ENDOCRINE PHARMACOLOGY: SELECTED TOPICS[1]

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Pharmacology, as the science of the interactions of chemicals with living systems, embraces the study of hormones, naturally occurring chemicals vital to life and health and often important drugs as well. From the time of John Jacob Abel, pharmacologists have made notable contributions to endocrinology. Yet the study of hormones, aside from the catechol amines, is not the major field in present day academic pharmacology that it should be. Research on hormones is so important, so attractive, and so broad in its scope that it is not surprising that almost every medical discipline has shared in it. Other basic sciences that precede pharmacology in the medical curriculum ordinarily relieve departments of pharmacology of much of the responsibility for teaching endocrinology, and the subject of hormones as diagnostic and therapeutic agents is often left to the clinical departments. These are some of the factors that have helped to determine the present restricted position of endocrinology in pharmacology.

Despite the importance of the hormones in the spectrum of pharmacological science, it is realistic to recognize that progress in endocrinology is being continuously reviewed in the *Annual Reviews of Biochemistry, Physiology,* and *Medicine.* In the future it may be in order to reallocate endocrine topics among the four *Annual Reviews;* for the present a minimum of overlap has been the objective. Furthermore, limitations of space and of the capacity of the reviewer have prohibited a comprehensive survey. A number of areas of significant research have been omitted entirely, for example, the mechanism of action of insulin. Since selection was necessary, the topics chosen reflect, naturally, the interests of the reviewer. Three subjects, androgens, parathyroid hormone, and the hypothalamic secretory factor for ACTH, have been reviewed in considerable detail, although not exhaustively. Seven other topics have been dealt with briefly by citation of recent reviews and a narrow selection of current papers. The survey of the literature was concluded, for the most part, in August, 1960.

Androgens

The defining characteristic of an androgen, typified by testosterone, is

[1] Abbreviations used in this chapter include: CRF (corticotropin-releasing factor); DHA (dehydroepiandrosterone); HSF-ACTH (hypothalamic secretory factor for ACTH); MSH (melanocyte-stimulating hormone); TPN (triphosphopyridine nucleotide).

its ability to stimulate growth of the male secondary sex organs and development of masculine secondary sex characteristics. Testosterone and a number of other androgens also inhibit secretion of the hypophyseal gonadotropins, both follicle-stimulating hormone and interstitial cell-stimulating hormone, and hasten closure of the epiphyses. They stimulate general body growth, particularly the muscles and long bones, hence the term, anabolic steroid. In large doses, androgens may be palliative drugs in the treatment of inoperable breast cancer. Psychic and behavioral attributes associated with masculinity have also been ascribed to the androgens. *Androgens,* by Dorfman & Shipley (1), a comprehensive survey of the literature through 1953, provides a good basis for the appreciation of more recent developments.

No nonsteroidal androgen, with the possible exception of one phenanthrene derivative (2), has been discovered. Within the category of steroids, however, the structural requirements for androgenic activity are rather loose, with a broad range of specific activities. Therapeutic and commercial interest has led to a search for steroids that are anabolic or effective against breast cancer yet less androgenic than testosterone. Little effort has been devoted to the search for intrinsically more potent androgenic steroids, although there has been interest in the preparation of esters with a more extended duration of action. Testosterone and many other androgens are poorly active by the oral route of administration. On the other hand, methyltestosterone, highly active by mouth, is somewhat toxic to the human liver. The chemical-pharmacological approach toward the elimination of this undesirable side-effect will no doubt be pursued further, although the failure of the dog to exhibit the alterations of liver function tests seen in man may hamper laboratory investigations (3).

Biological assay.—Studies of the relation of structure and activity rest on methods of biological assay. The two principal classic types of assay method for androgenic activity are based on the growth of the comb of the capon, or more conveniently, the baby chick, and the growth of the prostate and seminal vesicles of the castrated rat. Munson & Sheps (4) have refined the baby-chick comb method by applying the test steroid directly to the comb in a minute volume (.01 ml.) of ethanol. The effect of as little as 0.05 μg. of the reference standard, androsterone, daily for seven days, was readily detectable. The delicacy of the method makes possible the assay of extremely weak androgens and of rare steroids available in very small quantity. The mean index of precision (λ) was 0.32.

Methods in current use for the evaluation of the anabolic activity of steroids use castrated male rats and are based on the gain in total body weight, the nitrogen balance, or the weight of the levator ani muscle. Because of its convenience, the levator ani method has been used most extensively. As usually conducted (5), after seven daily injections of test steroid, the ventral prostate and seminal vesicles as well as the levator ani muscle are weighed, giving indications of both androgenic and anabolic activities in the same test animal. Ratios of the two activities relative to a standard an-

drogen may be computed for the test steroid. In practice, statistical evaluation of the results often has been uncritical. Both specific and general criticisms have been levelled at the method. In contrast to the immediate cessation of growth of the prostate and seminal vesicles, the levator ani muscle continues to grow for several weeks after castration. The results may be misinterpreted, according to Gordan (6), if the assay is started immediately (5) rather than three weeks after castration as in the original method of Eisenberg & Gordan (7). Gordan (6) also recommended a longer period of administration in order to evaluate steroids with a delayed onset of action or cumulative effects. Kochakian & Tillotson (8) have observed that the effect of androgens on muscular growth varies not only from species to species but also from muscle to muscle within a species. The possibility must be considered that certain muscles, the levator ani among them, are sex-linked, and that growth of such muscles may be unrelated to more general anabolism. In man no thoroughly studied (nonphenolic) anabolic steroid has failed to be androgenic in a large proportion of patients if administered for a considerable period of time.

The procedure of Kochakian *et al.* (9) for demonstrating the anabolic activity of testosterone by production of a positive nitrogen balance in castrated male rats has been adapted for use as a quantitative biological assay method by Arnold *et al.* (10). A similar but more elaborate method in monkeys has been developed by Stucki *et al.* (11). Schedl *et al.* (12) and Liddle & Burke (13) have studied the relative anabolic activity of steroids in man under carefully controlled conditions. The retention of urinary nitrogen was measured during four-day periods of steroid administration in comparison with control periods preceding and alternating between treatment periods. Clear-cut differences and similarities in nitrogen balance that permitted gross comparisons between steroids were observed by Schedl *et al.*, but two-fold differences in dosage could not be discriminated. Liddle & Burke expressed their results as the difference in urinary nitrogen between the control and treatment periods. Forty-two tests with methyltestosterone resulted in rather widely scattered data, but there was a significant log dose-response curve. The index of precision (λ) was estimated at 0.4, indicating, as the authors acknowledge, that precise estimates of relative potency would require large numbers of observations.

Newly discovered effects.—Effects on androgen-dependent target organs include inhibition of the spontaneous contractions of the seminal vesicle of the castrated rat [Grunt and co-workers (14, 15, 16)] and increased rate of incorporation of labelled glucose and glucurone into the comb of the capon [Balasz *et al.* (17)]. New anabolic effects were reported by Kochakian (18), who showed that the negative nitrogen balance and loss in body weight produced by thyroxine in castrated rats could be overcome by simultaneous administration of testosterone propionate, and by Bajusz *et al.* (19) who were able to prevent, in part, the muscle atrophy following nerve regeneration after crushing injury in rats by feeding methyltestosterone or ethyl nor-

testosterone. Effects on bone were demonstrated by Kowaleski & Emery (20), who found that ethyl nortestosterone increased the rate of uptake of ^{35}S by the callus of healing fractures of rat humeri, and by Urist & Deutsch (21), who reported that androgen administration protects laying hens against the usual spontaneous osteoporosis. The hyperthermic effect in man of etiocholanolone, a major urinary steroid previously thought to be biologically inert, was first reported in 1957 (22). Further investigation of the structural requirements of a steroid for this effect will be hampered by the fact that of all animal species tested (mouse, rat, guinea pig, dog, cat, rabbit, monkey, and man) only man responded (23). Androsterone, the major androgen of human urine, has now been found by Hellman *et al.* (24) to be highly effective in reducing the level of serum cholesterol in patients with hypothyroidism, in other patients with hypercholesterolemia, and in normal subjects. The authors suggest that the elevated serum cholesterol in hypothyroidism may be related to the low levels of androsterone associated with this disorder. Weisenfeld & Goldner (25) found that several but not all androgenic steroids tested prevented the hyperglycemic effect of glucagon in human subjects. The most effective steroids possessed an alkyl substituent in the 17α-position, a structural characteristic of steroids with liver toxicity. No consistent correlation with androgenic or anabolic activity was observed, except that estrogens, progestagens, and glucocorticoids were inactive. Hilz & Utermann (26) have reported that testosterone ($10^{-7}M$) stimulates the oxygen consumption and synthesis of polysaccharide sulfate in the isolated normal rat aorta.

Relation of chemical structure to androgenic activity.—A comprehensive survey of the relative androgenic potency of steroids by different assay methods would help to clarify the currently confused status of the subject. Extensive new data and an exhaustive review, restricted to the effect of esterification of androgens with a wide variety of organic acids on the duration of androgenic action in the rat, have been published by Junkmann & Witzell (27).

Rosemberg *et al.* (28, 29) compared the androgenic activity of 4-androstene-3,17-dione with its 11β-hydroxyanalogue and the 9-hydroxy and 9α-halo-11β-hydroxy analogues by local application to the baby chick comb. The parent compound is a highly active androgen, while the 11β-hydroxy derivative is only very weakly active and, furthermore, exhibits a rather flat dose-response line. The 9α-F, 9α-Br, and 9α-Cl derivatives of 11β-hydroxy-androstenedione, also the 9β-11β-oxido and the 9α,11β-dihydroxy compounds were not detectably androgenic at the relatively high doses tested. These observations are in harmony with the unpublished results of Munson and Sheps, who found, in addition, that the 11-oxo- and Δ9-11 derivatives of highly active androgens were intermediate in potency between the parent androgens and the 11β-hydroxy derivatives. These characteristics of the structure-activity relationship of growth-promoting steroids are, of course, in marked contrast to the enhancing effect of 11-oxygen and 9α-halo substitution on

activity in the catabolic glucocorticoid series. An exception to these generalizations is the reported high androgenic activity of fluoxymesterone (9α-fluoro-11β-hydroxy-17α-methyl-19-nortestosterone) and its 11-oxo analogue by oral administration (30).

Alibrandi *et al.* (31) tested a series of acyl esters of testosterone and of dihydrotestosterone by the procedure of Hershberger *et al.* (5) and found that the esters were considerably more effective in oil solution than in aqueous suspension, whether administered orally or subcutaneously. Gleason & Parker (32) have reported a new long-acting androgenic steroid, the benziloyl hydrazone of testosterone heptanoate.

Anabolic steroids.—Extensive bibliographies of laboratory and clinical data on anabolic steroids have been published (12, 33). Berczeller & Kupperman (33) have discussed the principal anabolic steroids generally available for clinical trial. In addition to the classic testosterone esters and methyltestosterone, their list comprised methyl-androstenediol (5-androstene-3β,17β-diol), norethandrolone (17α-ethyl-19-nortestosterone), and norethandrolone phenylpropionate. Fluoxymesterone, methandrostenolone (17α-methyl,17β-hydroxy-1,4-androstadien-3-one), vinyl and ethynyl nortestosterone, and androstanazole [17α-methyl-17β-hydroxy-androstano-(3,2-c) pyrazole] should be added to the list. Contrary to the prediction from the levator ani tests in rats, Schedl *et al.* (12) found vinyl and ethynyl testosterone to be anabolic in man. Androstanazole was indicated to be a potent anabolic agent in rats both by nitrogen retention and levator ani test (10, 34). Lerner *et al.* (35) found nortestosterone benzoate, by the method of Hershberger *et al.* (5), to be much less (18 to 42 times) androgenic than testosterone propionate, but almost equivalent in its effect on the levator ani muscle. However, the dose-response curve for nortestosterone benzoate on levator ani weight was very flat.

The nature and mechanism of the anabolic effect of the androgenic and related steroids is still quite imperfectly understood 25 years after discovery of the phenomenon. A valuable paper by Kochakian & Endahl (36) clarifies the relationship between dosage of testosterone propionate, castration, food intake, and gain or loss of body weight in rats. The additive effect of testosterone proprionate and growth hormone on body weight gain previously shown in mice (37) was confirmed in castrated rats (38), indicating that the two anabolic actions are independent in both species. Kochakian & Costa (39) studied the effect of testosterone propionate in depancreatized castrated dogs given sufficient insulin to maintain life but not enough to prevent glycosuria. Administration of testosterone propionate, 25 to 100 mg./day, resulted in a decrease in excretion of glucose accompanied by retention of nitrogen and a continuous increase in body weight. The androgen is known to be without effect on the blood glucose level in dogs with pancreas intact. A review by Kochakian (40) on mechanisms of androgen action and two new papers (41, 42) are concerned principally with changes in enzyme activity of various tissues of animals treated with androgens. It seems fair to

say that the work on enzyme content of tissues stimulated by androgens has not yet developed any clear lead to the understanding of the mechanism of action of androgens.

Recently, a mixture of steroid esters formulated by Gassner *et al.* (43) for administration to feeding stock (steers) has been shown to act as an anabolic agent of potentially great economic importance. The mixture consists of testosterone enanthate 60 mg., 17α-hydroxy progesterone 60 mg., and estradiol valerate 24 mg., and is injected as a paste in polyglycol suspension into the ear of the animal. Estrogen could not be found in extracts of the edible parts of the carcass, and the delicacy of the bioassays employed excluded the presence of more than one-billionth part of the estrogen injected. The rather slow rate of disappearance of the androgen and the progesterone derivative from the circulation and the lack of delicacy of available tests for these steroids in tissues forced the conclusion that additional studies would be needed before a definitive decision could be made concerning the public health aspects of treatment of cattle with the anabolic steroid mixture (44).

Inhibition of gonadotropin secretion.—The ability to inhibit the secretion of anterior pituitary gonadotropins may be at least in part dissociated from other activities of androgens. Thus both fluoxymesterone (45) and 2α-methyldihydrotestosterone propionate (46) were shown to be as effective or more effective than testosterone propionate as anticancer agents, yet induced little or no reduction in gonadotropin excretion. On the contrary, although nortestosterone propionate resulted in a marked decrease in gonadotropin excretion (significantly less marked, however, than similar doses of testosterone propionate), it was virtually inactive as an anticancer agent (47). Epitestosterone propionate was inactive in both respects (48). An exhaustive compilation of tests, in animals as well as in man, for inhibition of gonadotropin secretion by various steroids would be helpful.

Nortestosterone propionate (*vide supra*) and methyl nortestosterone have recently been shown to inhibit gonadotropin secretion in man, as shown by reduction in urinary gonadotropins (47, 49, 50, 51). Both ethyl nortestosterone and ethynyl nortestosterone, the latter more than the former, inhibited gonadotropin secretion in young male rats as shown by the effect on spermatogenesis and on the weight and histology of the testis (52).

Glenn *et al.* (53) have shown that 9,11-epoxy-methyltestosterone, 11α-hydroxy-methyltestosterone, 2-methylnortestosterone, and 9α-F,11β-hydroxy-17-methyltestosterone are effective inhibitors of the growth of the Huggins tumor (a benign mammary fibroadenoma) in female mice, with little stimulating effect on the preputial glands (an indicator of androgenic activity in the female). All members of the group were relatively weak inhibitors of gonadotropin secretion as tested by the effect on growth of the testes.

Adrenal androgens.—The significance and even the identity of the adrenal androgens are not yet known with certainty. The term itself, adrenal androgens, has been used rather loosely to include steroids isolated from

adrenal extracts, steroids synthesized by adrenal tisue *in vitro,* steroids presumably secreted because they have been identified in adrenal venous blood, and androgenic metabolites (originating from adrenal precursors) found in peripheral blood, nonendocrine tissues, and urine or other excreta. In addition, species differences undoubtedly exist. All the so-called androgens of urine are present in urine as biologically inactive conjugates, sulfates and glucosiduronates; androgenic activity is manifested only after hydrolysis to the free steroid. Finally, a distinction should be made between adrenal androgens from normal and pathological adrenal glands. Short (54) has summarized the limited information through 1958 about the identity of the adrenal androgens.

A marked increase in the quantity of urinary dehydroepiandrosterone (DHA), a steroid known to be of exclusively adrenal origin, is commonly associated with but is not certainly responsible for the virilizing syndromes arising from adrenal pathology in women and children. DHA is approximately one-half as potent an androgen as testosterone when tested by direct application to the comb of the baby chick, but it is relatively weak in all other animal assays, and not clearly proved androgenic in man. The concentration of free, as distinct from conjugated, DHA in peripheral blood has not been determined either in adrenal virilism or in normal subjects.

In normal physiology the significance, if any, of the adrenal androgens is quite unclear. It is obvious that the adrenal androgens are inadequate to maintain the normal status of the secondary sex glands and secondary sex characteristics in castrated men and other male animals (55) or to exert well-defined effects in the normal female. However, there are recurring suggestions that an increased supply of adrenal androgen may be responsible for at least some cases of idiopathic hirsutism (56) and that adrenal androgens may be of importance in the maintenance of normal libido (57, 58). The normal rate of secretion of DHA has recently been estimated by Vande Wiele & Lieberman (59) to be to 25 mg. a day, an amount larger than for any other steroid secreted. Partly attributable to this high rate of secretion and partly to a rather slow rate of disappearance of its principal conjugate, the sulfate, from the circulation (half-life of 128 and 98 minutes respectively in two subjects) associated with a slow rate of clearance by the kidney (60), DHA is quantitatively the major conjugated 17-ketosteroid in peripheral plasma (61). DHA is also the third most prominent 17-ketosteroid of human urine. Furthermore, the two more abundant urinary 17-ketosteroids, androsterone and etiocholanolone, have DHA as their sole adrenal precursor. All these facts encourage further investigation of the adrenal androgens in general and DHA in particular.

The special pharmacology of DHA is poorly known, lending added interest to the recent investigation by Howard (62), in which the effects of subcutaneous compressed pellets of DHA, 11β-hydroxy-androstenedione, and corticosterone on the mouse adrenal gland and reproductive tract were compared. The reduction of adrenal weight by DHA resulted from an effect

on the X-zone exclusively, whereas the effect of corticosterone was on the zona fasciculata. DHA stimulated marked growth of the preputial glands and phallus of both sexes with only minimal stimulation of growth of the seminal vesicles. The effects of DHA were approximately duplicated by 11β-hydroxy-androstenedione, at a dose level three to four times as large as that of DHA. This work suggests that the various androgenic steroids may have differential effects on the different secondary sex organs.

Additional androgenic steroids have not been isolated from adrenal extracts since Short's review (54), which listed 4-androstene-3, 17-dione (androstenedione), adrenosterone, 11β-hydroxy-androstenedione, and 11β-hydroxy-epiandrosterone from cattle and hog adrenal extracts, and testosterone, DHA, and androsterone (incompletely identified) from human adrenal tumors. The androgenic activity of 17α-hydroxy-progesterone, also listed (54), is dubious.

Dorfman (63) has tabulated the androgens isolated from adrenal perfusates and incubates of slices and homogenates. Androstenedione, adrenosterone, 11β-hydroxy-androstenedione, and DHA make up the list. The same four steroids have been identified with varying degrees of certainty in adrenal venous blood of several species (54). In man the isolations have been from patients undergoing adrenalectomy for cancer or adrenal disorders. In addition, androsterone was identified in the adrenal venous blood of a woman suffering from virilizing adrenal hyperplasia (64). Short (54) identified androstenedione, 11β-hydroxy-androstenedione, and DHA in adrenal venous blood samples after administration of ACTH to three postmenopausal women with breast cancer. No testosterone, androsterone, or adrenosterone could be detected. Lombardo *et al.* (65) isolated 11β-hydroxy-androstenedione from adrenal venous blood of 10 of 12 elderly women with breast cancer, and DHA from only one of the 12. It has been suggested (59) that the age and debility of the patients were responsible for the failure to find DHA more consistently. Oertel & Eik-Nes (66) were unable to detect DHA with certainty in the adrenal venous blood of dogs, but, following perfusion of pregnenolone, high levels of DHA were found in the systematic plasma. They reported, furthermore, that the isolated hind leg of the dog converted pregnenolone and 17α-hydroxy-pregnenolone to DHA, obtained as DHA phosphate, which is also found in systemic plasma of normal human subjects infused with ACTH (67). It was concluded that DHA is formed outside the adrenal gland from pregnenolone and 17α-hydroxy-pregnenolone of adrenal cortical origin. This new concept will require further study and confirmation before it becomes generally acceptable.

Little is known about the normal levels of the free circulating androgens. Oertel & Eik-Nes found 1.4 μg. testosterone/100 ml. men's plasma after administration of chorionic gonadotropin, but none in normal plasma (68). Injected testosterone disappears from the circulation with great rapidity. Extracts of acidified human plasma contain DHA as the principal 17-ketosteroid, but this undoubtedly represents hydrolyzed DHA sulfate. An in-

genious new method by Finkelstein *et al.* (69) for the analytical determination of as little as 0.05 μg. of testosterone will permit a new attack on the problem. Plasma is extracted under conditions that protect steroid conjugates from hydrolysis, and the testosterone is isolated by paper chromatography and converted to estradiol and estrone by a placental enzyme preparation. The estrogens are purified by paper chromatography and estimated fluorometrically. In three normal men the values were 0.4, 0.2, and <0.1 μg./100 ml. In two normal women they were 0.1, and <0.1 μg./100 ml. In two women with virilizing syndromes, one with an adrenal adenoma and one with an ovarian hilus-cell tumor, values of 1.3 and 2.0 μg./100 ml., respectively, were obtained, suggesting that the steroid responsible for the virilization was testosterone. However, the data are too few to be certain that testosterone is consistently present in plasma from normal women.

On the basis of a variety of indirect evidence it has long been assumed that in the normal human male one-third of the urinary 17-ketosteroids represent testicular precursors and two-thirds represent adrenal cortical precursors. Relegating DHA and the 11-oxygenated 17-ketosteroids to the adrenal cortex exclusively, a reasonable estimate of the source of the principal urinary 17-ketosteroids, androsterone and etiocholanolone, has been one-half from the testis and one-half from the adrenal cortex. A bombshell has been dropped on this widely accepted concept by Vande Wiele & Lieberman (59), who concluded, with impressive although limited supporting data, that DHA from the adrenal cortex is the precursor of essentially all of the androsterone and etiocholanolone of human urine, men's as well as women's. This conclusion is based on the observation that when DHA-7-^{3}H was administered to two normal young men the specific activities of the three steroids (DHA, androsterone, and etiocholanolone) isolated from urine were essentially identical, indicating that dilution by unlabelled endogenous precursor was the same for all three steroids. The corollary conclusion, that testosterone contributes insignificantly to the urinary androsterone and etiocholanolone, is in apparent contradiction to the results of Harrison, Munson, and co-workers (70, 71, 72), who found that the excretion of urinary androgens (measured by biological assay) by elderly men with prostatic cancer was markedly reduced by orchiectomy. Additional unpublished work has extended the data to include 16 patients, all but two of whom exhibited a fall in androgen excretion after removal of the testes. The mean fall, including the two negative results, was 47 per cent, in excellent agreement with the assumption that half of the total urinary androgen in these men was of testicular origin. (The androgen content of suitably prepared urine extracts is essentially equivalent to the androsterone content.) A possible explanation of the disagreement in the results from the two laboratories is related to the decline in excretion of androgens and 17-ketosteroids with age.

Although it should be obvious that the fall in androgenic steroid excretion with age is so great in men that a decrease in secretion of the adrenal precursors must occur also, this inference has been emphasized only rarely

(73) in the literature. The less extensively documented fall in androgen excretion in elderly women and the hypothesis of Vande Wiele & Lieberman also require that urinary adrenal androgens must fall with increasing age. If the mean urinary androsterone in normal young men is taken as 6.0 mg./24 hrs. and the variability of the data of Lieberman & Vande Wiele allow for a 10 per cent testicular contribution to the urinary androsterone, then as much as 0.6 mg. of androsterone per 24 hours may be of testicular origin. In the elderly patients with prostatic cancer studied by Harrison & Munson, the mean daily excretion of androsterone before castration was only 0.96 mg., declining to 0.51 mg. after castration. The decrease resulting from castration (0.45 mg.) was therefore within reasonable agreement with the postulated testicular component of urinary androsterone of young men. This attempt to harmonize the two sets of data implies either that the decline in secretion of DHA with age is much greater than that for testicular testosterone, or that the relative size of the testicular contribution to the urinary androgens is much greater in men with prostatic cancer than in healthy men of the same age.

In patients with virilizing congenital adrenal hyperplasia attributed to a deficiency in the 21-hydroxylating enzyme, the secretion of 21-deoxycortisol and the excretion of 11-oxygenated-17-ketosteroids are greatly elevated, because of increased ACTH stimulation resulting from cortisol deficiency. Since, however, administration of 21-deoxycortisol does not lead to increased excretion of the 11-oxygenated-17-ketosteroids, the origin of the latter must be through a different pathway (74).

It now appears that in normal subjects, as well as in patients with congenital adrenal hyperplasia, androstenedione, rather than cortisol, 21-deoxycortisol, or 17α-hydroxy-progestrone, is the principal precursor of the urinary 11-oxygenated 17-ketosteroids (75, 76), which also suggests that the highly androgenic androstenedione may be a major adrenal secretory product. The studies of Wilson *et al.* (77) on the urinary steroids of four hypophysectomized women during ACTH administration also favor separate biosynthetic pathways for cortisol and adrenal precursors of 17-ketosteroids.

Wade *et al.* (78) found that rectal administration of cortisol was followed by a 100-fold increase in the excretion of 11β-hydroxy analogues of androsterone and etiocholanolone. The increase was abolished by administration of neomycin, implicating microbial action as the cause of the increased urinary metabolites, and suggesting the possibility that the minor normal conversion of endogenous cortisol to 11-oxygenated urinary 17-ketosteroids may occur in the intestine.

Antiandrogens.—Dorfman & Dorfman reviewed the literature on antiandrogens and described tests for such substances (79, 80). The comb of the baby chick was stimulated to grow by feeding testosterone with the diet or by a single subcutaneous injection of testosterone enanthate, and the test substance was given daily either by subcutaneous injection or by direct application to the comb. A number of estrogens, corticoids, and progesta-

tional substances, with 17α-ethynyl-nortestosterone the most active, appeared to inhibit the androgen-induced growth of the comb. In most cases the effect of the inhibitor was not clearly related to the dose. Lerner *et al.* (81) demonstrated the antiandrogenic effect of A-norprogesterone in both the chick and the rat. In confirmation of Hertz & Tullner (82), the carcinogen, methylcholanthrene, also inhibited the effect of androgen on the comb, and benzpyrene acted similarly (79). Previously shown to be antiandrogenic in the rat (83), 2-acetyl-7-oxo-1,2,3,4,4a,4b,5,6,7,8,9,10,10a-dodecahydrophenanthrene was found to be an active inhibitor in chicks. Dihydroisosteviol is another nonsteroidal substance reported to have an antiandrogenic effect on the chick comb (84).

Androgens in target organs.—Greer (85) injected rats with testosterone-^{14}C and plotted the disappearance rates from blood, muscle, and prostate gland. In both intact and castrated rats the ratio of activity, prostate to muscle, was usually about 2.5, and the activity found in the prostate gland was consistently although only slightly higher than that in the blood. Pearlman & Pearlman (86) infused male rats intravenously with 2.3 to 3.4 mc. of 4-androstene-3,17-dione-7-^{3}H (specific activity, 3.8 mc./mg.) at a constant rate for two to three hours. The ratio of radioactivity between extracts of ventral prostate and systemic plasma was 1.55; that between muscle and plasma was 0.72. In addition to androstenedione, radioactive metabolites, testosterone, androsterone, etiocholanolone, and androstanedione, possibly of significance for understanding the mechanism of action of androgens, were identified and quantitatively estimated in the prostate and plasma. Ofner *et al.* (87) identified radioactive 17β-hydroxy-1-androsten-3-one as a product of the incubation of testosterone-4-^{14}C with minces of human prostatic tissue.

Parathyroid Hormone

Research on parathyroid hormone, relatively neglected for a number of years, has flourished since 1955, and important advances have already been made in the purification of the hormone, quantitative methods for its biological assay, and studies of its sites and modes of action. There are several relatively recent reviews of earlier work (88 to 92), and a symposium (held in Houston, February, 1960) on current research on the parathyroid glands has been published in book form (93). The essay by Aurbach (94) is also of interest.

Purification.—The status of purification of the hormone was reviewed briefly in April, 1960 (95). The starting extracts for the current purified preparations include the classic hot hydrochloric acid extract (96), 0.1 N hydrochloric acid at 4°C. (97), hot acetic acid (98), and phenol (99). The first report of a substantial purification of parathyroid hormone was made by Rasmussen & Westall (100). They fractionated the ultrafiltrate of a hot dilute hydrochloric acid extract on Dowex 50 resin and obtained "Parathormone A," a product containing 100 units/mg., about six times the specific activity of the *United States Pharmacopeia* preparation marketed by Eli

Lilly and Co. Starting with the ultrafiltrate of the hot acetic acid extract of Davies & Gordon (98), Rasmussen isolated a different and apparently homogeneous peptide ("Parathormone B": specific activity, 220 units/mg.) by zonal electrophoresis on polyvinyl chloride (101). Friedman & Munson fractionated the hot acid extract with ammonium sulfate (102) and purified it by gradient elution on carboxymethylcellulose to obtain a final product containing 200 units/mg. (103). Application of the same procedure to the cold hydrochloric acid extract increased the specific activity to 1300 units/mg. (95). Aurbach (104) revived the use of phenol for preparing the crude extract, which he then fractionated with trichloracetic acid and organic solvents and purified by countercurrent distribution. The specific activity of the final product was estimated to be 2800 units/mg. Rasmussen & Craig (105) used a similar procedure and obtained a product of equivalent potency, identified as "Parathormone C." It appeared to be homogeneous by countercurrent distribution, ultracentrifugation, and dialysis. Amino acid analyses of Rasmussen's A, B, and C peptides indicated the presence of 16 or 17 amino acids; cysteine was not found consistently and methionine was present in all three (106). The calculated minimum molecular weights of peptides A, B, and C were, respectively, 3800 to 5600, 6512, 9267. Peptide C, although it has the largest molecular weight, also has the highest specific activity. The amino acid analyses also indicate that the three peptides do not represent mere polymers. Partial degradation of peptide C with accompanying loss of activity is the explanation favored by Rasmussen (106). Rasmussen announced the identification of the N-terminal amino acids of his peptides B and C at the First International Congress of Endocrinology, Copenhagen, in July 1960. Publication of the details of this work is awaited with interest.

Study of the stability of the purified preparations is complicated by variable times of onset and durations of action in test animals following subcutaneous injection. Rasmussen discovered that his preparations were reversibly inactivated by treatment with hydrogen peroxide (107), somewhat similar to the observations of Dedman *et al.* (108) on ACTH. Like ACTH in the hands of Dedman *et al.*, the activity was restored or possibly increased above the pretreatment value by treatment with cysteine at elevated temperatures. Others, however, found that the activity of crude as well as purified extracts, not intentionally inactivated, was enhanced markedly by the mere addition of cysteine without heat (109). Rasmussen has reported that the reversible inactivation with hydrogen peroxide is associated with the formation of methionine sulfoxide (106).

Biological assay.—For standardization of the *United States Pharmacopeia* extract, the venerable dog method is still official (110). It depends on the rise in blood calcium in intact dogs 18 hours after subcutaneous injection of extract. Too cumbersome for following the purification of extracts, it has been supplanted in the research laboratory by the method of Munson *et al.* (109, 111, 112), which employs young male rats given a calcium-free diet for four days then injected subcutaneously with extract immediately after para-

thyroidectomy. Six hours after parathyroidectomy the serum calcium of untreated rats has fallen to about 6 mg. per cent. The degree of maintenance of the serum calcium above this level (at six hours) bears a linear relationship to the log of the dose. The mean index of precision (λ) is 0.25 (109) considerably better than that of the method of Davies *et al.* (113), in which rats on a stock diet were parathyroidectomized three to 21 days before the assay and the serum calcium was determined 21 hours after injection. The six-hour time interval is also preferable to 21 hours for assessment of the potency of the shorter-acting purified preparations.

The limitations and shortcomings of the assay method of Munson *et al.* have been discussed in considerable detail (109). The addition of various substances (107, 109, 114) to parathyroid extract (cysteine, for example) or the employment of certain vehicles such as gelatin or oil (109, 114, 115, 116) greatly enhances the activity of crude as well as purified extracts although to varying degrees. The prevailing custom, in pa
juvant to the unknown only and not to the standard may lead to misinterpretation of the results. Although Aurbach (116) and Rasmussen (114) have stated that the activity of the *United States Pharmacopeia* extract is not affected by addition of cysteine or gelatin, the extensive data of Munson (109) can leave little doubt that marked enhancement of activity will be observed under carefully controlled conditions with adequate numbers of animals.

Clark *et al.* (117) have developed an assay method for parathyroid hormone based on the withdrawal of ^{45}Ca from the skeleton of the intact rat given ^{45}Ca six weeks prior to the assay. The assay method of Kenny & Munson (118) is based on the phosphaturic effect in rats during the first six hours after parathyroidectomy and subcutaneous administration of extract. Other assay methods published prior to 1958 were reviewed by Greep & Kenny (91) and Munson (109).

The small amounts of parathyroid hormone required to maintain the serum calcium of the parathyroidectomized rat and dog at a normal level indicate that the concentration of hormone in blood and urine is low (112, 119). A more delicate assay method than is yet available, coupled with a simple efficient method for concentrating the hormone from blood and urine, will be needed for adequate quantitative assay of these fluids. Davies (120) reported the detection of parathyroid hormone in the urine of hyperparathyroid patients. The urine was subjected to preliminary fractionation, and the resulting product was injected into intact mice. The presence of hormone was inferred from the increased excretion of inorganic phosphate in the urine. The precision of the assay method was low so that even with large numbers of mice the limits of error were broad. Buckner & Nellor (121) have attempted to estimate parathyroid hormone in serum of hyperparathyroid rats, but the positive results reported were borderline and of dubious quantitative significance.

Mode of action.—It is now well established, contrary to the classic

hypothesis of Albright & Ellsworth (122), that the parathyroid hormone acts to maintain the level of plasma calcium by some means independent of the kidney and of the level of plasma inorganic phosphate, almost certainly by a direct effect on bone. The evidence for this newer concept has been reviewed recently (95). The hormone also has a phosphaturic effect on the kidney, but this effect and the effect on bone appear to be relatively independent of one another. Additional effects of the hormone for which new evidence has been presented include increased reabsorption of calcium by the kidney (123), other renal effects involving bicarbonate, sodium, potassium, and chloride (124), stimulation of absorption of calcium by the gut (125, 126), effects on the distribution of phosphate between fluid compartments (127, 128, 129), and changes in the composition of milk in rats (parathyroidectomy is followed by decreased concentration of water and increased concentration of calcium; the hormone restores the composition toward normal) (95, 130).

New studies of direct effects on bone *in vitro* have been reported by Gaillard (131). Other recent work on the mechanism of action of parathyroid hormone on bone, with particular reference to the significance of citrate, was well summarized by Kenny (132), also by Freeman (133).

The general significance of the effect of parathyroid hormone on the excretion of phosphate by the kidney, now that this effect can no longer be accepted as the mechanism underlying regulation of the blood calcium level, has not yet been clarified. The reality of a phosphaturic action of parathyroid hormone is no longer in any doubt. It occurs with purified as well as crude preparations (104, 134, 135, 136) and is independent of changes in the glomerular filtration rate, which may be increased by crude extracts. The effect of the hormone when injected intravenously is extremely rapid. Gershberg *et al.* (137) measured glomerular filtration rate, renal plasma flow, and serum and urinary inorganic phosphate before and at short intervals after intravenous injection of *United States Pharmacopeia* parathyroid extract in 42 normal human subjects and patients. In all cases except two with hyperparathyroidism, the percentage of filtered phosphate reabsorbed fell within 15 minutes after the injection. Beutner & Munson (138) studied the effect of parathyroidectomy and parathyroid extract on the urinary phosphate in unanesthetized rats infused with physiological salt solution in order to provide urine samples at frequent intervals. A significant fall in urinary phosphate occurred within 25 to 40 minutes after parathyroidectomy. One to two hours later, after the urinary phosphate had reached a very low level, parathyroid extract was injected intravenously. In every case a rise in urinary phosphate occurred within eight minutes. The rapidity of the response to the hormone in both rat and man suggested that there is a direct effect on the renal tubule. Lavender (136) and Pullman *et al.* (139) injected small doses of purified parathyroid hormone into one renal artery of the intact dog while collecting urine from the two kidneys separately. In the majority of dogs studied the phosphaturic effect was confined to the injected kidney, direct evidence for a local action of the hormone on the kidney.

The site of action of parathyroid hormone within the renal tubule is in

dispute. Pitts *et al.* (140) and Malvin *et al.* (141) localized the proximal tubule as the principal site of phosphate reabsorption by the mammalian kidney; active secretion of phosphate was not observed. Nicholson & Shepherd (142) studied the sites of phosphate transport in the tubule of the dog with the aid of differential nephrotoxic agents and concluded that there was active secretion of phosphate in the distal tubule as well as reabsorption in the first third of the proximal tubule. Studies with parathyroid extract (143) in similarly treated dogs seemed to show that the principal mode of action of the hormone on the kidney was to stimulate active secretion of phosphate in the distal tubule with little or no effect on reabsorption. On the other hand, Samiy *et al.* (135), in a preliminary report of experiments with the stop-flow technique in parathyroidectomized dogs, found no evidence of net active secretion anywhere along the tubule. The principal site of reabsorption of phosphate, about which there is no controversy, was again found to be in the proximal tubule, and it was concluded that the effect of administration of purified parathyroid hormone was exerted in both proximal and distal segments. Samiy *et al.* (unpublished) have concluded from stop-flow experiments on additional parathyroidectomized dogs that the major action of the hormone is on reabsorption in the proximal tubule. It may be difficult to resolve the differences in the results obtained by the two techniques. There is little question that active secretion of phosphate occurs in the kidney of the chicken (144), but the work of Nicholson & Shepherd is virtually alone in concluding that a similar phenomenon exists in mammals. Komarkova *et al.* (145) observed a marked increase in the citric acid content of the rat kidney two hours after subcutaneous injection of a small dose of crude parathyroid extract. Since there was little or no effect one hour after the injection, it might appear that the effect was too slow to be of importance in the more rapid phosphaturic effect just described. However, the route of administration may have been responsible for the delay in response, and for clarification it would be necessary to measure the time course of both effects simultaneously after intravenous as well as subcutaneous administration.

The possibility that there is more than one parathyroid hormone has been suggested repeatedly, particularly with respect to the separate effects on bone and on the kidney. The purified hormone preparations of Aurbach, Rasmussen, and Munson have all been found to possess both calcium-mobilizing and phosphaturic activities (104, 134, 135, 136). Although the eventual separation of two hormones is still conceivable and can hardly be eliminated as a possibility until the native peptide has been synthesized, the evidence available at present favors the possession of both activities by a single hormone. Purified preparations have not yet been tested for the other less thoroughly studied effects enumerated above.

Hypothalamic Secretory Factor for ACTH

The biosynthesis and secretion of two of the three major classes of adrenal cortical steroids, glucocorticoids and the adrenal androgens, are dependent on and regulated by the anterior pituitary through ACTH. The

third major type of adrenal cortical activity, regulation of electrolyte metabolism, to which both aldosterone and the native glucocorticoids contribute (146), is at least partially dependent on ACTH. The rate of secretion of ACTH itself is held in check by the level of circulating glucocorticoids, and thus the entire system is regulated in part by a feed-back mechanism familiar in endocrinology. However, a wide variety of changes in the external and internal environment can also affect the secretion of ACTH without being mediated through alterations in the blood cortical steroid level. Most, if not all of the known effective stimuli for increased ACTH secretion are also "stressful." Today, it is less certain than it seemed 10 years ago that the hypophyseal and adrenal cortical responses to stresses are beneficial or even physiologically significant, but, notwithstanding, the phenomenon is a striking one that has attracted widespread attention and critical analysis.

Much indirect evidence has accumulated in support of the concept that stressful stimuli act through the central nervous system, more specifically the hypothalamus, which secretes a neural hormone into the hypothalamico-hypophyseal portal vessels to reach the anterior pituitary where it increases the rate of secretion of ACTH. The classic evidence in support of this concept was marshalled by Harris (147). More recent developments have been thoroughly and critically summarized from five differing points of view by Sayers *et al.* (148), Saffran & Saffran (149), Ganong & Forsham (150), Fortier & de Groot (151), and Vogt (152). Additional useful reviews were cited by these authors.

The experiments of Egdahl (153) may necessitate modification of this almost universally accepted hypothesis. Leaving the pituitary intact, the brain of the dog anterior to the inferior colliculus, including the hypothalamus and median eminence, was removed. During the three to five days of subsequent survival the adrenal venous 17-hydroxy-corticosteroids were measured under resting conditions, following a severe burn applied to a leg, and after a large dose of ACTH. The resting level of 17-hydroxy-corticosteroid secretion was found to be much higher than that of intact dogs, and the absence of the hypothalamus did not prevent increased steroid secretion following the burn, although this increase was less than that produced in the same dogs by ACTH or that following a burn in anesthetized intact dogs. The stimulus from the burned leg was transmitted over neural pathways rather than through the circulation, since the response was abolished by nerve transection and was unaffected by occlusion of the veins from the leg (154). Egdahl (153) concluded, therefore, that afferent neural stimuli traveled from the leg to the midbrain, where they stimulated elaboration of a neural hormone transmitted to the anterior pituitary through the systemic circulation. Absence of a center in the forebrain that normally inhibits ACTH secretion was suggested to explain the high resting steroid secretion in the operated dogs. Further investigation of the postulated hypothalamic secretary factor for ACTH, based on extensive evidence from many laboratories, is not likely to be abandoned immediately as a result of Egdahl's ex-

periments. When they are confirmed and extended, however, it must be acknowledged that at least one type of stress can result in increased ACTH secretion in the absence of the hypothalamus.

Biological test systems.—Whether the postulated hormone is identified as one of the known constituents of the hypothalamus or whether it exists as an unknown component of hypothalamic extracts, a specific biological test is needed to establish this identity or to help in the isolation of the new substance. All of the tests that have yet been devised are open to criticism of one type or another. Since there are many drugs and many experimental procedures that provoke increased secretion of ACTH indirectly through the mediation of the hypothalamus, the fundamental requirement for specificity in a test system is that the hypothalamus must be removed or damaged or its function must be inhibited in a manner adequate to distinguish between nonspecific stimuli that act on the hypothalamus and the hormone, which acts directly on the anterior pituitary. There are four principal categories of tests in current use, each of which attempts to fulfill this requirement in a different way. In one, rats with extensive median eminence lesions are used. In another, hypothalamic function is inhibited by administration of cortisol or other glucocorticoid substance. In a third, suitable drugs are used as hypothalamic inhibitors. Finally, rat anterior pituitary separated from the hypothalamus is incubated *in vitro* with test substances. In all the tests a positive result requires the demonstration of increased secretion of ACTH, either inferred from adrenal cortical responses in the original test animal or by assay of the blood or incubation medium in a second test system. A second requirement of a specific test system is that it must be able to discriminate between ACTH and the hypothalamic secretory factor for ACTH. Before consideration of these test systems in detail, the justification for their use will be examined.

Studies of the location and the extent of lesions in the hypothalamus that are effective in eliminating the ACTH response to nonspecific stimuli have been reviewed previously (148, 150). In the rat the most effective lesion is a large, more or less obliterative lesion of the median eminence introduced by McCann (155). In spite of interference with the blood supply to the pituitary, this lesion does not prevent increased ACTH secretion in response to the injection of hypothalamic extracts (156, 157).

When a sufficiently high dose of a glucocorticoid is administered to rats the ACTH response to nonspecific stimuli may be markedly inhibited or abolished. Porter & Jones (158) demonstrated that doses of cortisol that are effective in inhibiting the response of rats to nonspecific stimuli permit ACTH secretion following injection of extracts of hypophyseal portal blood. Other evidence had suggested that both the hypothalamus and anterior pituitary are directly inhibited by glucocorticoids (150).

A number of drugs have been shown to inhibit the ACTH response to stressful stimuli under specified experimental conditions. The drugs, paradoxically, are capable of acting as nonspecific stimuli for ACTH secretion under

other experimental conditions. In several cases, morphine (159, 160) and reserpine (161) for example, the initial injection of the drug provokes increased ACTH secretion, but adaptation occurs after treatment for several days, and the stimulating action is replaced by inhibition. Kitay *et al.* (162) reported that repeated administration of epinephrine or reserpine eventually resulted in a decreased amount of ACTH in the pituitary and suggested that the smaller supply of ACTH was responsible for the failure to respond to normal stimuli. They did not demonstrate that a moderate reduction in pituitary ACTH content necessarily results in decreased sensitivity to stimulation of ACTH secretion. Furthermore, the assays for rat pituitary ACTH were based on the *in vitro* adrenal slice method (163, 164), which estimates approximately three to 10 times as much ACTH in rat pituitary tissue as is indicated both by the classic adrenal ascorbic acid method (165, 166, 167) and the new method based on plasma corticosterone (168).

Morphine is the drug in current use for inhibiting hypothalamic function in tests for hypothalamic hormone. It is administered to rats previously anesthetized with pentobarbital; used in this way, it does not itself stimulate ACTH secretion (159, 160) and inhibits the ACTH response to a wide variety of stressful stimuli (159, 169, 170). However, increasing the intensity of the stimulating procedure or the dose of the nonspecifically stressful substance will overcome the morphine inhibition (159, 160, 171). Nalorphine antagonizes the inhibition of ACTH secretion by morphine in anesthetized rats (172) as well as the stimulation of ACTH secretion in unanesthetized rats (173).

In the test system used by Royce & Sayers (156), young male rats that excrete 75 ml. or more of urine in the 24 hours following electrolytic destruction of the median eminence are selected for test 48 hours after the operation. The decrease in adrenal ascorbic acid 30 minutes after removal of one adrenal and intravenous injection of extract is taken as a measure of the ACTH secretion produced by the extract. Corrections for contamination of the extract are obtained by separate bioassays for vasopressin (174) (which, in high doses, stimulates ACTH secretion in the lesioned rat) and ACTH (175). In the hands of Royce & Sayers, there was no significant difference between the response to ACTH in rats 48 hours after median eminence lesions and that in 24-hour hypophysectomized rats, while others (157, 176) required three or more times the dose of ACTH in the lesioned rat to produce the same effect as that obtained in the hypophysectomized rat.

The procedure just described is a simplification of the rather cumbersome method recommended earlier by Sayers (177), in which extracts were tested in decerebrate rats with median eminence lesions following bilateral adrenalectomy two or more weeks earlier to allow time for development of the increased level of blood ACTH required for quantitative assay. After injection of the test substance the concentration of ACTH in the blood was estimated by bioassay in hypophysectomized rats.

In the procedure of Porter & Jones (158), intact rats are pretreated

with a large dose of cortisol acetate (6 mg./100 gm.) four hours prior to removal of one adrenal gland and intravenous injection of test extract. A second injection is made 30 minutes later, and 60 minutes later the second adrenal is removed. As in the procedure of Royce & Sayers (156), the effect on ACTH secretion is indicated by the difference in ascorbic acid concentration between the two adrenal glands.

The assay system developed by Leeman (178) and Leeman & Munson (179) involves the use of three procedures in each of which reduction of adrenal ascorbic acid of rats is used as the indicator of increased ACTH in the circulation. First, the intravenous dose-response curve for the extract in intact rats anesthetized with pentobarbital is obtained. Second, the relative effect of a submaximal dose of the extract in the anesthetized rats is determined with and without pretreatment with morphine. Lack of inhibition of the effect by morphine is an indication of the absence of nonspecific active substances, and the extent of noninhibited effect is taken as a measure of the amount of hypothalamic hormone. Use of submaximal doses of extract is important to avoid misinterpretation of a strong nonspecific stimulus that has surmounted the morphine inhibition for a hormonal effect that is not inhibited by morphine. The extent of contamination of extracts with ACTH must be measured in hypophysectomized rats in the usual way (175, 180, 181). The intact rat given pentobarbital and morphine cannot discriminate between hypothalamic hormone and ACTH; indeed, the response to intravenous ACTH was quantitatively identical in the three types of test rat, within the limits of experimental error. Extracts free of nonspecific stimulating substances and ACTH may be assayed reliably in anesthetized intact rats without morphine.

Guillemin *et al.* (176) have modified this test system in several important ways. Fifteen minutes after intravenous injection of extract into rats pretreated with pentobarbital and morphine, a blood sample is collected for fluorometric estimation (182, 183) of plasma corticosterone. Previous studies (168) had shown that a maximum effect of ACTH was attained in hypophysectomized rats 15 minutes after injection, with a linear log dose-response curve. A similar relation held between the log dose of a purified posterior pituitary extract (Fraction D) and the response (176). Determination of plasma corticosterone was preferred to adrenal ascorbic acid as a measure of ACTH secretion because the former is more obviously related to the principal function of the adrenal cortex than the latter, and because the minimum dose of extract required for an effect on plasma corticosterone appeared to be lower than that for an effect on adrenal ascorbic acid by a factor of 20 or more. (The ratio of the two minimum effective doses for ACTH was not consistently greater than two.) One criticism of the test system, its response to vasopressin (minimum effective dose: 100 milliunits), and presumably other nonspecific substances given in a large enough dose to surmount morphine inhibition, could be met by demonstrating equivalent responses to extract by test rats with and without

morphine, as in the method of Leeman & Munson. The methods of Royce & Sayers (156) and of Porter & Jones (158) are open to a similar criticism and a similar correction.

The *in vitro* assay system originating with Saffran and co-workers is based on the increased amount of ACTH found in the medium following incubation of rat pituitary tissue with test extracts. As described in its most recent form by Schally *et al.* (184), after "preincubation" of pairs of vessels, each containing half of a rat anterior pituitary, for one hour, the media are discarded, fresh medium alone is added to the control vessel and medium plus extract to the other. At the end of one additional hour of incubation, the relative ACTH content of the media in the paired control and experimental vessels is estimated by bioassay. A ratio > 1.0 at P.05 is considered to indicate the presence of hormone in the extract. (In order to correct for contamination of the medium with ACTH from the extract, extract is added to the control vessel at the end of the second incubation). Under favorable conditions a log dose-response relationship has been demonstrated (185), but apparently this has not been a consistent finding. Ordinarily, the minimum effective dose of the extract is estimated by testing serial dilutions. The "preincubation" part of the procedure was introduced in order to decrease and make more consistent the amount of ACTH released by control tissue. The ACTH in the media is assayed by an *in vitro* method (163), in which the production of ultraviolet-absorbing material by incubated quartered rat adrenals is linearly related to the log dose of ACTH. The *in vitro* system used in the laboratory of Guillemin (186) for assay of hypothalamic hormone is essentially identical with the one described by Schally *et al.* (184), except for omission of preincubation.

The assay system just presented has been subjected to several criticisms. The *in vitro* assay method for ACTH responds nonspecifically to plasma and plasma fractions (187) although their effects, unlike those of ACTH, are not related to the dose (162). Furthermore, the method estimates the ACTH content of rat pituitary tissue to be three to 10 times greater than that found by other methods (165, 166, 167), raising the possibility that part or all of the increase measured may not be ACTH. However, as exemplified by Swingle *et al.* (188, 189), increases in the ACTH content of the medium of incubated rat pituitary are not dependent on the *in vitro* method for ACTH assay; they may also be demonstrated by the classic adrenal ascorbic acid method. Lack of precision of the Saffran method seriously restricts its usefulness for quantitative bioassay. However, the most important limitation of the method is its lack of specificity. Fortier & Ward (190) found activity in the peripheral plasma of rats with no correlation between the effect and the endocrine status of the animal. Saffran (185) obtained positive results with synthetic oxytocin, Pitressin, purified lysine vasopressin, and synthetic arginine vasopressin, and, in addition, extracts of brain cortex, substance P from horse and cow gut, and hydrolysates of substance P and of Pitressin were found by others (186, 189)

to be active. Saffran (185) suggested that the scattered activities might be explained as an overlap of activities between peptides of similar structure, like the analagous behavior of oxytocin and vasopressin in other test systems. A more skeptical position was taken by Fortier & Ward (190), who stated that a positive result may mean either that the added material (*a*) contains ACTH, (*b*) potentiates the action of ACTH in the assay method for ACTH, (*c*) protects ACTH from inactivation, (*d*) actually stimulates ACTH release, or a combination of these effects. Items (*a*) and (*b*) were eliminated by adding the test material to the control medium at the end of the incubation before assaying for ACTH. Item (*c*), also suggested by Barrett & Sayers (191) was shown not to occur in experiments by Saffran (185) and by Guillemin & Schally (192), who demonstrated that no loss of ACTH activity occurred during the incubation period, either with or without the addition of extract.

The significance of the various purified extracts of hypothalamus or posterior pituitary must be evaluated in the light of the limitations of the test systems employed for identification and estimation of relative potency.

Purification of extracts.—Saffran *et al.* (193) were the first to obtain a substantial purification of the presumed hypothalamic secretory factor for ACTH. They applied the term corticotropin-releasing factor (CRF) to the postulated neural hormone. The term was appropriate for the *in vitro* test system employed; it has been used widely and is generally accepted. The reviewer prefers hypothalamic secretory factor for ACTH (HSF-ACTH) because, although somewhat more cumbersome, it denotes the physiological activity of the factor under study without restriction to a particular assay system.

Protopituitrin, an intermediate fraction in the commercial preparation on posterior lobe extracts, was chosen for further study by Saffran and coworkers because of its high CRF activity and because it was more readily available than hypothalamic extracts as starting material. Schally *et al.* (184) purified protopituitrin by serial paper chromatography in four different solvent systems. In this way an active fraction was repeatedly obtained which was separated from ACTH, oxytocin, and arginine and lysine vasopressins, and which was stated to be active in the *in vitro* test system at 0.001 μg., less than one-tenth the amount required of any other product yet reported. Amino acid analyses of two such products showed the presence of the amino acids of lysine vasopressin, plus serine, histidine, alanine, and arginine. Quantitatively, the two preparations differed considerably in per cent amino acid composition, suggesting, since they were equipotent, that neither one was homogeneous. The yield from 3 gm. of protopituitrin was 0.6 mg., an insufficient quantity for extensive further studies.

In more recent collaborative work by Schally & Guillemin (194, 195, 196) the paper chromatographic approach to purification has been superseded by procedures more adaptable to large-scale preparations. A continuing problem has been the separation of CRF from other peptides, partic-

ularly vasopressin and melanocyte-stimulating hormone (MSH), which when they themselves are tested in pure condition are not without effect on the *in vitro* test system.

Protopituitrin or "pitressin intermediate" was first treated three times with oxidized cellulose to remove the major portion of ACTH present. Inactive material in an acetic acid solution of the product was precipitated by acetone. Addition of ether precipitated the activity, which was applied to a column of carboxymethylcellulose. Eluates were tested for CRF by the *in vitro* method and also for MSH, ACTH, oxytocin, and vasopressin. Oxytocin, β-MSH, arginine vasopressin, and ACTH were well separated, but the CRF activity resided in a single peak that also contained α-MSH and lysine vasopressin. The eluates containing CRF were subjected to countercurrent distribution and, in confirmation of Gros & Privat de Garilhe (197), two peaks were separated, both containing CRF activity. One contained lysine vasopressin and the other α-MSH, poorly separated from CRF activity. Attention was first directed to the CRF activity accompanying α-MSH. In a preliminary report with insufficient detail for critical evaluation it was stated that the CRF, denoted α-CRF because of its association with α-MSH, could be separated from α-MSH by paper chromatography or by column chromatography on carboxymethylcellulose. It was thought to consist of a single peptide or a mixture of peptides containing all the amino acids of α-MSH plus threonine, alanine, and leucine. It had ACTH activity of 0.1 unit/mg., vasopressin activity of 0.1 to 0.2 unit/mg. and about 1/10 to 1/15 the MSH activity of α-MSH. It was stated, without documentation, to be active *in vivo* and *in vitro* at dose levels of 0.5 to 2 μg. The material studied previously (194), called β-CRF, differed from α-CRF by the presence of cysteine, a 50 to 100-fold higher potency, and the absence of ACTH activity. Because of its higher potency, β-CRF obtained by countercurrent distribution was restudied (196). A somewhat improved but still unsatisfactory separation from lysine vasopressin was obtained by differential paper chromatography. A differentiation between the two substances was claimed on the basis of the concentration of CRF activity in the descending limb of the nitrogen curve, and biological activity *in vitro* and *in vivo* at 0.05 to 0.1 μg., in contradistinction to lysine vasopressin, which requires much larger doses. The task of clear separation of the two substances, however, still lies ahead.

Porter & Jones (158) observed that intravenous injection of plasma collected from the sella turcica of the dog following removal of the pituitary stimulated ACTH secretion in rats pretreated with cortisol. After fractionation of the plasma by the alcohol procedure (198), the activity was restricted to Fraction II + III (199); in the zinc precipitation method (200) it was found in Fraction III_0 (201). The latter fraction was separated into three zones by starch-column electrophoresis: the middle zone contained the activity (202). Under special conditions, the activity was dialyzable, suggesting dissociation from a carrier protein. Contamination

with ACTH, vasopressin, norepinephrine, epinephrine, histamine, and serotonin was minimal or undetectable, and systemic plasma was inactive. Rumsfeld & Porter have also reported (203) the fractionation of an acetone extract of bovine hypothalamus. Material obtained from the aqueous phase after partition of the acetone extract between ether and 0.9 per cent NaCl, containing no detectable ACTH activity, was fractionated first by ion exchange chromatography then by countercurrent distribution, resulting in the isolation of two active fractions, distinct from lysine vasopressin by paper chromatography. However, the minimum effective dose level was not stated.

Royce & Sayers (204) prepared an extract of acetone-dried and defatted calf median eminence and pituitary stalk tissue by refluxing in glacial acetic acid. The activity was precipitated with acetone and ether, extracted with 0.01 *M* ammonium acetate buffer at pH 5.8, and applied to a column of carboxymethylcellulose. In the median eminence-lesioned rat, marked hypothalamic hormone activity, substantially free of ACTH and vasopressin, was consistently found in one set of eluates at a dose level of 5 μg., equivalent to one-third of a median eminence-stalk section. In an earlier study (156) the initial extract was prepared with 0.2 *M* acetic acid and much of the ACTH was removed by treatment with oxycellulose. This relatively crude extract was active in the lesioned rat at a dose equivalent to as little as one-fortieth of a median eminence, suggesting that a considerable loss of activity was associated with the purification described above. McCann & Haberland (157) prepared and tested similar crude extracts from bovine and rat stalk-median eminence tissue with results in essential agreement with Royce & Sayers (156). McCann & Haberland concluded that the presence of a hypothalamic hormone had been demonstrated in the crude extracts, since the amounts of contaminating ACTH and vasopressin were insufficient to account for the activity in rats with median eminence lesions.

Leeman & Voelkel (205) homogenized fresh calf hypothalmus with 0.1 N HCl, lyophilized the supernate and re-extracted the product with distilled water in the cold. The extract reduced the adrenal ascorbic acid in anesthetized intact rats, contained little ACTH and vasopressin, and the effect was not inhibited by morphine, indicating that the extract contained HSF-ACTH. Further steps in the purification of the extract have not yet been reported.

Vasopressin as the hypothalamic secretory factor for ACTH.—Since several if not all of the known biologically active constituents of the hypothalamus [epinephrine, norepinephrine, acetylcholine, serotonin, histamine, substance P, and γ-aminobutyric acid (206)] can stimulate secretion of ACTH if injected in adequate amounts, the possibility that one or another of them is the postulated hypothalamic secretory factor for ACTH has been considered. The case for vasopressin has been forcefully presented by Rothballer (207), McCann (171), and a number of others. Intravenously

injected vasopressin, both natural and synthetic, is active in all the *in vivo* test systems described above for the assay of hypothalamic hormone; it is also active in the method in which halved pituitaries are incubated *in vitro*. Morphine inhibits the effect of vasopressin in the intact rat, but the inhibitory effect is surmounted by a fourfold increase in dose (178). Evidence against vasopressin as the secretory factor for ACTH was reviewed by Ganong & Forsham (150). One of the objections raised, the rather large doses required for positive effects, is true only for vasopressin administered systemically. Kwaan & Bartelstone (208) have shown that as little as two milliunits of synthetic arginine vasopressin injected into the third ventricle of the intact dog produce a marked increase in the adrenal venous 17-hydroxy-corticosteroids; the same dose of synthetic oxytocin had no effect. However, there is still a sound basis for the current consensus of opinion that vasopressin is not the hypothalamic secretory factor for ACTH, chemical evidence in the separation of active fractions of hypothalamic and posterior pituitary extracts from vasopressin by several groups of investigators, as well as the dissociation of the ACTH-secretory from the antidiuretic effects of vasopressin in physiological experiments (150).

Other Topics

Biological assay of ACTH in blood.—Methods of biological assay for ACTH in plasma, their limitations and applications, particularly in the assay of human plasma, have been reviewed by Nelson (209) and Munson (210). The concentration of ACTH in normal human plasma is so low in relation to the dose requirement of currently available assay methods that concentrates of large volumes of plasma, prepared by as yet unperfected chemical procedures, must be administered in order to produce a minimum effect. However, rough quantitation of the elevated concentrations in the plasma of patients with Addison's disease or after bilateral adrenalectomy is possible. Several promising new assay methods, still under development, that require smaller amounts of ACTH were also described. The stability of ACTH in human and dog blood and plasma was studied by Meakin *et al.* (211, 212). Activity was lost rapidly over a period of several hours, but it was stable for several months if stored frozen. The inactivating system in plasma was destroyed by heat, confirming Pincus *et al.* (213), and was antagonized by L-cysteine. McFarland *et al.* (214) reported values much higher for ACTH in cavernous sinus blood (technique for collection described) of conscious sheep after electric shock than in peripheral blood which was collected simultaneously, confirming the results of Ganong & Hume (215) in the dog, but the unusually high values reported for peripheral blood raise some doubt about the reliability of the assays. Persky *et al.* (216) assayed extracts of blood from anxious patients and normal subjects for adrenal-stimulating factors in hypophysectomized rats. By the classic adrenal ascorbic acid method, ACTH was detected ($>$ 1.5 milliunit/100 ml.) in two of 11 patients and in none of the 15 controls. The adrenal

weight-maintenance factor [reviewed in (210)] was detected in eight of the patients and in only one of the controls.

Mechanism of action of ACTH on adrenal cortex.—The work of Haynes and co-workers, recently reviewed by Haynes *et al.* (217), Sutherland & Rall (218), and by Tepperman & Tepperman (219), has provided a rational biochemical basis for the effect of ACTH on the biosynthesis of the cortical hormones. ACTH was found to stimulate the accumulation of adenosine-3′,5′-monophosphate and the activation of phosphorylase in adrenal slices. The cyclic adenosine monophosphate mediates the effect of ACTH on phosphorylase in the adrenal cortex just as it mediates the effect of epinephrine and glucagon on phosphorylase in the liver. However, ACTH has no such effect on liver or muscle, and glucagon and epinephrine are ineffective in the adrenal cortex. The hypothesis outlined by Haynes states that ACTH, by increasing adenosine-3′, 5′-monophosphate and, in turn, active phosphorylase in the adrenal cortex, stimulates the breakdown of glycogen to glucose-1-phosphate, thence to glucose-6-phosphate. The further metabolism of glucose-6-phosphate by the dehydrogenase system leads to an increased supply of reduced TPN, known to be important in hydroxylating reactions in the biosynthesis of the adrenal cortical steroids. However, the predominant action of ACTH on steroid biosynthesis had previously been localized at the stage of conversion of cholesterol to progesterone, rather than in the later hydroxylations. The work of Halkerston *et al.* (220), therefore, showing the requirement of reduced TPN for sidechain cleavage of cholesterol was particularly important in establishing the pertinence of Haynes' hypothesis.

It is not yet clear how relevant the effect of ACTH on the formation of cyclic adenosine monophosphate is for the many other actions of the hormone on the adrenal cortex, including secretion of steroids and ascorbic acid and the stimulation of increased size of the gland. Other interesting data and speculations on the mechanism of action of ACTH on the adrenal cortex have been presented by Hayano *et al.* (221) and Hechter & Lester (222).

Inhibitors of adrenal function.—In 1955 Hertz *et al.* (223) reviewed the remarkable spectrum of biological effects possessed by amphenone B [1, 2-bis-(p-amino-phenyl)-2-methyl-propanone-1], including progestational, antithyroid, and anesthetic actions, and in the rat the production of marked adrenal hypertrophy with deposition of increased amounts of cholesterol. It is now clear from studies in the dog and in man that amphenone B interferes with the biosynthesis of the adrenal cortical steroids; adrenal hypertrophy results from increased ACTH secretion in response to the declining level of circulating glucocorticoids. Tests on a series of 61 analogues of amphenone B prepared by Bencze & Allen (224) showed that it was possible to vary the potency and quantitative pattern of activities. Sixteen of the active analogues were examined in some detail by Chart & Sheppard (225), for the effect on adrenal and thyroid weight, natriuresis-

and hypothermia in rats, the adrenal venous cortical steroids in the dog, and the action on *in vitro* corticosteroid production by rat adrenal slices. A pyridine analogue, methbipyrapone [1,2-bis-(3-pyridyl)-2-methyl-1-propanone] (SU-4885), relative to amphenone B, was more active as an inhibitor of adrenal function, possessed a narrower range of biological activities, and was less toxic. Primarily it inhibits the 11β-hydroxylation of steroids, thus eliminating or drastically reducing the biosynthesis and secretion of cortisol, corticosterone, and aldosterone. Chronic administration, however, may eventually lead to sodium retention, through compensatory increase in ACTH secretion and a marked overproduction of the active 11-deoxy mineralocorticoids, Substance S (11-deoxycortisol) and cortexone (deoxycorticosterone). In clinical use the abnormal steroid production may be avoided by simultaneous administration of a potent glucocorticoid to inhibit excessive ACTH secretion. SU-4885 is also useful in the differential diagnosis of pituitary-adrenal disorders, since if the anterior pituitary ACTH-secreting mechanism is capable of functioning normally, the administration of the drug is followed by a rise in 11-deoxy cortical steroid levels and by natriuresis. Additional details and an excellent review are included in the paper by Chart & Sheppard (225).

Aldosterone antagonists.—The native hormones, progesterone and testosterone, are weakly natriuretic and also show some antagonism to the action of mineralocorticoids. However, a series of synthetic steroidal spirolactones synthesized and studied by Cella & Kagawa (226) proved to be more highly effective aldosterone antagonists, active in man (227) as well as in experimental animals. Except for a very weak progestational effect, they are apparently without other significant biological actions. The effective daily dose in man of the original compound studied, 3-(3-oxo-17β-hydroxy-4-androsten-17α-yl)-propionic acid-7-lactone (SC-5233), was rather high—400 mg. intramuscularly or several grams orally. However, the 17-nor and doubly and triply unsaturated analogues were more potent. Spironolactone, the 7-acetyl thio derivative of the original compound, is available for clinical use and is effective in a daily dose of 100 mg. orally.

The spirolactones antagonize the retention of sodium, chloride, and water and the excretion of potassium induced by mineralocorticoids, but are without detectable renal effects in the absence of an excess of these steroids. The evidence that the mechanism of action of the spirolactones is a direct antagonism of the renal tubular action of aldosterone and other mineralocorticoids is as follows. They have little or no effect on urinary electrolytes in adrenalectomized animals or patients with adrenal insufficiency in the absence of mineralocorticoid therapy. The electrolyte excretion pattern produced by spirolactones in patients with intact adrenals is similar to that following abrupt withdrawal of aldosterone. The spirolactones do not act by interfering with the biosynthesis or secretion of aldosterone or other adrenal cortical steroids, since they have been shown not to cause significant changes in the rate of secretion of aldosterone, or in the

amounts of aldosterone, tetrahydro-aldosterone, 17-hydroxy-corticoids, or 17-ketosteroids in the urine. Therapeutically, the spirolactones used in conjunction with more conventional diuretic agents tend to combat the tendency of these drugs to induce kaliuresis through increased aldosterone secretion. Aldosterone antagonists have been reviewed recently by Beyer (228), Gaunt *et al.* (229), Kagawa (230), and Coppage & Liddle (231), and a symposium on the subject has been published in book form (232).

Chemical structure and biological activity of insulin.—A review of the chemistry of insulin by Sanger (233) includes a section on the relation between biological activity and chemical structure. That free amino groups are not essential is indicated by the absence of effect of acetylation on activity. On the other hand, since esterification leads to loss of activity, one or more of the six carboxyl groups must be essential. It is not the C-terminal alanyl carboxyl on the phenylalanyl (B) chain, since enzymatic removal of the alanine does not affect activity. Furthermore, C-terminal alanine (B chain) in bovine, pig, sheep, horse, and whale insulin is replaced by serine in rabbit insulin and by threonine in human insulin without detectable difference in activity. C-terminal asparagine (A chain), however, is essential as shown by loss of activity following its enzymatic removal. The importance of the phenolic hydroxyl groups of tyrosine was demonstrated by loss of activity following iodination. Species differences in the amino acid sequence inside the disulfide ring of the A chain—Thr · Ser · Ileu (pig, whale, rabbit, human); Ala · Ser · Val (bovine); Ala · Gly · Val (sheep); Thr · Gly · Ileu (horse)—without change in biological activity demonstrate the unimportance of the exact structure of this part of the molecule. It was concluded from the results of studies on the effect of leucine aminopeptidase that the first six (N-terminal) residues of the B chain are not essential for activity. After rapid splitting off of C-terminal alanine, trypsin slowly splits the bond between arginine and glycine in the B chain, yielding a heptapeptide, which is biologically inactive, and the residual molecule (DHA insulin). DHA insulin retained about 15 per cent of the activity of intact insulin, indicating that the absent fragment, although not essential, contributed materially to the activity. Recent work has suggested some modification of Sanger's statement that the disulfide bridges must remain intact for activity. After splitting the interchain disulfide bonds of insulin with sulfite, Cecil & Leoning (234) partially resolved the product into A and B chains. A sample of A chain containing approximately 20 per cent of B chain as impurity was tested for insulin-like activity in the isolated perfused rat heart (penetration of L-arabinose) (235) by Fisher & Zachariah (236) and found to be active, although less than 10 per cent as active as intact insulin. Whether or not unchanged insulin had been eliminated from the sample tested was not clear. Langdon (237) cleaved the disulfide bonds of insulin with thioglycollate or *n*-propyl mercaptan and separated the two resulting peptide chains. The A chain was biologically inactive. The phenylalanyl chain, as the phenylmercuric derivative, was obtained in crys-

talline, electrophoretically homogeneous form. It was active in stimulating the metabolism of rat adipose tissue in two *in vitro* test systems (238, 239). The amounts tested, however, were 200 times or more than the equivalent amount of insulin required for similar effects, and the test systems are not specific for insulin. Therefore, although care was taken to exclude the possibility that unchanged insulin contaminated the test sample, some doubt remains as to the significance of the results.

Orally active hypoglycemic agents.—A detailed critical review by Duncan & Baird on the pharmacology of the orally active hypoglycemic agents, with emphasis on the current status of knowledge of mechanisms of action, was published in March, 1960 (240). There are shorter reviews by Ashmore (241) on mechanisms of action and by Dunlop (242). The paper by Mahler (243) is concerned principally with the relation between chemical structure and biological activity.

Relation of chemical structure to biological activity of analogues of thyroxine.—The approximate relative potencies of 55 active analogues of thyroxine in three types of biological assay, oxygen consumption, growth and differentiation, and prevention of thiouracil-induced goiter, have been tabulated and evaluated by Pitt-Rivers & Tata; 70 inactive analogues were also listed (244). The data were obtained from the reports of many different investigators; it was hardly feasible to express the results in the language of biostatistics. Nevertheless, certain conservative generalizations were possible. The basic diphenyl ether structure is essential but not in itself adequate for activity. At least two halogen substituents are necessary; the highest activity being associated wtih iodine substitution, bromine less, and chlorine still less. Trihalogenated compounds substituted in the 3, 5, and 3′-positions are almost invariably more active than the corresponding tetrahalogenated compounds. The position of the halogen substituents, the length of the side chain, the phenolic group and its degree of ionization, and the presence of a free carboxyl group in the side chain are additional important structural features.

The data reported by Stasilli *et al.* (245) concerning the relative potency in the goiter-prevention and calorigenic assay methods of 45 compounds related chemically to thyroxine were included in the summary by Pitt-Rivers & Tata. Subsequently, the same compounds were tested by Money *et al.* (246) for their inhibitory effect on collection of ^{131}I in the rat thyroid. Twenty-seven compounds were tested by Kumaoka *et al.* (247) for their effect in depressing the growth of a thyroid-stimulating hormone-secreting pituitary tumor in mice; thyroxine and triiodothyronine were the most potent compounds tested, and in general there was a high correlation with results obtained in the goiter-prevention assay. The lactic acid analogues of 3, 5-diiodothyronine, 3, 5, 3′-triiodothyronine and thyroxine were found to be less potent in the goiter-prevention assay than the corresponding parent compounds and acetic and propionic analogues (248). All analogues of thyroxine tested depress serum cholesterol at lower dose levels

than are required to increase oxygen consumption *in vivo* in the rat and in man (249, 250) or by excised tissues after previous treatment of the whole animal (249). However, the recent discovery that androsterone depresses serum cholesterol (24) should turn the search for compounds with this property in a new direction.

Pittman & Barker (251, 252) discovered that the weakly thyromimetic compounds, 3, 3′, 5′-DL-triiodothyronine and 3, 3′-DL-diiodothyronine, and their propionic acid analogues, in doses 100 times that of thyroxine, completely and reversibly antagonized the calorigenic effect of thyroxine in thyroidectomized rats. At the same dose levels, the acetic acid analogues were less active. Previously known antagonists, reviewed by Selenkow & Asper (253), were much weaker, with effective doses 250 to 2000 times that of thyroxine. The limited supply of 3, 3′, 5′-triiodothyronine has precluded extensive tests in man. In three of four treated hypothyroid patients, the compound significantly depressed the basal metabolic rate. It was inactive in one euthyroid subject and in one patient with hyperthyroidism (254). At higher doses, however, it reduced the basal metabolic rate in one patient with Graves' disease (255). Jorgensen *et al.* (256) reported four alkyl-substituted analogues of thyroxine that antagonized the antigoitrogenic effect of thyroxine at dose levels 100 to 200 times that of thyroxine. In intact rats they did not antagonize the calorigenic effect of thyroxine; they were not tested in thyroidectomized rats.

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