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COMMENTARY

PREGNENOLONE—FROM SELYE TO ALZHEIMER AND A MODEL OF THE PREGNENOLONE SULFATE BINDING SITE ON THE $GABA_A$ RECEPTOR

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Hans Selye from 1936 to 1976 literally created the field of neuroendocrinology based on insightful clinical observations about the relationships between the stresses of living and disease. His sweeping hypotheses were accompanied by ingenious experiments with rats in which he attempted to mimic pathology observed in human diseases believed to be caused by stress [1]. What a tribute it is to him that his ideas, made at a time of sparse knowledge, sparked so much work [see Refs. 2 and 3 for literature citations] that led to the discovery of substances (e.g. steroid anesthetics [4]) and phenomena that, while sometimes falsifying his original detailed speculations, illuminated the whole biological landscape! The overall pattern he discerned was correct (Fig. 1). The heuristic appeal of his proposals directly encouraged some younger scientists of his day, the author among them [5–7], to pause occasionally in their frenetic molecular-reductionist activities to think integratively about the organism as a whole. Those who, like Selye, make integrative efforts and face the critical barbs that inevitably follow may take solace from Einstein's words, "Man seeks to form for himself, in whatever manner suitable to him, a simplified and lucid image of the world, and so to overcome the world of experience by trying to replace it to some extent by this image."

Living systems are pattern-recognizing or generalizing entities, from single cells to complex human organizations. When a living system, whether unicellular or international, is presented with a novel informational pattern in its environment (external and internal), it is activated in a unique fashion. The types of impinging influences and their sequences, intensities, and rates of change result in an activation pattern that is likely to be different from any experienced previously. It is this change in pattern, not in a single variable in the environment, which is the stimulus (stress, pressure, forcing function) for a biological system.

Even in the resting state, all of the subunits of a particular living system participate in many mutually shaping interactions that range from physical forces they exert on each other to exchanges of varieties of trophic and/or inhibitory substances. These interactions are accelerated by increased activity, during which all relevant cellular components derive from each other and from available external sources supplies of the smorgasbord of factors they require optimally to modulate metabolic activities and extents of genomic transcription needed for adaptive responses to take place [5].

Consideration of inhibitory processes in the nervous system and of the roles of inhibition, in general, led to the following generalization [6]: *Living systems are tonically inhibited, autonomous optimizers, and disinhibition coupled to variability generation is the major organizing principle. Progressive disinhibition is coupled to increased variability generation in such a manner that the probability of making an optimally adaptive choice of a response option from among those available remains approximately constant over a wide range of increasing force parameters.*

It is surmised that increases in activities with increases in stimulus strength occur according to the principles of non-linear dynamics. It would be expected, according to the latter, that with progressively increasing stimulus strength, each living system considered and every subunit thereof show three characteristics of behavior: smooth, periodic (oscillatory), and turbulent (chaotic). With full participation of inhibition and relatively tight coupling between degrees of disinhibition and variability generation, the region of smooth flow (efficiently adaptive behavior) would extend over a much greater range of force parameter than in their absence. Variable and fluctuating combinations of the above types of behavior may be present in different regions of a particular complex system during the performance of adaptive behavior by the system as a whole.

In all living systems, dynamic tension exists between influences that maintain the relative freedom of interactions within them and those that tend to synchronize activities of their constituent subunits or to isolate them from each other functionally. The health and survival of cells, individuals, and nations

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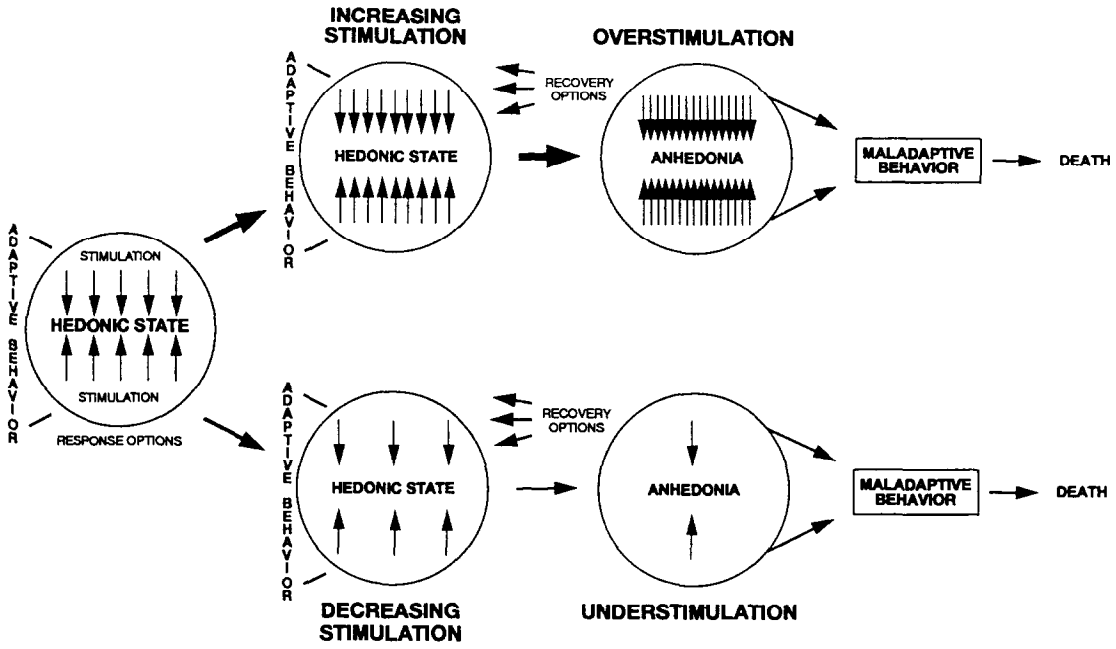


Fig. 1. The organism as an hedonic optimizer. I interpret Selye's ideas as follows: The organism is an hedonic optimizer. In its waking condition, it seeks the optimal state of well being, which in the human being is associated with comfort, pleasure, ease, satisfaction, and absence of anxiety and boredom. Displacement from such a state, the set points for the detection of which are variable, may result in feelings of anxiety, discomfort, boredom, or pain. Continual changes taking place in the external environment and in the internal metabolism (stress) generally act counter to the maintenance or achievement of an hedonic state and tend to displace the organism away from it. If the stimulus input is too weak or too great and complex for the organism to handle, maladaptive responses and pathological tissue changes take place that eventually could lead to the actual destruction of the organism. If the organism survives, return to an adaptive pattern may occur through new learning and/or simplifying or increasing the complexity of the environmental input. Intervention is possible with drugs, hormones, surgical techniques, and electroshock. Psychotherapeutic approaches may be effective, particularly those that disclose the existence and encourage the use of previously unused potentially adaptive behavioral options. The efficacy of any therapeutic approach must be evaluated at this level of organization.

often are precarious because the respective systems are walking tightropes between states of freedom without license, tyrannical authority, and anarchy. Sometimes it may take but little additional input to tip the balance in catastrophic fashion from one to another of these modes. The "cure" of a diseased or injured system is similar, in principle, to the restoration of a fallen acrobat to his tightrope.

At the outset of injury or disease, many cybernetic adjustments necessary for maintaining homeostatic integrity take place so that transactional states different from those found before the disturbance exist at cellular, tissue, and organismal levels. Recovery may take place or damage may progress until breakdown occurs, leading to progressive deterioration and finally irreversible and fatal loss of adaptive function.

When it is desired to improve suboptimal function of a living system or to accelerate repair of damage as a result of disease, injury, or aging, it is necessary to facilitate adaptive coupling among relevant processes by relieving rate-limiting constrictions in mutually shaping interactions among intracellular, intercellular, and extracellular components within an organism and between the organism and the external environment. The various steroids that abound in tissues and tissue fluids play key multifactorial roles in facilitating such coupling, from membrane to genomic expression. Although mechanisms of effects in particular instances largely have not yet been defined at the molecular level, the overall experimental results and clinical observations suggest the roles of steroids to be those of pleiotropic facilitators of coordinative processes that enable immune, neural, and metabolic systems, separately and together, to cycle freely through their operational modes in solving problems of survival and reproduction and in achieving rebalancing when malfunctioning occurs.

Non-genomic and genomic effects are exerted by the non-covalent association of steroids with sites on macromolecular entities, affecting their conformations and, therefore, their functional states. Individual substances to which steroids bind may have several potential binding sites with varying affinities for different steroids. At the genomic level, there is extensive overlap in binding site competition among substances that bind to various steroid

receptors as well as promiscuity in binding of different steroid–receptor complexes to the same DNA sequence. Modulation of some genes by steroids may involve combinatorial interactions. A continuum of binding specificities may exist from virtually complete promiscuity, such as is found in induction of the mammary tumor virus, to the extreme exclusivity of the induction of some secondary sex characteristics. A high degree of binding specificity of a particular steroid–receptor complex to a DNA region near a particular gene, the transcription of which it regulates in target tissues, might be conferred by the nearby binding of other transcriptional regulatory proteins, including other steroid–receptor complexes [8–14].

This commentary focuses only on a few aspects of the biology of one steroid, PREG*, in the context of the above formulation, with full awareness that the roles that may be ascribed to PREG are played out in environments in which many steroids coexist; and that at any time it is the pattern of distribution of these substances that helps determine regional functional states and behavior of the organism as a whole, and not just the level of one particular steroid.

Sketch of steroid metabolism

To serve for reference in what follows, in Fig. 2 is shown a skeletal outline of the steroid metabolic scheme as it may occur in the human organism as a whole. If one were to homogenize the *entire* organism and extract the homogenate appropriately, one could expect there to be the substances shown in Fig. 2 and many others derived from them, known and still unknown, and to be able to demonstrate the activities of enzymes that catalyze the required interconversions. However, it is highly unlikely that any single tissue or tissue fluid contains all of the substances and/or enzymes; and among those tissues in which they do exist, marked differences in levels would be found. All cells in the body probably require for regulation of their functions some or all of these steroids, whose combined activities range from modulation of membrane excitability to regulation of genomic transcription.

The biosynthesis of steroid hormones begins with cholesterol, from which the sex steroids, glucocorticoids, and mineralocorticoids all derive (Fig. 2). PREG is the major precursor for the steroid hormones. It is formed from cholesterol in mitochondria of tissues that produce steroid hormones. The rate of steroid synthesis is controlled by the delivery of cholesterol from cytoplasmic inclusion droplets to the inner mitochondrial membrane, where steroidogenesis begins by production of PREG from cholesterol by action of cytochrome P450scc (side-chain cleavage enzyme), a reaction found in peripheral tissues as well as in brain. Formation of PREG is regulated by pituitary hormones, such as luteinizing hormone and follicle-stimulating hormone in ovaries and testes, ACTH and one or more non-ACTH pituitary peptides in adrenals, and possibly hypothalamic hormones at the latter and other sites. PREG can go directly to progesterone and thence to aldosterone (route A, Fig. 2) or to 17α-OH PREG, which is a precursor for cortisol formation (route B, Fig. 2) and for sex-related steroids (route C, Fig. 2). Route A can contribute to route B and route B to route C, as shown. DHEA, the first product in route C, can inhibit the flow through routes B and C by inhibiting conversion of PREG to 17α-OH PREG. PREG is of major interest because it lies at the branchpoint at which decisions are made as to how the subsequent metabolic flow is fractionated between the mineralocorticoid, androgen → estrogen, and glucocorticoid pathways.

I eschew further discussion of steroid metabolism, in general, since reviews, books, and symposia on all aspects of the subject abound [see Refs. 16–23 and literature citations therein]. Special aspects will be discussed as the need arises.

The "charm" of PREG as a potential therapeutic agent

Long before any of the details of its metabolism had been worked out, PREG was being tested for effects in animals and in humans. Early on after its synthesis, PREG was tested in animals for estrogenic, progestational, and adrenal cortical activity with negative results. Selye's subsequent work with rats, performed only under unusual experimental conditions with high doses of PREG, made it possible to attribute a number of classical hormonal actions to PREG, none of them particularly impressive [24–26]. However, Selye's remarkably intuitive interpretation of his data and his suggested scheme for a possible route of biogenesis of the different types of hormonal steroids presaged current biochemical knowledge by several years: "It is very probable that the inability of earlier workers to detect these manifold activities of PREG was due to the fact that in most respects the compound is quantitatively not very potent. It distinguishes itself from other steroids, however, because it possesses so many different activities. Thus the compound possesses—at least in traces—every independent main pharmacological action which has hitherto been shown to be exhibited by any steroid hormone. In the light of these observations it was tempting to speculate on the possible role of the compound as an undifferentiated hormone-precursor from which the organism may—according to its needs—produce compounds in which one effect is particularly developed at the expense of other activities of the multipotent parent substance."

Since PREG now is known to be the precursor of all of the known steroid hormones, it is not surprising that a variety of typical hormonal effects is produced by administration of PREG. PREG may exert its own relatively specific effects, e.g. on $GABA_A$ and NMDA receptors (see below), as well as serving as precursor for the formation of a panoply of different steroids and steroid derivatives, of which probably

* Abbreviations: PREG, pregnenolone; PREG-S, pregnenolone sulfate; DHEA, dehydroepiandrosterone; GABA, γ-aminobutyric acid; NMDA, *N*-methyl-D-aspartate; AD, Alzheimer disease; MS, multiple sclerosis; NSAIDs, non-steroidal anti-inflammatory drugs; COX, cyclooxygenase; and HMGCoA, 3-hydroxy-3-methylglutaryl coenzyme A.

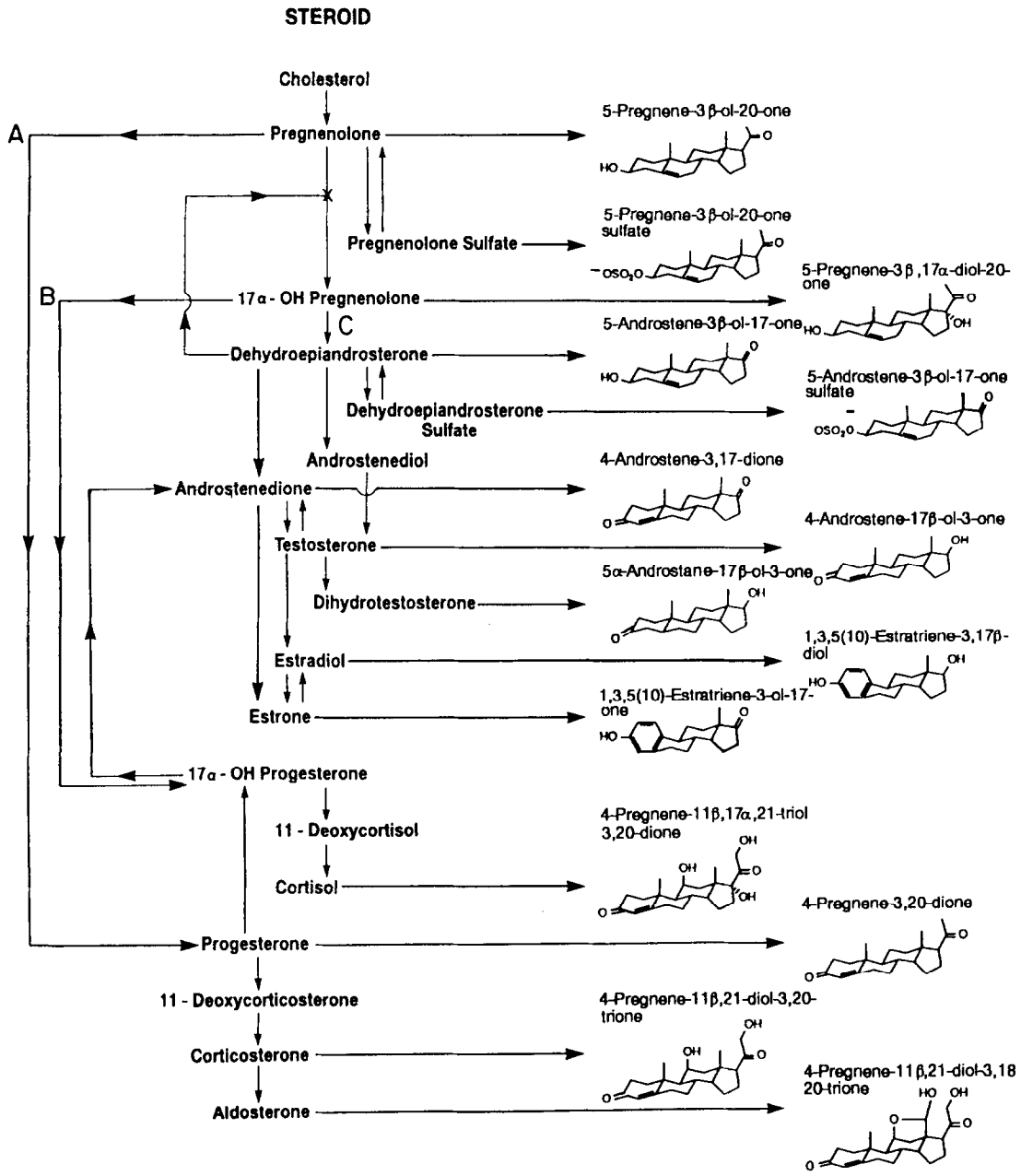


Fig. 2. Skeletal outline of steroid metabolism. Structural formulae are shown only for those substances that had been tested previously for effects on memory retention in mice [15]. Of the latter, all were memory-enhancers, with the exception of progesterone, estrone, and estradiol. A number of references may be consulted for orientation in the field of steroid metabolism [16–23].

only a small fraction are known. It may be imagined that PREG, the parent steroid, also can play synergic roles with other steroids at genomic and non-genomic sites, facilitating their actions in helper-like fashion through allosteric effects exerted by binding at different loci to the same entities. An adequate supply of locally synthesized and/or externally supplied PREG could ensure near-optimal modulation of non-genomic and genomic processes at sites at which enzyme activities exist for the subsequent conversion of PREG to tissue-specific patterns of steroids.

With aging, continued stress, or disease, decreases in amounts or incoordination in rates of formation of PREG and steroids arising from it can occur globally throughout the organism or locally at specific tissue sites, which may lead to failure of maintenance of optimal steroid patterns and, therefore, to decreased capacity to respond adaptively to demands of everyday life [27]. Current results [unpublished]

show that in normal individuals mean blood serum levels of PREG and PREG-S decrease with age, those of PREG-S showing a 60% reduction at a mean age of 75 years compared with the values observed at a mean age of 35 years.

Restoration of normal steroid patterns by administration of PREG alone or together with much smaller than currently employed amounts of other steroids is likely to be less physiologically disturbing than is administration of arbitrarily selected amounts of more potent substances that derive from it, e.g. cortisone, sex steroids, or aldosterone, because myriad feedback inhibitory loci exist in steroid formation beginning with the synthesis of PREG from cholesterol, which in different tissues may be under the control of different pituitary hormones, and because there exists widespread competition of steroids for binding to receptor and allosteric sites. For example, DHEA, the first substance in route C (Fig. 2), retards metabolite flow through routes B and C by inhibiting conversion of PREG to 17α-OH PREG. Results consistent with such an inhibition [27] are shown in data from two individuals with AD receiving a single oral dose of DHEA (Table 1). There were marked elevations in serum PREG and reductions in 17α-OH PREG after ingestion of DHEA, results consistent with inhibition of 17α hydroxylation of PREG by DHEA. The increased levels of 17α-OH PREG at 24 hr after DHEA ingestion indicate that a rebound synthesis and/or secretion occurred when the greatly elevated serum levels of DHEA and its sulfate, DHEA-S, at 1–2 hr returned to near-normal values at 24 hr (data not shown).

For as long as they have been known, androgens and estrogens have been administered to males and females, respectively, to retard one or another feature of aging or to achieve other therapeutic effects. Problems often arise that preclude prolonged use of these hormones. Endometrial bleeding, prostatic hypertrophy, and carcinogenesis are among the several danger signs along the road. Obviously, cybernetic control mechanisms can be overwhelmed by exogenously imposed hormonal thrusts. In some instances in which sex hormones are required, it might be better to give PREG and DHEA rather than to administer the sex hormones, themselves. DHEA is a normally occurring precursor of androgens, which in turn are precursors for estrogens. Upon penetration of DHEA and PREG to androgen- or estrogen- synthesizing sites in the various tissues, conditions existing at these sites would determine quantities and rates of androgen and estrogen synthesis. Presumably, the presence of PREG would allow smaller amounts of DHEA to be given to achieve a particular effect than without it, because PREG could serve as precursor of indigenous synthesis of DHEA as well as possibly play a helper role, as suggested above. Smaller quantities of DHEA would be less likely to inhibit synthesis of 17α-OH PREG in those tissue compartments that may utilize it mainly for glucocorticoid synthesis rather than for sex steroid biosynthesis (Fig. 2). By furnishing substances along both glucocorticoid and mineralocorticoid paths, PREG would help maintain a more balanced steroidal environment, minimizing disturbances in allosteric and transcriptional relationships that large excesses of particular sex steroids might bring about by competing with other steroids. In those instances in which desired therapeutic goals cannot be attained without actual administration of the sex steroids, themselves, co-administration of PREG with relatively small amounts of sex steroids might give the same physiological effects as would administration of larger amounts of the latter alone. This would minimize risk of feedback inhibition of formation and/or release of pituitary factors that play a role in steroid hormone synthesis and thus attenuate the consequent homeostatic disturbance that would occur upon cessation of administration of steroid or a reduction in dosage.

The strategy of co-administration of PREG with low levels of highly active steroids to achieve desired therapeutic goals otherwise attainable only with

Table 1. PREG and 17α-OH PREG levels in two male AD patients before and after a single preprandial dose of DHEA (400 mg)*

	Patient			
	67-years-old		75-years-old	
Time (hr)	PREG (ng/mL)	17α-OH PREG (ng/mL)	PREG (ng/mL)	17α-OH PREG (ng/mL)
0	0.35	0.37	0.33	0.25
1	0.81	0.20	2.20	0
2	1.25	0.19	2.01	0.22
3	2.21	0.39		
6	1.83	0†	2.18	0
12	0.73	0	1.58	0
24	0.54	1.30	0.74	0.53

* Data from Ref. 27. Means and ranges, for population control values derived from sera of 50 healthy individuals of both sexes, 20–50 years of age: PREG, 1.11 (0.46 to 2.25); 17α-OH PREG, 1.24 (0.53 to 3.57).

† Not detectable.

much higher levels of the latter alone could be extended to treatments with glucocorticoids and mineralocorticoids.

Studies with PREG in humans

Fatigue and endurance. ". . . it appears that marked variations in the abilities of men to withstand fatiguing ordeals is related to their adrenal cortical functions and it was natural to ask if the administration of suitable steroids might increase one's ability to withstand the type of measurable stress we were investigating. We have accordingly tested several steroid substances and have found one in particular, Δ^5-PREG, a synthetic compound supplied to us by the Schering Corporation, that has improved and prolonged scoring ability on our pursuit meters without having any deleterious after affects." Thus began the study of PREG in humans [28–31].

Below is cited a summary [32] of the remarkable series of meticulously controlled studies on fatigue and performance by Pincus, Hoagland, and co-workers. These seminal and potentially clinically useful discoveries have lain fallow and unmentioned for 50 years, while relatively little has been done to alleviate problems of chronic fatigue that plague so many.

"Hoagland showed that fatigue, produced by a standardized method which simulated the operation of an airplane in flight, was objectively measurable in terms of the urinary elimination of 17-ketosteroids. The latter could be correlated with the number of errors made in tests, with time actually spent in the air, or with anoxia, in that the more stressed activity was reflected in a higher excretion of 17-ketosteroids. Fatigue was found to induce an increase in 17-ketosteroid excretion which could be plotted on a line of given slope. When PREG, 50 mg daily by mouth, was administered, the slope of this plot was reduced to one half its original value. Adrenal cortical extracts and progesterone were not found to have this effect. PREG was further described as improving the scores obtained in test runs, and as leading to a diminution in the feeling of fatigue.

Pincus and Hoagland trained 5 college students to constant performance on a fatiguing-machine, and then studied the effect of oral adrenal cortical hormone, progesterone, by injection, and PREG by mouth. In three-hour runs, only PREG appeared to show a significant influence on the scores. Using a target meter (an automatically-scoring device operated like the stick of an airplane), 14 subjects were tested under treatment with 50 mg of PREG per day, orally, in an elaborate scheme of deceptions calculated to rule out suggestibility. It was concluded from the scores that PREG had a cumulative effect during two weeks of administration, persisting several days afterward, consisting of an obvious improvement in performance. Seven of the subjects were pilots concurrently engaged in active flying: some of these volunteered the information that they felt better in their work, and some appeared able to distinguish positively when the placebo was being substituted. These investigators then administered 25 mg to 75 mg of PREG daily by mouth to 8 leather cutters, 12 lathe operators, and 77 optical workers, using as criteria of effect their productivity, wastage of material and perfection of the finished product. Some subjective responses were noted, consisting of a feeling of well-being, a tendency to tire less easily, and a sense of being better able to cope with the job. As to productivity, no effect was seen when the work was unhurried and unstressed (e.g., leather cutting) or when the worker had little enthusiasm for what he was doing (e.g., young men about to be conscripted); but in 2 groups of optical workers, a clear-cut improvement was evident with 75 mg, less with 25 mg, and none with the placebo. The effect outlasted the treatment. The least noticeable effect was observed in a department working at leisure, i.e., not 'under pressure.' "

Without being aware of the work cited above, recently two groups independently found in short-term open studies that oral ingestion of DHEA, which is formed from PREG (Fig. 2), decreased fatigability and generally improved the quality of life in a substantial proportion of patients of both sexes with MS without there being improvement in neurological assessments. One of the studies [33] reported such positive effects in 3 of 9 female patients and 7 of 12 male patients receiving 90 mg/day (approx. 1.3 mg/kg/day) for 14 weeks, which effects were not increased when the dose was doubled for an additional 14 weeks and which disappeared gradually after cessation of DHEA intake. The other study [34] achieved similar results in 9 of 14 MS patients ingesting huge amounts of DHEA (40 mg/kg/day) for 3 months. There was some evidence in both studies of incipient virilization in a few female patients.

The above preliminary results warrant the mounting of much more sophisticated controlled studies in larger numbers of patients for longer periods of time, particularly with a view to determining whether or not the progressively downhill course of MS can be attenuated with DHEA. More importantly I raise the question as to whether or not the anti-fatigue effects of PREG cited above are attributable to the action of PREG alone, to a combination of effects of the PREG administered and the DHEA formed from it, or solely to the effects of the DHEA formed. In this light, it is suggested that potential long-term virilizing side-effects of DHEA in women might be obviated by the administration of PREG alone, if PREG should prove to be as effective as DHEA. It has been stated [32] that ". . . pregnenolone will also reduce the excessive ketosteroid excretion of pathologic origin which occurs in virilism in woman." A combination of PREG and small doses of DHEA might show a synergistic effect in relieving fatigue in MS without virilization.

Above all, attention is called to the need for further study of the anti-fatigue effects of both PREG and DHEA. The interaction found between anti-fatigue effects and motivational state with PREG suggests that development of therapies for chronic fatigue syndromes might combine PREG and/or DHEA administration with increasing motivation.

Importantly, no toxic effects were observed in experimental animals following large doses of PREG

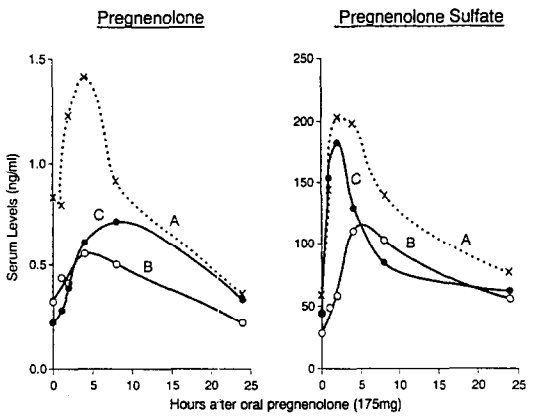


Fig. 3. Serum levels of PREG and PREG-S before and after preprandial oral ingestion of a capsule containing 175 mg of PREG. Oral administration of PREG produced rapid and large increases in serum levels of PREG and PREG-S above fasted control levels after oral ingestion of a capsule containing 175 mg of PREG (A, 35-year-old female; B and C, 45- and 46-year-old males, respectively). Even at 24 hr after ingestion, the serum levels of PREG-S were still elevated above control levels in all three individuals. PREG levels, on the average only 1.7% of PREG-S levels, were elevated in two instances and reached peak values *after* the peak values of PREG-S values had been attained and in one instance at the same time. In two instances, PREG levels were lower than controls at 24 hr. Taken together, the data are congruent with the interpretation that orally ingested PREG is converted to PREG-S in the intestine, which is rich in steroid sulfate-conjugating enzyme activity, and that PREG-S is absorbed rapidly into the blood. Various tissues and blood cells take up PREG-S and use it as such or convert it to PREG by widely occurring, tissue-specific steroid sulfate-splitting enzymes. A small amount of the PREG that is formed from PREG-S enters the bloodstream [35–38]. The prolonged elevation of blood PREG-S by comparison with PREG is attributable to the fact that the latter is cleared about 3 times more quickly than PREG-S in both humans and rabbits [39].

by oral, subcutaneous, or intraperitoneal routes [32]. PREG also was found to be without observable toxicity in humans when given parentally or orally in a number of older studies [e.g. see Refs. 28–32]. The lack of toxicity in humans has been confirmed in a current study with Alzheimer patients on daily oral intake of 525 mg for 3 months; and orally administered PREG has been shown to be readily absorbed (Fig. 3).*

Rheumatoid arthritis and inflammation. The following citation in Ref. 32 of unpublished observations made long ago gives an account of how PREG came to be tested in rheumatoid arthritis after the publication of reports of its anti-fatigue effects [28–31].

"Lansbury was the first to suggest the use of PREG in the treatment of rheumatoid arthritis in December 1946, and gave three reasons for concluding that it might be useful: 1, The well marked and often prolonged fatigue which precedes and accompanies the disease. 2. The recently established relationship between the adrenal cortex and the immunity mechanism of the body. 3. Some data which I have obtained on patients by using crude extracts of the whole adrenal cortex."

In March of 1947 Lansbury reported in a private communication [32] the following effects of the oral administration of PREG: "In three cases of rheumatoid arthritis there was subjective improvement with less fatigue, a gain in strength, less pain, and in two cases an actual measurable reduction in swelling. In one case there was relief of a constant dull pain in the eyes which had been present for many months and a relief of nervousness and an increased ability to sleep so that sedation could be discontinued. In considering these effects I feel that there is a suggestive lead here. There is no evidence that PREG is curative but I think further trial is justified. It seems to me, as I think over the cases, that the fundamental thing which has occurred is a lessening of swelling possibly due to alteration of permeability of the cell membranes. If this is true, then PREG might be of use in many conditions where swelling or slowed up transmission of fluid across membranes is a factor."

A number of subsequent studies with many patients reported between 1949 and 1951 [32, 40–46] confirmed that PREG, indeed, possessed anti-arthritic effects. It was optimally effective on daily oral ingestion of approximately 500 mg. Daily intramuscular injection of 50–150 mg of PREG acetate in oil to patients with ankylosing spondylarthritis resulted in "a striking relief of subjective and objective evidences of arthritis, including pain, muscle spasm, spasm, and limitation of motion." In a limited number of cases, PREG showed promise in the treatment of lupus erythematosus, malignant exophthalmos, scleroderma, and psoriasis. PREG was effective chiefly in patients at earlier stages of rheumatoid arthritis in whom the pathologic processes had not progressed too far and inflammatory processes were clearly evident. PREG was ineffective in treatment of osteoarthritis, in which little inflammation existed. It also was ineffective in treatment of Addison's disease, showing that its anti-inflammatory effect could not be attributable entirely, or possibly even in part, to adrenal production of cortisone from it. This did not preclude, however, local production of cortisone from PREG in cells at sites of inflammation.

When improvement was noted with PREG, it lasted much longer after cessation of treatment than that following cessation of treatment with cortisone or ACTH. In some instances, PREG prolonged remissions produced by cortisone treatment after cessation of the latter. Treatment with PREG can be maintained indefinitely without apparent harmful effects and is much less expensive than with ACTH or cortisone or with other anti-inflammatory steroids. Its use in patients is simple because it is absorbed rapidly when ingested in ordinary gelatin capsules.

Dissection of events occurring during inflammatory processes and the recent development of more specific and less toxic NSAIDs than hitherto available offer opportunities to extend knowledge of the mechanism of action of PREG and its potential

* Roberts E, Raum WJ and Miller BL, manuscript in preparation.

clinical utility [47–51]. Until recently some confusion existed in the devisal and use of NSAIDs because in almost all instances a balance had to be sought between the desired therapeutic effects and undesirable side-effects, e.g. stomach ulcers and renal damage.

Normal activity or injury leads to activation in tissues of membrane-located hydrolyzing enzymes that liberate free fatty acids from phospholipids, among which is the polyunsaturated fatty acid, arachidonic acid, from which in turn are formed and released prostaglandins, prostacyclin, and thromboxanes. Prostacyclin is antithrombogenic when released by endothelial cells and cytoprotective in gastric mucosa, in which latter tissues it is produced by a constitutive cyclooxygenase (COX1). However, another form of cyclooxygenase (COX2) is induced by cytokines released upon tissue injury and by actions of various exogenous immunostimulants upon interaction with receptors on blood monocytes and macrophages resulting in release from them of a variety of inflammatory arachidonic acid metabolites, e.g. eicosanoids and thromboxane A_2, as well as other substances that are pro-inflammatory, such as nitric oxide, interleukin-6, proteases, and reactive oxygen intermediates. Although the cascade of events leading from activation to release of inflammatory cocktails can be interrupted pharmacologically at several points, the best single strategy might be to nip it in the bud, so to speak, by blocking the induced prostanoid release by inhibiting *de novo* synthesis of COX2. This can be achieved by administration of relatively large amounts of glucocorticoids, e.g. dexamethasone, which, however, pose potent dangers of their own upon prolonged use.

The earlier work on the anti-inflammatory effects of PREG in rheumatoid arthritis cited previously and the likelihood that these effects may be only partly attributable to formation of cortisone from PREG suggest the possibility that PREG, itself, may play an important role in modulation of transcriptional and/or translational events in the formation of COX2. Examination of this possibility with techniques of modern cell biology would be a worthwhile endeavor and might help make public the hitherto relatively private molecular life of PREG.

Opportunities exist for improvement of *therapies for rheumatoid arthritis and other conditions in which inflammatory processes may play an initiating role in pathogenesis, including Alzheimer disease (see below)*. Clinical trials are just now beginning in patients with rheumatoid arthritis receiving BF389, a potent, non-toxic orally administered COX2 inhibitor [48–51]. PREG given orally with BF389 or with a substance with similar properties might exert powerful synergistic anti-inflammatory effects, the combination possibly simultaneously imposing rate-limiting constrictions on the production of COX2 and on its catalytic action. PREG and BF389 are essentially non-toxic, can be taken orally, and are readily absorbed. If, in some instances, it should be found that cortisone still is therapeutically necessary, amounts far below toxic levels could be used.

Alzheimer disease. Because most often AD occurs coextensively with aging, many pathological features of decyberneticization are shared. However, AD cannot be considered in the same category as usual attritional types of aging because, in addition to the general types of cerebral aging, changes, such as accumulation of lipofuscin, loss of neurons and shrinkage of dendritic trees, microvascular abnormalities, and gliosis, there are some special features consisting of occurrence of neurofibrillary tangles, neuritic (senile) and amyloid plaques, and granulovacuolar degeneration. The problems in AD are particularly difficult because they relate not only to molecular and cellular properties of the brain but also to emergent properties of the whole system, such as memory, cognition, and participation in everyday life.

Because such complex changes are found in brains of individuals with AD, it has been difficult to identify a specific initiating process that could lead to the development of curative or attenuative therapeutic modalities. However, the first description by Alzheimer and his students of the neuropathology in the disease that now bears his name and much work in recent years have suggested that continuing, low-grade inflammatory processes, however provoked (viral or bacterial infections, deposition of aluminum silicate or amyloid, injuries to the head, etc.), may be initiators, in some instances, of a characteristic self-perpetuating progression of degenerative processes, while preventing effective self-repair from taking place [see literature citations in Ref. 52].

Based on the inflammatory hypothesis, prednisolone (10 mg/day) was tested on 4 patients with AD and found to cause severe behavioral problems [53], a finding not surprising in view of the known hippocampal neurotoxicity of glucocorticoids [54, 55]. In a preliminary trial with the NSAID indomethacin, individuals receiving 100–150 mg/day over a 6-month period showed no decline in performance in a battery of 4 cognitive tests, while a matched placebo-treated group showed sufficient cognitive decline during this period for there to be a significant difference between the two groups [53]. Expectedly, the study was plagued by the occurrence of side-effects, largely of a gastrointestinal nature. Whatever else it may do, indomethacin is a powerful inhibitor of COX1 [49], the action of which gives substances necessary for maintenance of gastric mucosa, while only weakly inhibiting COX2, which produces substances that cause inflammation. When indomethacin is given in doses necessary to prevent inflammation, the gastric mucosa is at risk of damage. Substances with more favorable relative efficacies on COX1 and COX2 may prove to be more effective in AD and less toxic than indomethacin.

Based on its reported anti-inflammatory activity in rheumatoid arthritis [32, 40–46], lack of toxicity and natural occurrence, ready absorption when taken orally (Fig. 3), potential alerting effects [28–31] and remarkable memory-enhancing effects [15], PREG is being tested in a double-blind crossover study in 20 patients with AD receiving oral doses of 525 mg/day during the drug period, a dose that was reported to produce optimal improvement in symptoms in responsive patients with rheumatoid

arthritis. Although the code has not yet been broken, it already is apparent that there has been no toxicity, whatsoever, in those patients who have finished both drug and placebo periods.

As matters stand today, therapeutic expectations in AD should be modest. Early diagnosis and subsequent attenuation of progression of the disease are worthy goals. Many studies performed to date have been designed to identify curative agents. Attenuation of the disease seems like a more realistic goal. PREG and NSAIDs specific for COX2, given alone or together, may be useful in attenuating the progression of AD, particularly after early diagnosis.

Cholesterol deficiency in brain and in cranial and peripheral nerves. Levels of PREG many times higher than in plasma are found in human brain and in cranial and peripheral nerves [56, 57], at which sites PREG is present in oligodendrocytes and Schwann cells [58], respectively, and where it is formed independently of the peripheral endocrine system. Cholesterol used for PREG synthesis may come either from preformed cholesterol delivered to these sites or may be furnished by indigenous synthesis of cholesterol from acetyl coenzyme A [59–64]. Ordinarily, facile alterations might be made in rates of flow of cholesterol to sites of PREG synthesis through adjustments in rates of transport and biosynthetic pathways so that an adequate supply of precursor is maintained. However, in aging, injury, and in other conditions, it could be that either one or both sources of precursor supply could become rate-limiting, and the ability to form and release PREG and substances formed from it could become compromised. If both lines of supply of cholesterol were constricted, as would occur in the case of treatment with blood cholesterol-lowering drugs that act by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis, inability to synthesize PREG and its derivatives in nerve tissue and to release them at a sufficiently rapid rate could seriously handicap whatever functions they regulate or modulate, and adverse effects might occur in individuals taking such drugs.

In the latter regard, studies have shown that treatments with HMGCoA reductase inhibitors, while ameliorating cardiac symptoms, actually have resulted in increases in non-cardiac deaths, including cancer and nervous system-related phenomena, such as accidents, suicides, and violence [65; see also 66]. Perhaps administration of PREG together with cholesterol-lowering drugs might prevent such undesirable effects from occurring. Detailed evaluation of *brain* steroid metabolism, cognitive function, and behavior in patients receiving such drugs for prolonged periods is recommended.

When lovastatin was given to young beagle dogs in amounts up to 180 times the maximum therapeutic dose in humans, there was clinical evidence of neurotoxicity in 37% of the animals given the drug for 11 days or longer [67]. Endothelial degeneration and hemorrhagic encephalopathy were observed in the CNS, and axonal degeneration with neuronal chromatolysis was found in the optic system. No gross evidence of nervous system damage was observed in dogs maintained for 2 years on lower doses of the drug. However, sensitive behavioral measurements were not reported.

PREG and PREG-S and the GABA and NMDA neurotransmitter systems

Inhibitory projection and local-circuit neurons play crucial roles in information processing in the nervous system [68]. They are the elements of the nervous system that prevent its lapse into a tyrannical synchronic state of paroxysmal discharge, total inactivity, or chaos by adjusting the timing, sensitivity, and versatility of the processes by which information is received, interpreted, and acted upon. They determine in neuronal assemblies at any particular moment which neurons shall act as groups and which shall act alone, and the frequencies and sequences of these activities. Adequate function of the GABA-releasing system of neurons, the major inhibitory system in the vertebrate nervous system, is essential for maintenance of appropriate information-processing states in various functional modes.

The nervous system is highly restrained, inhibitory neurons acting like reins that serve to keep the neuronal "horses" from running wild. In coherent behavioral sequences, innate or learned, pre-programmed circuits are *released* to function at various rates and in various combinations. This is accomplished largely by *disinhibition* of pacemaker neurons whose activities are under the dual tonic inhibitory controls of local circuit GABAergic neurons and of GABAergic projection neurons coming from neural command centers. According to this view, disinhibition is permissive, and excitatory input to pacemaker neurons has mainly a modulatory role. Disinhibition, acting in conjunction with intrinsic pacemaker activity and often with modulatory excitatory input, is the major organizing principle in nervous system function.

By acting on a particular class of receptors ($GABA_A$), GABA produces enhanced permeability to Cl^- that is measured as an increase in membrane conductance. GABA also produces increases in K^+ conductance by action on another distinct class of receptors ($GABA_B$) that are not colocalized with $GABA_A$ receptors. In general, neural inhibitory mechanisms act like chemical voltage clamps, accelerating the rate of return to the resting potential of all depolarized membrane segments which the transmitter contacts and stabilizing underpolarized membrane segments by decreasing their sensitivity to depolarizing influences. The most commonly observed inhibitory action of GABA is dependent on extracellular/intracellular Cl^- ratios in the usual physiological range. If the ratio should become sufficiently low, GABA could act as an excitatory or depolarizing transmitter. Coordinate enhancement occurs in GABAergic inhibitory function with progressive acidification because GABA formation and its anion channel-opening efficacy are increased, while its metabolic destruction by transamination and removal by transport are decreased. Diminution occurs upon alkalinization. On the contrary, acidification decreases post-synaptic efficacy of glutamate, the major excitatory neurotransmitter [68].

In the above manner the delicate balance between excitation and inhibition in the brain is maintained

within the adaptive range in response to local or global activity that acidifies the environment in which it occurs. Accelerated metabolism following nerve activity results in accelerated formation of CO_2 and lactic acid, the accompanying acidification applying physiological "brakes", so to speak, slowing down neural activity while recovery takes place. This keeps the system from "overheating", thereby preventing structural and functional damage from taking place. When GABAergic-glutamatergic relations are unbalanced by glutamatergic overactivity, seizures may occur. Overbalancing in favor of the GABA system can lead to maladaptive decrement in neural activity, serious behavioral disorders [69], and even to coma. The properties of the GABA system make it eminently suitable to guide the brain to function in a "civilized" manner. The Yin–Yang between the glutamatergic excitatory and GABAergic inhibitory systems is delicately balanced.

PREG in the brain exists in the free form and as sulfate, sulfolipid, and fatty acid esters [21]. PREG-S is both a negative modulator of the GABA receptor complex and a positive modulator of the NMDA excitatory receptor complex [70–76]. The molar potencies of PREG-S in *in vitro* measurements made individually on one or the other of the above receptor systems seem too low to suggest an important *in vivo* role for PREG-S. However, by affecting both neurotransmitter systems as described above when liberated into synaptic regions, PREG-S could exert a remarkable synergistic amplification of excitatory transmission at much lower concentrations than would be expected from effects of PREG on either the inhibitory or excitatory system alone. This might help explain the powerful effect of PREG-S in enhancing in mice retention of footshock active training, significant effects being observed on post-training intracerebroventricular administration of 3.5 fmol/mouse [15]. Remarkably, only 100–1000 molecules of PREG-S was required to give enhancement in recent studies employing regional intracerebral injection [manuscript in preparation]. Since PREG-S greatly enhances Ca^{2+} entry during stimulation of NMDA receptors [77], an increased release of consequent cascades of reactions required to achieve the plastic changes associated with memory could account for the amplificatory effect of PREG-S. It would be expected that under the proper circumstances similar effects on memory could be attained in humans by oral, intravenous, or, if required, intrathecal or intracranial [78] administration of appropriate amounts of PREG-S. However, to achieve such effects, too high a level of PREG-S must be avoided (see below).

Dual effects have been observed in many studies of memory enhancement by excitatory substances. In the effective dose ranges, progressively increasing doses first increased responses to a maximum, beyond which decreasing responses were observed until a dose was reached at which no significant effects were seen over the controls. A compendium of results in the literature showed that this phenomenon was observed for "27 memory-enhancing compounds given by seven routes of administration to six organisms in 15 laboratories" [79]. Although PREG-S has memory-enhancing effects in mice over a wide lower dose range, depending on brain area injected it loses effectiveness at levels between 10^{-14} and 10^{-17} moles/mouse. The oral dose currently being used in patients with AD, 525 mg/day, while possibly effective in ameliorating symptoms of rheumatoid arthritis through its peripheral effects, may be too high for memory enhancement. That much lower oral doses of PREG can exert CNS effects was shown in a study in which 1 mg of PREG given orally before sleep to male volunteers significantly increased the amount of time spent in slow wave sleep and depressed EEG sigma power [80].

A model for the PREG-S binding site on the $GABA_A$ receptor

It is time that the large number of interesting phenomenological observations being made with PREG and PREG-S be given form by knowledge at the molecular level of the nature of their interactions with macromolecular cellular constituents. Knowledge at the molecular level of the exact mechanism of action of GABA on $GABA_A$ receptors that results in the opening of ion channels will require kinetic measurements by stopped-flow rapid mixing techniques, complete sequence determination of the proteins involved, high-resolution crystallographic and NMR characterization of relevant constituents, and detailed analysis of their associations and interactions under physiological conditions. Although there now exist extensive sequence data on at least 15 $GABA_A$ receptor subtypes, present information

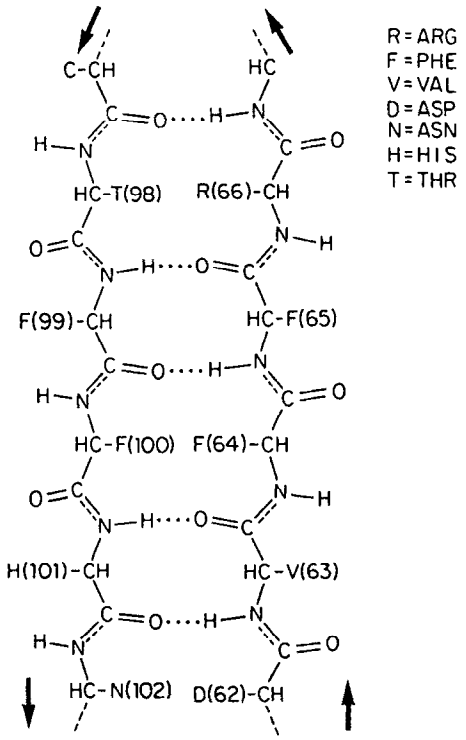
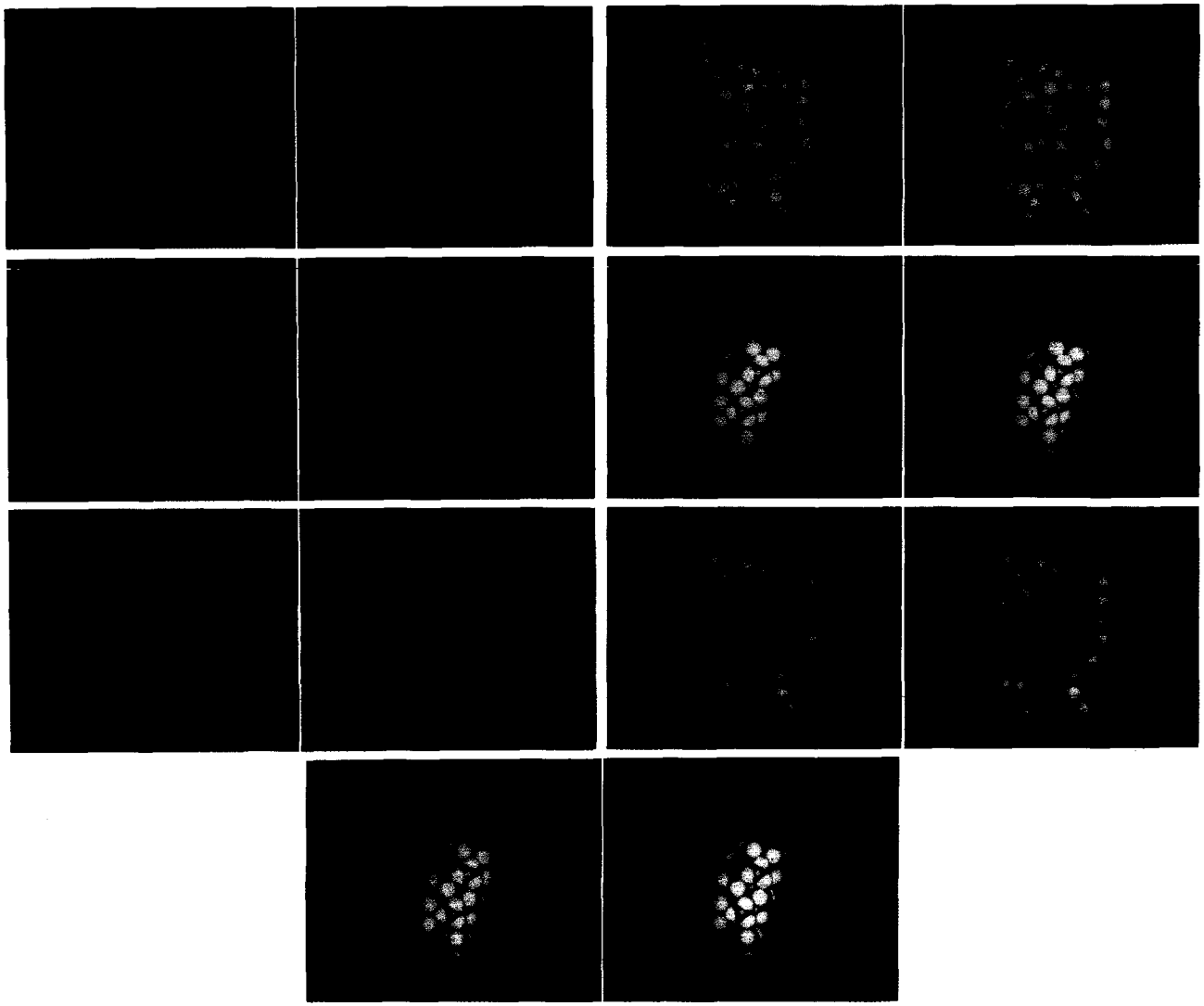


Fig. 4. Anti-parallel β-sheet formed from residues 62–66 and 98–102 of the α_1 subunit of mouse brain $GABA_A$ receptor.



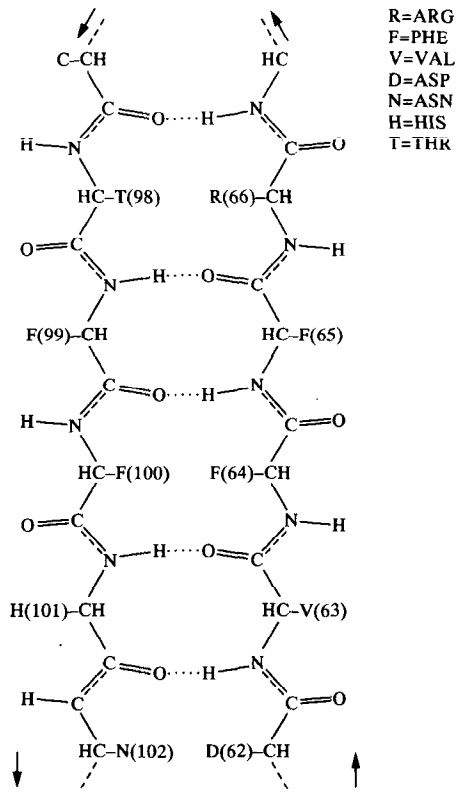


Fig. 5. Docking of conformer of PREG-S with a computer-generated model of a binding site formed by the amino acid residues on one side of the anti-parallel β-sheet described in Fig. 4. Dreiding (1a) and space-filling (Ab) models of the proposed receptor surface, respectively. Dreiding (2a) and space-filling (2b) models of PREG-S, respectively. Superimposition of Dreiding model of PREG-S on that of the proposed receptor surface (3a) and similarly of the space-filling models (3b and 3c). Atoms are color coded as follows: carbon, grey; oxygen, red; nitrogen, blue; hydrogen, white; and sulfur, yellow.

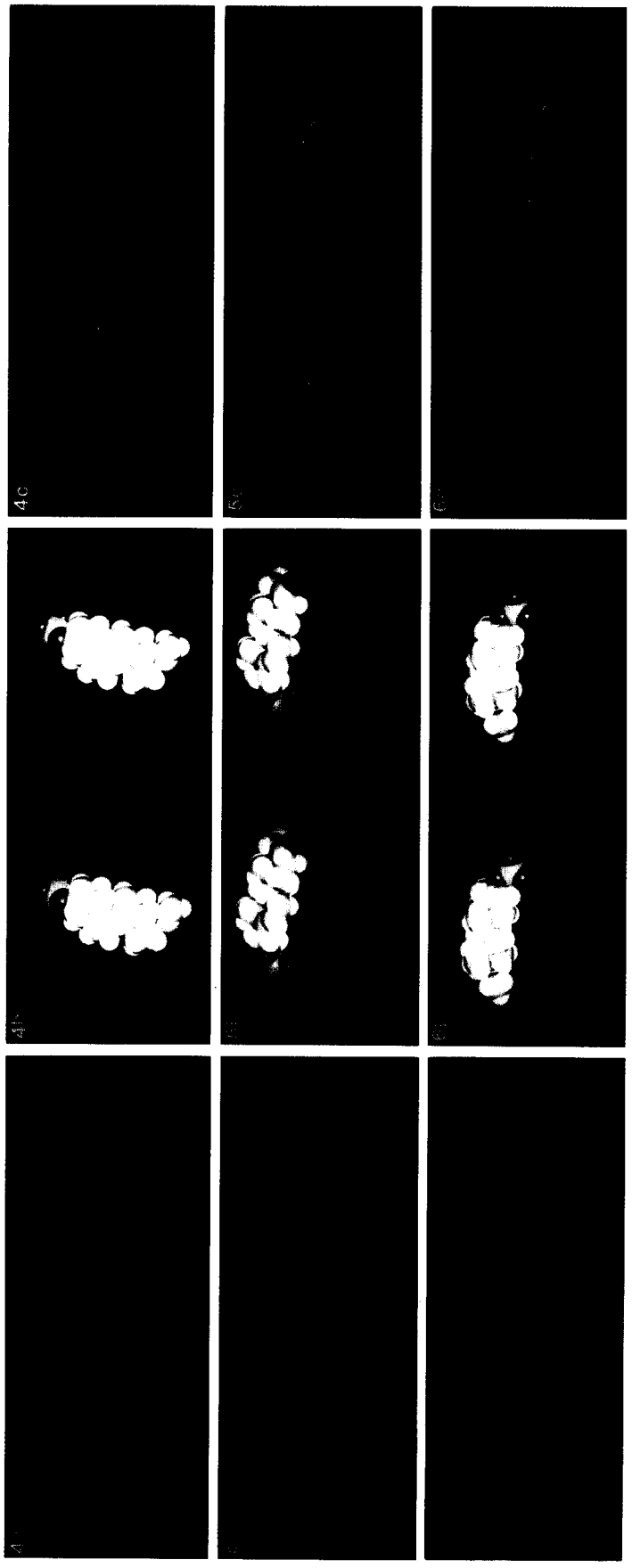


Fig. 6. Docking of conformer of PREG-S with the computer-generated model of a topographic representation of the proposed PREG-S binding site shown in Fig. 5. Topographic map of the PREG-S binding surface viewed from above and from left and right sides, respectively, with (4a–6a) and without (4c–6c) a Dreiding representation. The fit of the space-filling model of PREG-S to the map from above and from left and right side, respectively, is shown in 4b–6b.

has been insufficient to construct detailed molecular models of the pockets into which GABA fits to initiate the conformational changes that result in anion channel opening or onto which GABA mimetics, GABA inhibitors, or allosteric modulators might fit [81]. A particular difficulty in actively seeking to locate pockets into which GABA, muscimol, or other simple ligands might fit has been that the molecules are too small and simple to give sufficient specific information to search effectively among the alternatives existing at sites on various conformations of the receptor protein. The biochemical, electrophysiological and behavioral evidence that PREG-S possesses strong GABA-antagonistic properties as well as its large size and rigid structure recommended it as possibly an effective probe.

We focused on two specific small non-contiguous sequences of the N-terminal hydrophilic region of the α_1 subunit of the $GABA_A$ receptor that contain a Phe residue at position 64 [82] and His at position 101 [83], respectively. When Leu is substituted for Phe 64, the apparent affinity for GABA-dependent channel gating is greatly decreased [82]. When His 101 is replaced by Arg, affinity for benzodiazepines is greatly diminished [83]. Work with Corey-Pauling-Koltun (CPK) models of peptide sequences containing the above residues indicated that a particular apposition of them in an anti-parallel β-sheet structure (Fig. 4) would give a surface made up of alternating residues from the two sequences that could accommodate a large hydrophobic entity and would generally create an appropriate site for attachment of PREG-S. Computer modelling then was used to more accurately create a hypothetical binding site for PREG-S using the apposed sequences shown in Fig. 4. Two short segments of anti-parallel β-pleated sheet were built using standard peptide geometries. The receptor model was then energy minimized with constraints [84] to optimize β-sheet geometry. PREG-S was likewise built and then docked onto the hypothetical receptor surface. Another round of energy minimization then was used to optimize intermolecular interactions. Images were created using molecular modeling software marketed by Biosym, Inc. (San Diego, CA). Solvent accessible surfaces were calculated using the algorithm of Connolly [85]. The results of the modeling are shown in crossed stereo in Figs. 5 and 6, which can be viewed in three dimensions with an appropriate viewer (e.g. Taylor-Merchant Stereopticon 707).

In Fig. 5-1a and 1b, respectively, are shown the Dreiding and space-filling models of the surface of the proposed PREG-S binding site of the α_1 subunit of the $GABA_A$ receptor. Phe 64 and Phe 100 form an hydrophobic surface with which the hydrophobic core of PREG-S can associate. The cationic guanidino group of Arg 66 and the H-bonding hydroxyl group of Thr 98 lie opposite each other in such a manner that the anionic sulfate group of PREG-S (Figs. 5, 2a and 2b) can form an H-bond with the Thr 98 hydroxyl group and two H-bonds with the guanidino group of Arg 66, as well as interacting with the latter coulombically (Fig. 5, 3a–3c). The carboxyl group of Asp 62 lies opposite the amide group of Asn 102. The keto group at position 20 of PREG-S can H-bond with the amide nitrogen of Asn 102 (Fig. 5, 3a–3c). In Fig. 6 are illustrated topographic maps of the proposed PREG-S binding surface viewed from above and from left to right sides, respectively, with and without a Dreiding representation. The excellent fit of the space-filling model of PREG-S to the map is clearly evident.

A point mutation of Phe 64 to Leu greatly diminished the apparent affinity of GABA and GABA agonists for the $GABA_A$ receptor and also greatly diminished sensitivity of the receptor to competitive antagonists of GABA, whereas substitution of Phe 65 with Leu had hardly any effect on these affinities [82]. The latter, together with a number of other features of the model to be detailed elsewhere, suggests the possibility that the proposed PREG-S binding site also may be the site to which 2 molecules of GABA, muscimol, or other effective agonists may bind to initiate the conformational changes that lead to chloride channel opening. It is possible to begin to test this hypothesis by introducing a number of additional point mutations, singly and together, in the suspected regions in conjunction with measurements of channel properties of appropriate subunit combinations [82, 86]. An effort currently is in progress to determine whether it will be possible to compute the relative affinities of the several steroids tested for memory enhancement shown in Fig. 2 for the proposed binding site. Should the latter be feasible, a partial test of validity of our formulation would be to ascertain whether a good correlation exists between the calculated affinities and memory-enhancing efficacy in mice.

To date, other efforts to discern the molecular details at steroid-binding sites on proteins [e.g. see Refs. 87–89] have not led to development of as much structural detail as is proposed in Fig. 5 for the PREG-S binding site on the $GABA_A$ receptor. The model generated by apposition of two hydrophobic segments of the α_1 subunit of the latter in an antiparallel β sheet structure suggests a general strategy that might be useful for further study of PREG and PREG-S interactions with other proteins as well as in study of the interaction of other steroids with proteins to which they are known to bind.

Comment

The possibility of using the naturally occurring non-toxic PREG therapeutically, alone or together with other substances as suggested in the foregoing, is most appealing. The metabolic machinery for managing its disposition is in place at various tissue sites. Its administration in appropriate amounts in situations in which its availability is insufficient for normal function to take place would be less likely to create serious problems of physiological reorganization than would administration of complex synthetic drugs.

One wonders, therefore, why there was no follow-up of the promising early studies with PREG on fatigue, rheumatoid arthritis, inflammation, and autoimmune diseases in humans. Perhaps the answer to this query may have been furnished recently by an industrial scientist working in a related area, "When the CEO passes through, he tells us not to

work on natural substances because such substances generally are not patentable *per se* and use patents tend to have little commercial value." It is disquieting to think that our version of the free enterprise system might not generate sufficient variability to bring to utility substances to relieve pain and suffering just because it might not be profitable. A healthy social system must be able to overcome rate-limiting constrictions that embarrass its function.

There are many fascinating investigative avenues dealing with PREG and PREG-S that are wide open and ready for development. It is hoped that this commentary will encourage some others to enter this field. Selye would have liked it!

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