The Structure of Growth Controlling Peptides for Mammalian Cells in Axenic Culture*

George L. Tritsch and Otto Grahl-Nielsen

ABSTRACT: Two peptides were isolated in pure form from tryptic digests of the α chain of human hemoglobin. The smaller peptide, Val-Leu-Ser-Pro-Ala-Asp-Lys, was synthesized from L-amino acids and was identical to the one isolated. Both peptides were equipotent on a molar basis in their cytotoxicity to mammalian cells in axenic culture. Levels of 10^{-5}–10^{-7} M reduced the viable cell number in suspension culture without a lag period. Serum protein was able to counteract the toxicity of both peptides. These findings demonstrate that small peptides of known structure are able to alter the growth of mammalian cells.

The growth of mammalian cells in axenic culture can be controlled by proteins in the medium. Whole serum is generally used to stimulate growth (Harris, 1964) even though considerable variability in the biological potency of different samples has been recorded (Puck *et al.*, 1958; Fedoroff and Cook, 1959; Phillips and Andrews, 1960). The coexistence of cytotoxic and growth stimulatory materials has been demonstrated in a single sample of serum (Chang *et al.*, 1959), and cytotoxic proteins (De Luca *et al.*, 1964), lipoproteins, and lipids (De Luca *et al.*, 1966a) have been isolated. Enzymatic digests of various proteins have also been reported to possess growth stimulatory as well as inhibitory activity (Eagle, 1960; Metzgar and Moskowitz, 1960; Moskowitz and Schenck, 1965; Pumper *et al.*, 1965; De Luca *et al.*, 1966b). In no instance has the structure of the active entity been determined. Reported herein is the purification and amino acid sequence of two toxic peptides from tryptic digests of the α chain of human hemoglobin. The smaller heptapeptide has been synthesized from L-amino acids and was identical with the one isolated.

Experimental Section

The α chain of human hemoglobin was prepared by countercurrent distribution (Hill *et al.*, 1963) of globin isolated from human erythrocytes obtained from the Buffalo Chapter of the American Red Cross. Tryptic digestion was carried out as described by Guidotti *et al.* (1962). The entire digest was lyophilized, dissolved in the least amount necessary of 0.2 M acetic acid, and pipetted onto a 2.5 × 120 cm column of Sephadex G-25. The solvent was 0.2 M acetic acid. The flow rate was regulated by adjusting the height of the reservoir and never exceeded 2 ml/min per cm^2 of column cross-sectional area. Several gel filtrations were required before pure peptides were obtained. Fractions of 5 ml were collected and analyzed for absorbance at 280 mμ and for free amino groups by reaction with trinitrobenzenesulfonic acid (De Luca *et al.*, 1966b). Suitable fractions were pooled and the solvent removed by lyophilization.

Amino acid analyses were performed on a Beckman–Spinco amino acid analyzer with the accelerated system of Spackman (1963). Hydrolysis of 1-mg amounts of peptide was carried out in 1 ml of distilled, constant-boiling HCl under N_2 in sealed ampules for 24 and 48 hr at 107–108°. From the amino acid composition of the peptide, the amino acid sequence was deduced by consulting the structure of human hemoglobin (Braunitzer *et al.*, 1964).

The assay of the biological activity has been described in detail (Tritsch *et al.*, 1967). Cell line RPMI No. 2402 which originated from a carcinoma of the small bowel of the Syrian hamster was used (Moore *et al.*, 1963). The starting cell density in each assay was $2 \times 10^5 \pm 10^4$ cells per ml and the incubation temperature was 37°; 1 hr after inoculation, the pH was readjusted to 7.2 if necessary and a cell count was performed in a hemocytometer. The ability of the cells to exclude trypan blue was used to measure viability (Hoskins *et al.*, 1956). In practice, 0.5 ml of the cell suspension was mixed with 0.25 ml of 0.1% trypan blue in the balanced salt solution of Earle (1943). Counting in the presence of this dye therefore indicated not only changes in cell number but changes in viability as well.

The cytotoxicity will be expressed as "t," the time in hours required to reduce the number of viable cells to half of that present at the start of the experiment, *i.e.*, from 2×10^5 to 10^5 cells per ml.

The Synthesis of L-*Val*-L-*Leu*-L-*Ser*-L-*Pro*-L-*Ala*-L-*Asp*-L-*Lys*. The solid-phase method of peptide synthesis developed by Merrifield (1964) was used. The synthesis was carried out in the apparatus described by Grahl-Nielsen and Tritsch (1969) and was started with 3.30 g of α-t-butyloxycarbonyl-ϵ-carbobenzoxy-L-lysine-

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resin (containing 0.77 mmole of L-lysine). The following amino acid derivatives were coupled successively to the peptide chain: α-*t*-butyloxycarbonyl-β-benzyl-L-Asp, *t*-butyloxycarbonyl-L-Ala, *t*-butyloxycarbonyl-L-Pro, *t*-butyloxycarbonyl-*O*-benzyl-L-Ser, *t*-butyloxycarbonyl-L-Leu, and *t*-butyloxycarbonyl-L-Val.

To facilitate the synthesis of analogs of the heptapeptide in the future, approximately half of the resin was removed from the reaction vessel after the prolyl residue had been coupled to the peptide chain, and the synthesis was continued with the remainder.

When the last amino acid had been added, the peptide was cleaved off the resin by bubbling HBr gas through a suspension of the dried, blocked heptapeptide-resin (1.95 g) in 15 ml of trifluoroacetic acid for 5 min. The trifluoroacetic acid was sucked off the resin, the resin was washed three times with 5 ml of trifluoroacetic acid, and the combined filtrates were evaporated by passing a stream of dry air over the surface. The resulting yellow syrup was dissolved in 5 ml of methanol and the product was precipitated by adding a large excess of ether. The white precipitate was washed with ether and dried under vacuum over P_2O_5. The yield was 358 mg. The short deblocking time was used to avoid undesired side reactions. To check if any peptide was left on the resin, the treatment was continued for another 55 min. This resulted in only 24 mg of additional crude product.

The 5-min treatment might have been too short to cleave all the side-chain-protecting groups. As all these groups contain a benzene ring and the desired peptide does not, removal of protecting groups was checked by determining the ultraviolet absorption spectrum of the crude product on a Cary Model 14 spectrophotometer. The peptide showed no absorption in the 240–270-mμ region which is characteristic for aromatic compounds. A sample of the deblocked heptapeptide was hydrolyzed for 30 hr as described and subjected to quantitative amino acid analysis. The following molar ratios were found: Val, 1.12; Leu, 1.00; Ser, 0.81; Pro, 1.03; Ala, 1.10; Asp, 1.12; and Lys, 1.06.

The crude heptapeptide (120 mg) was dissolved in 1 ml of 0.2 M acetic acid and placed onto a 2.5 × 100 cm column of Sephadex G-15 equilibrated with 0.2 M acetic acid. The column was developed with the same solvent and 50 drops (3.5 ml) were collected in each test tube in a fraction collector at a rate of approximately 5 sec between drops. Only one major peak was observed after reaction of portions of the eluate with trinitrobenzenesulfonic acid. The volatile solvent was removed from the desired fractions by lyophilization. The purity of the resulting peptide was checked by thin-layer chromatography on a 0.25-mm layer of silica gel G (Merck) developed with 88% phenol–water–$CHCl_3$ (8:1:1, v/v). The plate was developed with ninhydrin. A major spot of R_F 0.20 was observed and two minor spots of R_F 0.12 and 0.40, respectively. Traces of at least four other ninhydrin-reactive compounds could also be seen.

The high resolution obtained with this thin layer system was used on a preparative scale. The crude heptapeptide (35 mg) was dissolved in 0.5 ml of methanol and applied as a streak along the starting line of a 20 × 20 cm plate with a 1-mm layer of silica gel H (Merck). The plate was developed with the same solvent as above for 3.5 hr when the solvent had migrated 17 cm. After drying the plate at room temperature, narrow sections on both sides of the plate were developed with ninhydrin, and the portion of the adsorbent which contained the major fraction was scraped off the plate. Three plates with a total of 105 mg of crude peptide were developed. The remaining phenol was removed from the silica gel by washing it several times with anhydrous ether. To free the peptide from the silica gel and at the same time to eliminate the last traces of phenol, the silica gel was suspended in 0.2 M acetic acid and placed onto the top of the same Sephadex G-15 column as described above. After developing the column and detecting the peptide with trinitrobenzenesulfonic acid, 25 mg of dry product was obtained after lyophilization. The purity was checked by the same thin-layer system employed above. Only one spot of R_F 0.20 was observed with ninhydrin.

A sample of the purified peptide was hydrolyzed for 40 hr as described, and by amino acid analysis the following molar ratios were found: Val, 0.94; Leu, 1.00; Ser, 0.95; Pro, 0.99; Ala, 1.02; Asp, 1.04; Lys, 0.97.

Results and Conclusions

Structure of the Peptides. The isolation of the peptides in pure form by filtration through Sephadex G-25 is illustrated in Figure 1. The peptides isolated were Tα1[1] Val-Leu-Ser-Pro-Ala-Asp-Lys and Tα9 Val-Ala-Asp-Ala-Leu-Thr-Asp-Ala-Val-Ala-His-Val-Asp-Asp-Met-Pro-Asp-Ala-Leu-Ser-Ala-Leu-Ser-Asp-Leu-His-Ala-His-Lys. Contrary to expectation, the smaller peptide Tα1 eluted earlier than the larger peptide. The amino acid sequence of these peptides was deduced from their amino acid composition and the known structure of human hemoglobin.

Cytotoxicity of the Peptides. Both peptides were found to decrease the number of viable cells at microgram per milliliter levels. Bioassay of the various pooled effluent fractions indicated that all the fractions other than tubes 51–60 as illustrated in panel A of Figure 1 were inert. Figure 2 illustrates the results obtained with peptide Tα1. Similar data were obtained with Tα9. The data fall on the familiar exponential die-away curve. The semilogarithmic plot of Figure 2 was selected to permit the fitting of a straight line rather than a curve to the experimental points. This plot shows that the loss in viable cell number began upon contact with the peptide and followed first-order kinetics without a significant lag period. During the first 24-hr period, the total cell number in all flasks remained relatively constant, *i.e.*, cell lysis and fragmentation were minimal. Only after longer periods of time was there lysis and cell fragmentation as evidenced by a diminution in total cell number. The ability to count nonviable cells is subject to considerable error since it is difficult to distinguish cell frag-

[1] Abbreviations used: Tα1, the heptapeptide consisting of residues 1–7; Tα9, the nonacosapeptide consisting of residues 62–90.

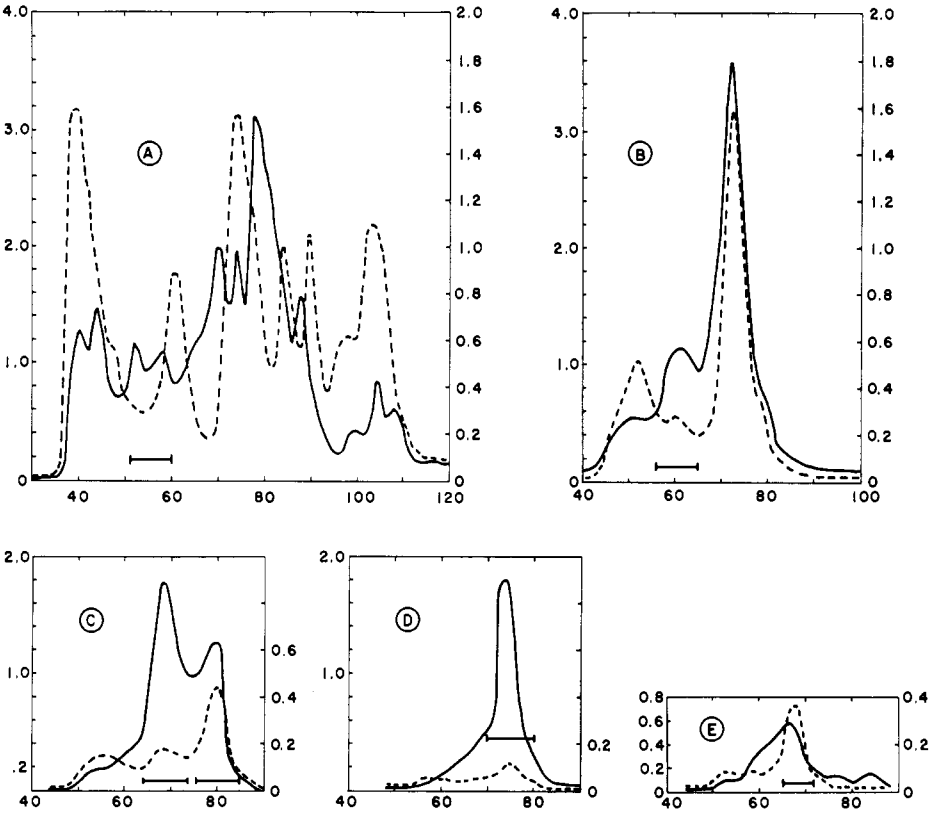

FIGURE 1: Purification of toxic peptides Tα1 and Tα9 from a tryptic digest of 425 mg of the α chain of human hemoglobin on a 2.5 × 120 cm column of Sephadex G-25. In each graph, the left ordinate (solid curve) is in terms of millimolar concentration of amino groups equivalent to leucine as measured with trinitrobenzenesulfonic acid, and the right ordinate (broken curve) is absorbance at 280 mμ, 1-cm light path, relative to 0.2 M acetic acid. The abscissa is the fraction number; in all instances, 5-ml volumes were collected. (A) Tryptic digest of the α chain. The contents of tubes 51–50 was pooled, lyophilized, and refractionated as shown in B. (B). The contents of tubes 56–65 was pooled, lyophilized, and refractionated as shown in C. (C) The contents of tubes 64–74 was pooled, lyophilized, and refractionated as shown in D to yield peptide Tα1 in tubes 70–80. The contents of tubes 75–85 of C was pooled, lyophilized, and refractionated as shown in E to yield peptide Tα9 in tubes 65–72.

ments from a nonviable and shrunken cell. The data in Figure 2 are not subject to this error since only the number of unstained viable cells was plotted.

Several formulations have been proposed to account for the relationship between the concentration of a toxic material to the magnitude of its effect (Hinshelwood, 1946). The duration of the lag phase, the maintenance of viability, the magnitude of the growth rate, and the maximum cell density attained should be taken into account. Since these may vary in complete independence in the presence of a toxic agent, no single mathematical expression can represent all aspects of the whole of the growth curve (Monod, 1942). Watson (1908) was able to account for similar data on the effect of different levels of phenol on paratyphoid bacilli (Chick, 1908) by the relationship $C^n t = K$ or $n \log C + \log t = K$, where C is the molar concentration of the material in question, n is a constant specific for the material in question, t is the time necessary for the attainment of a given effect, and K is a constant. From this equation it follows that a plot of $\log t$ *vs.* $\log C$ will be linear and the slope equal to $-n$. The data in Figure 3 show that over a range of concentration of 10^{-5}–10^{-7} M for both peptides, a plot of log C *vs.* t is linear with $n = 0.77$. This may be compared with values of n of 0.7–1.0 for $HgCl_2$, and 4–6 for phenol when tested with *Staphylococcus aureus* (Porter, 1946). From this plot it is clear that both peptides are equipotent at equal molar concentrations. At the lower concentrations tested, the effect approached the maintenance of viability in the absence of the peptides. A concentration of 10^{-6} M is approximately equivalent to 0.73 μg/ml for peptide Tα1 (molecular weight 729) and to 3 μg/ml for peptide Tα9 (molecular weight 3000).

Inhibition of Cytotoxicity by Serum Proteins. Serum protein was able to counteract the toxicity of both peptides. Figure 4 shows that over the entire range of calf serum protein concentration tested, peptide Tα1 was able to cause a diminution of the growth rate or a manifestation of toxicity at protein levels too low to stimulate growth. Diminution in cell number is indicated as a negative growth rate in Figure 4. The response to calf serum in the absence of Tα1 shown in Figure 4 is indistinguishable from previous experiments with this cell line and growth medium (Tritsch *et al.*, 1967; Tritsch, 1966; Rakowski and Tritsch, 1966).

Toxicity could be demonstrated with a cell line derived from a Burkitt lymphoma (Tritsch *et al.*, 1967). These cells require a different growth medium and relatively high levels of fetal calf serum as compared with the RPMI No. 2402 cells, and exhibit a lower viability

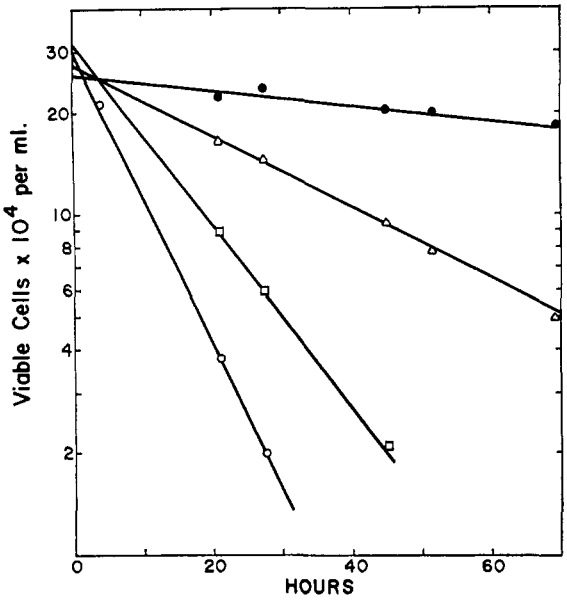

FIGURE 2: Cytotoxicity of synthetic peptide Tα1 in the absence of serum protein. The concentration of Tα1 was 1.4 (○), 1.12 (□), 0.112 (Δ), and 0.0 (●) × 10^{-6} M. The straight lines were drawn by eye. The data points at 0.5 hr were omitted for clarity.

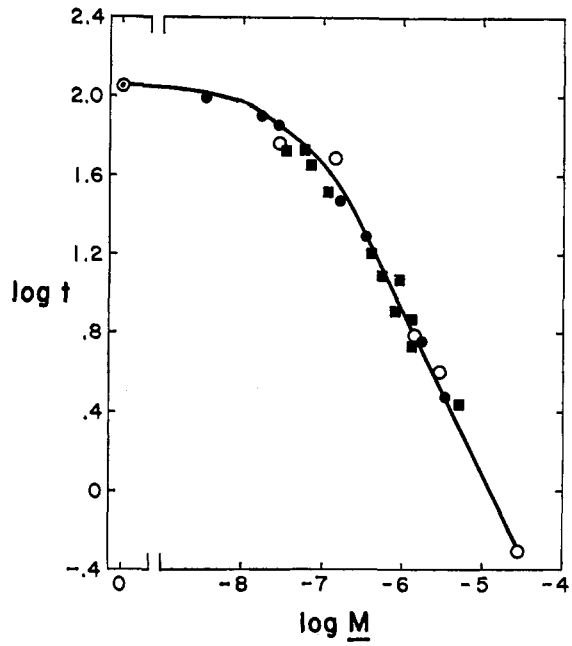

FIGURE 3: The relationship between concentration of peptide Tα9 (solid circles), isolated Tα1 (open circles), and synthetic Tα1 (solid squares), and the time required to reduce the number of viable cells to half of the inoculum. The curve was drawn by eye.

and maximum growth response. These cells are too fragile to permit experiments in the absence of serum proteins. Sufficient data for a plot similar to the one in Figure 4 were not accumulated, but serum protein could overcome the toxicity of synthetic peptide Tα1 in this system as well, even though different levels of protein and peptide were required to demonstrate this effect. The peptide prolongs the duration of the lag phase, reduces the maximum cell density attained, and slightly diminishes the maximum growth rate. In contrast to the RPMI No. 2402 cells, these cells showed a lag period even in the presence of serum protein. At higher levels of fetal calf serum protein (*i.e.*, 5.6 and 8.4 mg of protein per ml) the differences in the growth response in the presence and absence of peptide Tα1 were less pronounced but unequivocally indicative of growth inhibition by 7 × 10^{-6} M peptide.

In the experiments just described, serum protein and peptide Tα1 were present simultaneously. The effect of calf serum on cells exposed to the peptide alone for different periods of time will now be described. Replicate flasks which contained 0.2 μg of peptide Tα1/ml (0.27 × 10^{-6} M) were inoculated with 2 × 10^5 of RPMI No. 2402 cells/ml, and calf serum protein to a final level of 0.62 mg/ml was added after 0, 1, 3, and 10 hr (Figure 5). Later addition of calf serum resulted in continuous loss in viability with no growth. The later the addition of serum protein, the longer the lag period, the largest effect observed between 3 and 10 hr. In these four flasks (Figure 5) the maximum growth rate varied between a change of 580 × 10^3 and 760 × 10^3 cells per ml per 24 hr, and the maximum cell density was similar in all instances. In the absence of the peptide, no lag period was observed and logarithmic growth began upon inoculation. For purposes of the illustration, this was omitted from Figure 5. The principal effect of the delayed addition of protein (within the time limits specified above) was on the lag period.

Discussion

This paper is the first description of peptides of known structure which are able to effect the growth of mammalian cells. Further work will be required to determine whether this activity is confined to a few cell types or is exhibited toward many or possibly all mammalian cells. By implication, particular amino acid sequences may be able to alter the growth of intact animals as well.

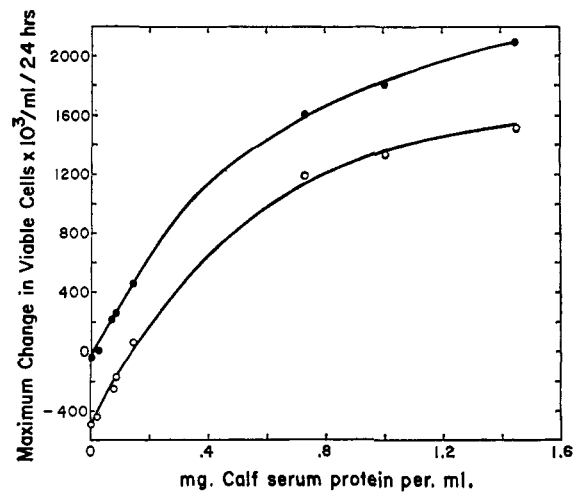

FIGURE 4: The ability of 1.03 × 10^{-6} M peptide Tα1 to diminish growth in the presence of calf serum. Open circles, Tα1 was present; solid circles, Tα1 was absent.

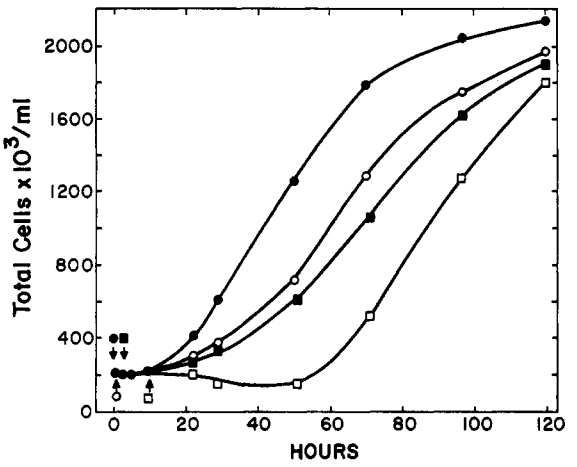

FIGURE 5: Effect of 0.62 mg/ml of calf serum protein added after an exposure to 0.27 × 10^{-6} M peptide Tα1 of 0 (●), 1 (○), 3 (■), and 10 (□) hr. The viability did not fall below 90% at any time in the flasks to which calf serum was added at 0, 1, and 3 hr; it was 70% at the time of addition of calf serum at 10 hr.

This has been suggested by several investigators (Woolley, 1946; Womack and Rose, 1946). Because cells in the intact animal are continuously bathed by fluids of high protein content, unequivocal demonstration of this effect *in vivo* has not been possible.

The knowledge of the amino acid sequence of cytotoxic peptides does not by itself shed light on the mechanism of the toxicity, or which portions of the peptides are responsible for the biological activity. Since both peptides are equipotent on a molar basis, they may act by similar mechanisms. The Ala-Leu-Ser sequence, which occurs twice in peptide Tα9, is similar to the sequence Val-Leu-Ser in peptide Tα1, and both peptides contain a proline residue near the middle. It seems doubtful, however, that these attributes constitute a common "active site."

The peptides reduced the viability (*i.e.*, the ability to exclude trypan blue) of randomly dividing cell populations without a lag period (Figure 2). However, damage to the cells occurred sooner than this measurement indicated: the experiment illustrated in Figure 5 shows that the viability of the cells was not altered appreciably by exposure to peptide Tα1 for 1–3 hr, yet the lag period was unquestionably extended. The cells which survived for a limited period of time (10 hr in the experiment in Figure 5) were able to respond to calf serum with growth rates independent of the time of exposure to the peptide, but with lower growth rates than would have been observed in the absence of peptide (compare growth rates in Figures 4 and 5). The levels of the peptides required for these effects were of the order of micrograms per milliliter. Growth stimulation required milligrams per milliliter of protein. These levels of peptide represent 10^9 molecules of Tα1/cell at the start of the experiments.

The toxic peptides herein described are products of enzymatic digestion of a protein. By inference, it may be that enzymes originating from the cultured cells may alter the serum proteins in the medium during growth to produce peptides which can alter growth. In this manner, the cells could influence their own growth. This has been observed in bacterial systems (Baudet and Cherbuliez, 1961). Materials toxic to cultured cells have been found in the serum of pregnant women (Rejnek *et al.*, 1963), schizophrenics (Fedoroff and Cook, 1959), and cancer patients (Saxen and Penttinen, 1965). The elaboration of such peptides may thus be of importance in the intact animal.

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Adrenal Cells in Tissue Culture. III. Effect of Adrenocorticotropin and 3′,5′-Cyclic Adenosine Monophosphate on 11β-Hydroxylase and Other Steroidogenic Enzymes*

J. Kowal†

ABSTRACT: Monolayer cultures of an adrenocorticotropin-responsive cell line from transplantable mouse adrenal tumors show an augmentation in maximum steroidogenic output in response to prolonged incubation with adrenocorticotropin and 3′,5′-cyclic adenosine monophosphate. This is associated with progressive increases in 11β hydroxylation of endogenously produced steroids. After 12–72-hr exposure to these agents, twofold or greater increases in the 11β hydroxylation of added [^{3}H]pregnenolone or [^{3}H]progesterone were found in stimulated cells. The continued presence of adrenocorticotropin was not required to elicit the stimulation. Adrenocorticotropin added *de novo* with the radioactive steroids did not stimulate the enzyme. In cells maintained in culture many months in the absence of adrenocorticotropin, as much as a tenfold stimulation could be obtained after 72-hr stimulation with adrenocorticotropin. Precise quantitation of this effect in the intact cell was obtained through the use of a sensitive radioactive assay. The properties of the enzyme in mitochondria obtained from these cultures were similar to those reported in bovine and rat adrenal systems. Reduced triphosphopyridine nucleotide was the optimum pyridine nucleotide cofactor, but Ca^{2+} (10 mM) was required to elicit the reaction. 11β Hydroxylation was also supported by pyruvate and a number of Krebs' cycle intermediates; the highest activity was obtained with isocitrate. Ca^{2+} inhibited 11β hydroxylation supported by Krebs' cycle intermediates. Cyanide was not inhibitory. Levels of stimulation comparable with that seen in the intact cells were obtained with mitochondria isolated from adrenocorticotropin-treated cells under all conditions which supported 11β hydroxylation. Adrenocorticotropin and 3′,5′cyclic adenosine monophosphate did not stimulate 3β-hydroxysteroid dehydrogenase or 20α-hydroxysteroid dehydrogenase.

The development of techniques for the maintenance of functional murine adrenal tumor cells in monolayer culture (Buonassisi *et al.*, 1962; Yasamura *et al.*, 1966) has provided the opportunity for *in vitro* studies of long-term, as well as immediate effects, of adrenocorticotropin on cell regulation. Additional advantages of this system are the ability of the cultures to maintain a basal output of steroids in the absence of adrenocorticotropin and the absence of an effect of adrenocorticotropin on cell growth. Measurable steroidogenic responses to adrenocorticotropin can be observed within 5–10 min (Kowal and Fiedler, 1968). These cells lack a 21-hydroxylase (Pierson, 1967) and possess a more active 20α-hydroxysteroid dehydrogenase than that present in mouse adrenals (Pierson, 1967). As a result, they elaborate a mixture of 20α-dihydroprogesterone[1] and 11β-hydroxy-20α-dihydroprogesterone instead of corti-

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† Faculty Research Associate of the American Cancer Society.

[1] The following trivial names have been employed: 20α-dihydroprogesterone, 20α-hydroxypregn-4-en-3-one; 11β-hydroxy-20α-dihydroprogesterone, 11β,20α-dihydroxypregn-4-en-3-one; desoxycorticosterone, 21-hydroxypregn-4-ene-3,20-dione; corticosterone, 11β,21-dihydroxypregn-4-ene-3,20-dione; preg-