$R_F = 0.73$; unknown, $R_F = 0.73$). In the second dimension, 1-butanol, saturated with water and ammonia (100 : 1, by volume), gave R_F values of 0.60 and 0.58 for the thymine standard and unknown compound, respectively. A chloroform, acetic acid (95 : 5, by volume) solvent system was used in the polyamide thin-layer chromatography. Both the unknown compound and thymine had R_F values of 0.60 in this system. The unknown was eluted from chromatograms with distilled water. In control experiments, uracil and paraformaldehyde, or paraformaldehyde and hydrazine, or uracil and hydrazine, under identical conditions gave negative results. These experiments excluded the possibility of contamination giving rise to thymine. Apart from the evidence obtained from chromatographic procedures, the unknown compound was also identified as thymine by its ultraviolet adsorption spectra at three different *p*H's—2, 10, and 12 (Fig. 1). An inspection of the ultraviolet spectra of uracil, 5-hydroxymethyluracil, and thymine at *p*H 10 will indicate the value of recording the spectrum of the unknown compound at this *p*H. Conclusive evidence was obtained by mass spectral analysis of the isolated, pure compound. The analysis shows a molecular ion of mass number 126 with three major fragmentation ions of mass numbers 83, 55, and 28. Thymine under the same conditions yielded a molecular ion of 126 and an identical fragmentation pattern. Molecular ions are observed for pyrimidines, and the fragmentation pattern is dependent on the nature of the substituents and their positions on the pyrimidine ring (*13*). This excludes the possibility that the unknown compound is an isomer of thymine.

The above experiments were carried out in air. To exclude the possibility that oxygen was involved in the reaction, a reaction was carried out in a solution purged with nitrogen prior to the addition of hydrazine. The reaction vessel was tightly stoppered, and the solution was heated for 3 days as usual. However, during the isolation of the unknown compound, air was not excluded. Thymine was isolated as in previous experiments.

The yield of thymine in this reaction was found to be 0.1 percent, as determined by the isotope dilution method. This yield is low but is in accord with other prebiotic syntheses. Oró and Kimball (*14*) obtained a 0.5 percent yield for adenine from an 11.1*M* solution of ammonium cyanide. The yields obtained for uracil in the condensation of β-aminopropionamide with urea in ammoniacal solutions at 135°C were also less than 1 percent (*2*). Sanchez *et al.* (*3*) obtained 5 percent yields of cytosine by heating cyanoacetylene with potassium cyanate in an aqueous solution at 100°C for 1 day. Fox and Harada reported yields of more than 10 percent in their synthesis of uracil (*1*).

This abiotic formation of thymine, subject to confirmation, completes the list of nucleic acid bases synthesized under prebiotic conditions. Of interest is the similarity of this particular abiotic synthesis to the normal biological pathway (*8*).

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Calcium as a Mediator of Adrenocorticotrophic Hormone Action on Adrenal Protein Synthesis

Abstract. *Calcium stimulates leucine incorporation into protein during incubations of sections and cell-free preparations of the rat adrenal. Like adrenocorticotrophic hormone (ACTH) action, calcium enhances the transfer of amino acid from transfer RNA to protein. Stimulation of leucine incorporation by ACTH and cyclic adenosine monophosphate is best observed when sections are incubated in limiting calcium concentrations.*

While it has long been recognized that calcium is required during the steroidogenic action of adrenocorticotropin (ACTH) (*1*, *2*), the role of calcium is obscure. Adenosine 3′,5′-cyclophosphate (cyclic AMP) apparently mediates the ACTH effect on steroidogenesis (*3*), and Lefkowitz *et al.* (*4*) have found a requirement for calcium in the activation of adenyl cyclase by ACTH; but, in addition, calcium must act *after* generation of cyclic AMP, since the steroidogenic action of the latter also requires calcium (*5*). I have observed a marked effect of calcium on adrenal protein synthesis (*6*), and this effect may largely explain the calcium requirement during stimulation of steroidogenesis, since the latter is known to require continued protein synthesis (*7*).

In addition to steroidogenesis, ACTH controls various metabolic processes in the adrenal cortex, including, and perhaps most fundamentally, adrenal growth. The mechanism or mechanisms whereby ACTH controls adrenal growth is not well understood. Administration of ACTH leads to increased protein synthesis in cell-free preparations of the rat adrenal, and this is due to increased activity in the supernatant after centrifugation at 105,000*g* (105,000*g* supernatant) (*8*) and in the microsomes (*9*). The increase in supernatant activity precedes the increase in microsomal activity (*10*) and is due to a macromolecular factor which enhances the transfer of amino acid from the aminoacyl–transfer RNA (tRNA) complex to protein (*8*) and appears to be identified with the so-called "transfer enzymes" (*11*). The mechanism whereby ACTH increases transfer enzyme activity is unknown.

In accord with the hypothesis that cyclic AMP is an intracellular "second messenger" for ACTH (*3*), Ney (*12*)

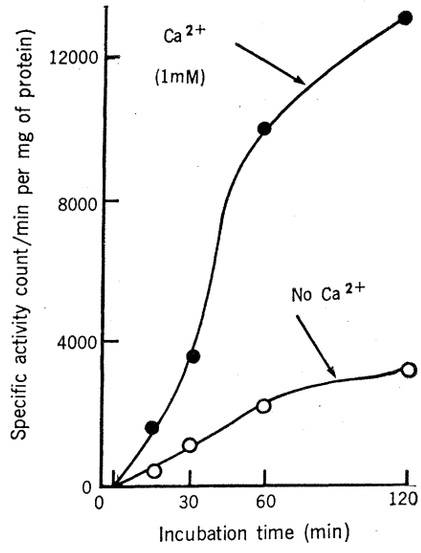

Fig. 1. Effects of calcium on incorporation of [^{3}H]leucine into protein of rat adrenal sections. The sections were first incubated for 60 minutes in 2 ml of calcium-free KRB (with 8 mM glucose and 0.76 mM aminoglutethimide, final concentrations) and 0.5 ml of saline containing 0 or 1 mM $CaCl_2$ (final concentration). The medium was replaced by fresh medium and [^{3}H]leucine (1 μc) was added, and the mixtures were then incubated for the indicated time.

has shown that cyclic AMP mimics ACTH in partially preventing adrenal atrophy after hypophysectomy. However, further evidence that cyclic AMP may be the mediator of the ACTH effect on adrenal growth is lacking [cyclic AMP does not directly stimulate either amino acid incorporation into protein or nucleotide incorporation into RNA in cell-free preparations of the rat adrenal (*13*)]. Indeed, it has been difficult to study early effects of ACTH and cyclic AMP on adrenal growth, since with in vitro systems, such as the commonly used rat adrenal section system, these substances have little or no effect on protein and RNA synthesis (*14*).

I now report that (i) calcium stimulates amino acid incorporation into protein of rat adrenal sections; (ii) calcium mimics the induced effect of ACTH by directly stimulating the transfer step (see above) of protein synthesis in adrenal cell-free systems; and (iii) it is possible to demonstrate consistent stimulatory effects of both ACTH and cyclic AMP on protein synthesis in rat adrenal sections by controlling the calcium concentration in the medium. My results suggest that calcium mediates the ACTH effect on adrenal protein synthesis, and this supports the hypothesis of Rasmussen (*15*) that cyclic AMP controls intracellular metabolism by regulating calcium flux.

The conditions for incubation of rat adrenal sections and measurement of incorporation of [4,5-^{3}H]leucine into protein have been described previously (*14*), the only exception being that the washed protein residues were dissolved in 1N sodium hydroxide and portions thereof were taken for protein determination and measurement of radioactivity by combining with Instagel (Packard; efficiency for tritium was approximately 23 percent). Synthetic β^{1-24}-ACTH (Organon) and cyclic AMP (Schwarz BioResearch) were in the incubations of sections, and aminoglutethimide (Sterling-Winthrop) was present in the media ($7.6 \times 10^{-4}M$) to prevent accumulation of corticosteroids and consequent inhibition of leucine incorporation (*14*).

The conditions for incubation of cell-free preparations of the rat adrenal have also been described (*8*), modified only by substituting [4,5-^{3}H]leucine (Amersham/Searle, specific activity = 1 c/mmole) for [^{14}C]glycine during the incubation, and by using the above-mentioned technique for measuring radioactivity in protein. The incorporation of amino acid into the RNA extract [that soluble in hot 5 percent trichloroacetic acid (TCA) after the cold TCA-precipitable material was heated for 15 minutes at 90°C] was used to assess the formation of the aminoacyl–transfer RNA complex in the cell-free system, and when unlabeled amino acid (*8*) was added and the incorporation of isotope was halted, the radioactivity in the RNA extract was associated with a rapidly turning-over intermediate in protein synthesis. The RNA extract was examined for RNA content (*8*) and for radioactivity by combining with Instagel.

As shown in Fig. 1, when adrenal sections were incubated in Krebs-Ringer bicarbonate buffer (KRB) containing 1 mM calcium or none at all, there was a marked stimulatory effect of calcium on incorporation of [4,5-^{3}H]-leucine into protein. The marked inhibition of amino acid incorporation due to the absence of calcium was fully reversible, and could not simply be explained by a generalized depression of cellular metabolism, since incorporation of [6-^{14}C]orotic acid into RNA (which also requires energy) was significantly stimulated when calcium was absent (*16*).

In experiments on the ionic requirements for ACTH action, Peron and Koritz (*2*) also noted that calcium promoted glycine incorporation into protein, but at that time it was not realized that protein synthesis is required during the steroidogenic action of ACTH

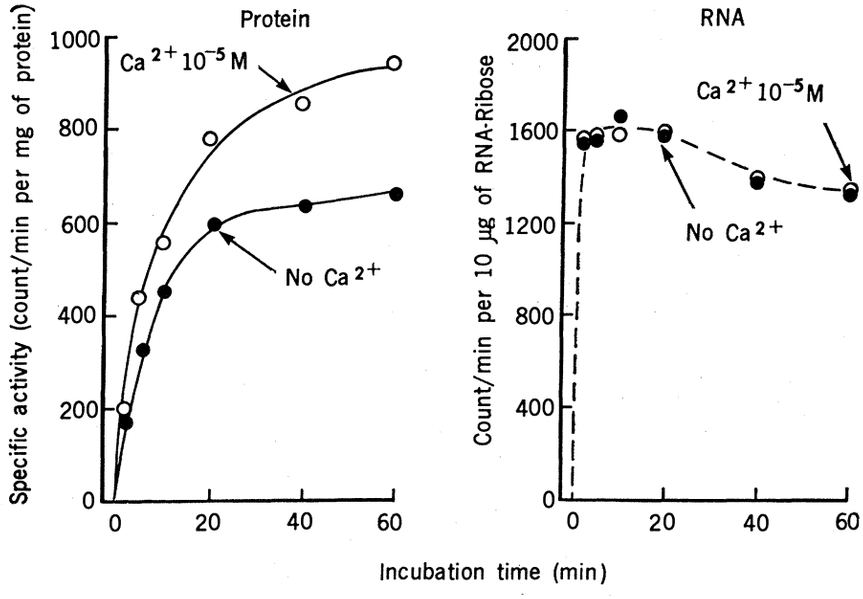

Fig. 2. Effects of calcium on incorporation of [^{3}H]leucine into protein and RNA fractions of cell-free preparations of the rat adrenal. Each incubation tube contained, in a total volume of 0.55 ml: 15,000g supernatant from 20 mg of control adrenal tissue (containing approximately 1.7 mg of protein and 12 μg of RNA-ribose); 0.5 μc of [^{3}H]-leucine; 0.5 μmole of ATP; 0.125 μmole of GTP; 5 μmole of phosphoenolpyruvate; 25 μg of phosphoenolpyruvate kinase; 0.213M sucrose; 0.006M $MgCl_2$; 0.021M KCl; and 0.043M tris buffer, pH 7.5. Comparable results were obtained in duplicate experiments.

and the relation of calcium to adrenal protein synthesis was not pursued. In experiments (*16*) in which steroidogenesis was not blocked by aminoglutethimide, the degree of inhibition of steroidogenesis induced by ACTH or cyclic AMP correlated well with the degree of inhibition of amino acid incorporation because of deleting or limiting calcium, and this suggested that the calcium requirement during steroidogenesis is largely explained by effects of the ion on protein synthesis.

As shown in Fig. 2, in the cell-free system calcium directly stimulated leucine incorporation into protein [peak effects were usually observed on adding $10^{-5}M$ $CaCl_2$ (*16*)], but had no effect on incorporation of leucine into the RNA fraction. Since the latter assesses the first two steps in protein synthesis, that is, amino acid activation and formation of the aminoacyl-tRNA complex (*8*), it would appear that calcium stimulates a subsequent step or steps in protein synthesis. In other experiments (*16*), calcium indeed enhanced the transfer of leucine from leucyl-tRNA to protein, and this was apparent if the 105,000*g* supernatant was obtained from control, but not ACTH-stimulated, rat adrenals. Thus, calcium, like ACTH, stimulates the transfer of amino acid from aminoacyl-tRNA to protein, and, moreover, this effect is apparent only if the transfer enzymes are not already maximally stimulated by ACTH. This suggests that calcium mediates the ACTH effect on transfer enzymes, and this is supported by other experiments (*16*) in which a calcium chelator (EGTA) was found to obliterate the difference in activity between supernatants from control and ACTH-stimulated adrenals.

Since these results suggested a role for calcium in the stimulation of adrenal protein synthesis by ACTH, it was of interest to return to incubations of rat adrenal sections. As mentioned above, it had not been possible to observe consistent stimulatory effects of ACTH or cyclic AMP on amino acid incorporation in this system. However, calcium was usually present in "excessive" or supraphysiological concentrations, 2 to 2.5 m*M*, and this may have masked hormonal effects if these revolved around calcium uptake. To bypass this problem and magnify calcium-dependent effects, adrenals were first depleted of calcium by incubation in calcium-free KRB, and then incubated in KRB containing 1 m*M* calcium. As shown in Fig. 3, and in (*14*), when adrenals were first incubated in KRB and then incubated in KRB with "excessive" (2.2 m*M*) calcium, there was no consistent effect of ACTH on amino acid incorporation into protein. However, when calcium was "limiting" (as described above), regular stimulatory effects of ACTH were observed. Cyclic AMP significantly stimulated leucine incorporation under conditions of "limiting" calcium, and this contrasts with the relatively small (virtually inconsequential) effect noted when calcium was present in "excessive" concentrations (*14*). This stimulatory effect of cyclic AMP, furthermore, required at least some calcium because, when calcium was deleted from both the first and second incubations, leucine incorporation decreased and the stimulatory effect of cyclic AMP was no longer apparent (Fig. 4).

The results suggest that calcium directly stimulates adrenal protein synthesis by enhancing the transfer of amino acid from aminoacyl-tRNA to

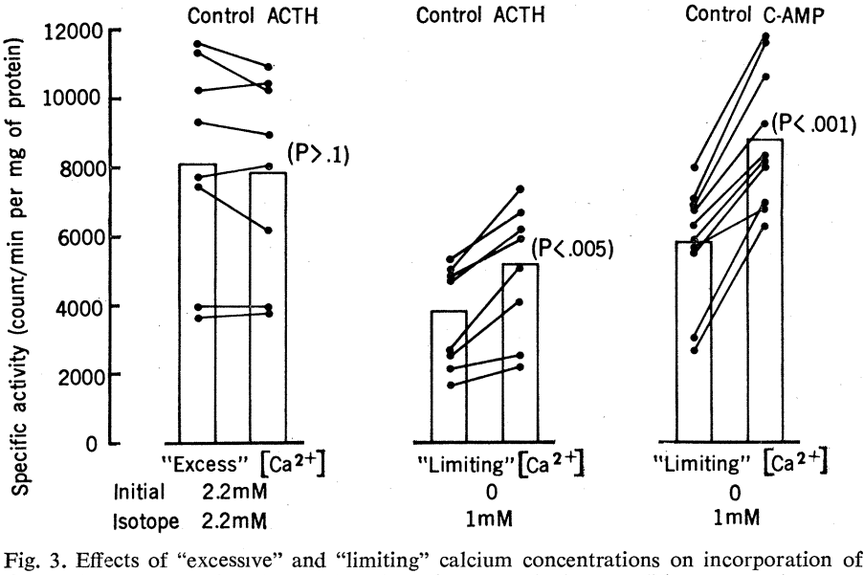

Fig. 3. Effects of "excessive" and "limiting" calcium concentrations on incorporation of [^{3}H]leucine into protein of rat adrenal sections. Incubation conditions were the same as described in Fig. 1, except that the cells were incubated in medium alone (*Initial*) for 60 minutes, and with fresh medium and isotope (*Isotope*) for 120 minutes at the calcium concentrations indicated; ACTH (25 μg) and cyclic AMP (5 m*M*) were present only during the incubation. The bars denote the mean values, and the points corresponding to control and stimulated values of each experiment are connected by lines. *P* was determined by *t*-test evaluation of the mean difference (paired data analysis). The experiments shown on the left (control compared to ACTH with "excessive" calcium) were conducted in parallel with the experiments shown at center (control versus ACTH with "limiting" calcium), so that in each experiment adrenal sections (quarters) from the same tissues (eight adrenals) were evenly distributed to flasks of all four experimental groups. In each experiment of the group shown on the right, adrenal sections (quarters) from four adrenals were evenly distributed to two flasks (control compared to cyclic AMP). All flasks had a total of eight adrenal sections.

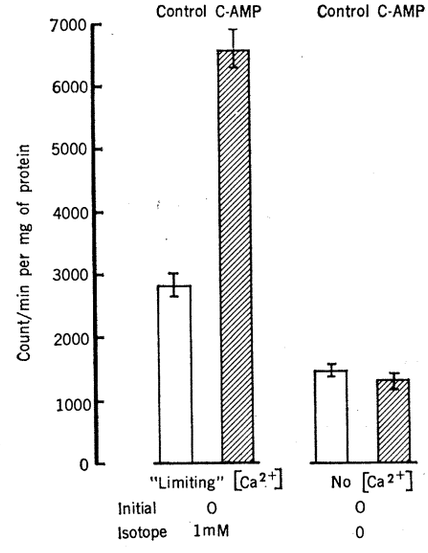

Fig. 4. Dependence of cyclic AMP–induced stimulation of [^{3}H]leucine incorporation on the presence of calcium. Adrenal sections (quarters) from eight rats were evenly distributed to eight incubation flasks (eight sections per flask), incubated without isotope for 60 minutes and then with isotope for 120 minutes in calcium-free KRB and saline containing calcium in the indicated final concentrations. Other experimental conditions are described in Figs. 1 and 3. Bars and brackets denote mean values and variations of duplicate flasks, respectively. Comparable results were observed in repeat experiments.

protein, and, since ACTH increases the adrenal uptake of ^{45}Ca (*17*), calcium may be responsible for the activation of the transfer enzymes by ACTH. Conceivably, this may be the initial or early step whereby ACTH stimulates adrenal growth, since the early in vitro effect of ACTH on leucine incorporation in rat adrenal sections appears to depend upon calcium. Since cyclic AMP (exogenous) also has calcium-dependent stimulatory effects on adrenal protein synthesis, it is possible that this nucleotide is ultimately responsible for the calcium-mediated stimulation of adrenal protein synthesis by ACTH. However, attempts to show that cyclic AMP enhances ^{45}Ca uptake by the adrenal have not been successful (*17*).

While the above findings suggest that calcium may (at least partly) mediate the effect of ACTH on adrenal growth, it should not be construed that calcium mediates the steroidogenic effect of ACTH. In other experiments (*16*), calcium *alone* stimulated adrenal protein synthesis, but had no effect on steroidogenesis unless ACTH or cyclic AMP was present. Thus, if ACTH induces the synthesis or activation of a metabolically labile, steroidogenic protein, the synthesis of this protein may require calcium to maintain optimum protein synthesis, but calcium itself does not appear to be the inducing factor.

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Melatonin and Abnormal Movements Induced by L-Dopa in Mice

Abstract. *Melatonin has blocked adventitious movements induced by* L-*dopa in intact mice. It has reversed the adventitious turning to the right, and it has induced running to the left in mice receiving* L-*dopa after a lesion in the right caudate nucleus.*

Administration of melanophore stimulating hormone (β-MSH) to patients with Parkinsonism darkened their skins and aggravated their tremor (*1*). The assumption was pursued that the darkening skin deprived the brain of neurotransmitters by sequestering their precursors (*2*). This led to control of Parkinsonism first with D,L- (*1*) and thereafter with L-dopa (*3*). We were much less impressed by therapy with an antagonist of β-MSH, melatonin (*4*).

At the peak of therapy with dopa, abnormal movements emerged (*1, 3*). These have been thought of [see (*4*)] as perhaps related to the diminution of cerebral serotonin by L-dopa (*5*). In turn, the concentration of serotonin in the brain was increased by administering melatonin (*6*).

The present experiments are not aimed specifically at treatment, but rather they serve to (i) illustrate a radical alteration of the effects of L-dopa by melatonin and (ii) settle, perhaps, some notions about treating Parkinsonism.

More than 500 intact male Swiss albino mice of the Hale-Stoner strain were tested. Under specified conditions (*7*), these mice developed abnormal movements from L-dopa (Nutritional Biochemicals Corp.), whereas those of another strain were highly resistant (*7*). In addition, 300 Hale-Stoner mice were included after partial suction of the right caudate nucleus. [The operations and the pretesting were performed according to Lotti (*8*)]. Immediately after operation, these animals turned their heads (and often their bodies and tails) to the right and retracted their limbs on the right under their bodies while extending those on the left (*9*). Over the next 10 days, they gradually resumed mobilities and postures similar to intact mice. Thereafter, apomorphine (Merck & Co.) given intraperitoneally (2 μg per gram of body weight) reproduced the adventitious turning and most of the other signs observed postoperatively. Animals previously tested with apomorphine were included in further experimentation a week later.

For the further experimentation, nonlethal doses were selected. The LD_{50} (lethal dose, 50 percent effective) of the orally administered melatonin (Sigma Chemical Co.; Regis Chemical Co.) was shown by probit analysis to be roughly 2.4 mg/g after 24 hours. The LD_{50} of melatonin given intraperitoneally was approximately 1 mg/g. Intraperitoneal injections of this hormone (0.4 to 0.8 mg/g) drastically diminished the mortality from oral doses of L-dopa [3 mg/g (*7*)], whereas orally administered melatonin failed to do so.

In intact mice, oral L-dopa (3.0 mg/g) induced the adventitious movements discussed earlier (*7*). Administration of melatonin (0.4 to 0.8 mg/g intraperitoneally or 1.0 to 2.5 mg/g orally), either together with L-dopa or 30 minutes prior to it, blocked the hypermobility while lessening piloerection and salivation (*7*). The duration of these effects appeared dependent upon the dose of melatonin. In the operated animals, furthermore, special combinations of melatonin with L-dopa induced the additional phenomenon discussed below. This is partially illustrated in Fig. 1.

Given alone to operated mice, L-dopa (0.2 to 0.6 mg/g) duplicated the turning to the right and often the other signs evoked by apomorphine, although L-dopa induced more hypermobility again (*7*). The apomorphine-like signs of L-dopa could be reversed by combinations with melatonin given either orally (each 1.0 mg/g) or intraperitoneally (each 0.4 mg/g). These combinations changed the effects of L-dopa. Instead of merely turning to the side of the lesion, the animals turned to the left and ran in that direction for ½ hour or longer. These specific combinations seemed critical to the evolution of the full phenomenon, but mere turning to the right could be blocked by other combinations of dopa with melatonin (Table 1), as was the case with the adventitious movements of intact mice.

In man, administration of L-dopa has markedly increased the excretion