculation, spermatozoa bind caltrins as well as other seminal vesicle products. Bound caltrins prevent calcium movement into the acrosome and thus prevent a premature acrosome reaction. The hydrolytic and proteolytic enzymes are retained until needed. By

![Fig. 4. Binding of guinea pig caltrin II to guinea pig epididymal sperm.](image)

A, immunofluorescence photomicrograph. Arrows designate caltrin binding to the sperm tails. B, corresponding phase-contrast photomicrograph. Reprinted with permission from Ref. 48.
preventing calcium uptake by the tail, caltrins keep the sperm moving forward. After some time the sperm will have moved up the female reproductive tract and encountered the egg(s); the phosphatidylserine will have been dissociated from the caltrin protein in the case of bovine and the carbohydrate residues hydrolytically removed in the case of rodents. The enhancer forms of caltrin then stimulate calcium uptake at the acrosome where it activates membrane discomposition and at the tail where it induces whiplash movement of the sperm tail. Species that produce two caltrins have one that acts at the acrosome and controls hyaluronidase and acrosin release; the other binds to the sperm tail and regulates motility.

Investigation of these proteins in our laboratory was dropped for lack of laboratory space on my reaching emeritus status in 1988 but is being continued by Carlos Coronel in Argentina.

**Dehydroepiandrosterone—A New Chapter**

The University of Wisconsin treats its retirees more hospitably than Columbia treated Professor Chargaff (62); I was allowed to retain a small laboratory, enough for chemistry but not for extensive metabolic research.

A problem that was inviting concerned possible active steroid hormones derived metabolically from dehydroepiandrosterone (DHEA). This steroid was known as an intermediate in the conversion of cholesterol to testosterone and estrogens. Administered in large amounts, it caused fat and weight loss in obese mice (63), rats, and dogs, decreased blood sugar in diabetic mice (64), decreased the incidence of spontaneous and carcinogen-induced tumors in mice (65),

![Steroid molecule diagram](image)
enhanced immune responses (66), and improved memory in old mice (67). We, like some others, assumed that DHEA was converted metabolically to more active steroids that exerted these beneficial effects. Beginning in the 1960s many investigators had studied the conversion of DHEA to other steroids by animals, humans, and tissue preparations, but only a few of the products were tested for any biological activity. We initiated a program of synthesizing derivatives of DHEA that were logical metabolites in the hope of finding one or more new hormones. Such a search requires an assay for biological activity, and our earlier research provided one. Administering extra thyroid hormone to rats induces the formation of mitochondrial glyceraldehyde phosphate dehydrogenase (GPDH) to 20 times the normal level in liver (15, 16) and somewhat less in other tissues (16). After Tagliaferro et al. (68) reported that DHEA enhanced metabolism and thermogenesis, we found this steroid induced the formation of hepatic GPDH but not that of other tissues (17, 69). Cytosolic malic enzyme is also increased by these hormones (70), and the response of these two enzymes to administered steroids thus provides a semiquantitative assay of activity. The two enzymes comprise a thermogenic system regulated by calcium and other factors (17, 18, 71, 72).

We found that hydroxylation of DHEA at any position other than 7 abolished the ability to increase the thermogenic enzymes (73). 7α-Hydroxy-, 7-oxo-, and 7β-hydroxy-DHEA were more active than DHEA. Because activity increased in that sequence we postulated that the same sequence was involved in converting DHEA to an active hormone. By incubating DHEA with liver homogenate fortified with ATP, NADPH, and malate and assaying the products at short time intervals, that sequence was indeed established (74) and is shown in Fig. 5. The detection and quantitative measurement of the many products formed from DHEA (Fig. 5, 1) by liver were possible because of the analytical prowess of Dr. Ashok Marwah (75–79). Several additional products remain to be identified including some glucuronides.

DHEA derivatives bearing oxo- or hydroxyl groups at position 7 do not serve as precursors of androgens or estrogens and therefore are potential therapeutic agents. 7-Oxo-DHEA had no detectable toxicity in rats (80) or monkeys (81) even in massive doses and in a phase I clinical trial was well tolerated by normal men given doses up to 200 mg/day for 28 days (82). Tested at that dose for 8 weeks in obese subjects (body mass index of 31.9 ± 6.2 kg/m²) who were restricted to 1800 calories/day, subjects receiving 7-oxo-DHEA lost significantly more body weight and fat than those receiving placebos (83). 7-Oxo-DHEA was far more effective than DHEA as an enhancer of memory in old mice and in restoring memory in mice treated with scopolamine (84). Androstenediol (Fig. 5, 6), one of the main products of DHEA metabolism in liver, has been known for many years to have estrogen activity. It also activates androgen receptor transcriptional activity in prostate cancer cells (85). This function is not inhibited by hydroxyflutamide or bicalutamide, two agents used for treating prostate cancer (86). This raises the question whether androstenediol, produced in adrenals and liver, accounts for the failure of orchidectomy to be an effective long term treatment for prostatic cancer. In a collaborative study, we have also found that DHEA has activity in adipose cells not displayed by its metabolites (87).

Structure/activity comparisons show that ring D of DHEA can be altered in several different ways without abolition of activity. The ring can be expanded by insertion of oxygen at 17α (88) with retention of the ability to induce the formation of both GPDH and malic enzyme. Hydroxylation at position 15 or introduction of 15–16 unsaturation, nearly abolishes the response of GPDH, but induction of malic enzyme is retained (89). Substitutions at position 16 yield steroids with varying activity; some are highly active and point the way to possible routes to true hormones. The search goes on.

Address correspondence to: halardy@facstaff.wisc.edu.

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