INDUCTION OF TYROSINE α-KETOGLUTARATE TRANSAMINASE BY STEROID HORMONES IN A NEWLY ESTABLISHED TISSUE CULTURE CELL LINE

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Our attention was drawn to the possibility of studying enzyme induction in tissue culture by a brief report from Pitot *et al.*[1] that the Reuber hepatoma in tissue culture showed an increase in tyrosine transaminase activity in response to treatment with hydrocortisone.[1] Through the courtesy of Dr. H. P. Morris of the National Cancer Institute, we obtained primary cultures from rats containing two lines of hepatomas in the ascites form. Each of these resulted in a permanent tissue culture line. In this paper we describe the characteristics of one of these lines in which glucocorticoids induce a rapid, substantial increase in the activity of tyrosine α-ketoglutarate transaminase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5). While being carried in serial transfer for over a year, this cell line (designated HTC for hepatoma tissue culture) has remained stable as to growth and inducibility. Furthermore, from single cells of the original line, clones have been isolated which continue to show the same characteristics.

Materials and Methods.—Sera were obtained from Microbiological Associates; the NIH medie unit prepared Swim's medium. Merck, Inc., very kindly supplied the dexamethasone phosphata (Dx). Porcine kidney p-hydroxyphenyl pyruvate keto-enol tautomerase (4.3 K units/ml) was purchased from Sigma Chemical Co., centrifuged at 20,000 *g* for 10 min, the pellet discarded, and the soluble fraction stored frozen.

The cell line described here came from an ascites tumor[2] which in turn had been derived from a solid hepatoma (#7288c) originally induced by feeding male Buffalo rats a diet containing 0.04% N,N'-2,7-fluorenylenebis-2,2,2-trifluoroacetamide for 12.4 months.[3, 4] Primary culture was carried out by sterile peritoneal puncture and withdrawal of 0.1 ml ascitic fluid which was placed in a T30 culture flask to which 5 ml growth medium was at once added. After an initial lag of a few days, a layer of epithelioid cells grew out. For the first 8 months, growth was maintained in tightly stoppered bottles in a standard laboratory incubator, but since then a humidified CO_2 incubator running with 3% CO_2—97% air has been used with the bottles stoppered loosely. The growth medium was Swim's medium 77 (S77). S77 has the same composition as S103 described by Swim and Barker,[5] except that hydroxyproline was omitted, the serine concentration was 0.2 mM, and choline bitartrate was substituted for choline chloride. S77, when used to support growth, was supplemented with 20% bovine serum and 5% fetal bovine serum. Penicillin G, 100 units/ml, streptomycin sulfate, 12.5 μg/ml, were added for routine culturing; however, periodically they have been omitted for several days and the medium was then cultured for bacterial contamination. Checks for PPLO contamination also have been carried out by plating on "mycoplasma agar" as described by Hayflick.[6] No mycoplasmas have been found.

Periodically, cells have been frozen in 5% glycerol—95% growth medium by standard techniques[7] and stored in liquid nitrogen. Upon thawing after as much as a year of such storage, HTC cells exhibited the same growth and induction as the original line. Chromosome preparations, stained with Giemsa, were carried out by a slight modification of the method of Tjio and Puck.[8]

For enzyme induction studies, growth medium was replaced by serum-free S77; then the cells were gently shaken free, pooled, and apportioned as needed for a given experiment.

Tyrosine transaminase was assayed as follows: Each aliquot of cells was centrifuged from the induction medium at 600 *g* for 5 min at 0°, twice washed and recentrifuged with aliquots of 0.15 *M* sodium phosphate, pH 7.9 at 0–4°, and the resulting pellet frozen. To the frozen pellet 0.5 ml

of the buffer was added and the cells were ruptured with a chilled probe sonicator, using two 10-sec bursts at 2 amp in an ice bath. The broken cell suspensions were centrifuged at 20,000 *g* for 10 min and the supernatant solution was assayed for tyrosine transaminase activity by the following modification of the method of Lin and Knox.[9] A total volume of 1.0 ml buffered with a final concentration of 0.5 *M* sodium borate, pH 7.8, contained 0–0.25 ml of centrifuged cell extract in phosphate buffer, 5×10^{-3} mmoles of tyrosine, 0.010 ml porcine p-hydroxyphenylpyruvate-enol-keto-tautomerase, 2×10^{-4} mmoles of pyridoxal phosphate, 0.01 mmoles of α-ketoglutarate, and 0.15 *M* sodium phosphate, pH 7.8, to volume. Enzyme activity was followed by the rate of increase of absorption of light at 310 mμ on a Gilford recording spectrophotometer. One unit of transaminase activity was defined as the quantity of enzyme which catalyzed the formation of 1 mμmole of the p-hydroxyphenylpyruvate-enol borate complex per minute. The molar absorbancy of the complex was taken to be 10,700 at 310 mμ.[10] Specific activity was expressed as units per mg protein. Proteins were estimated by the method of Lowry *et al.*[11] using bovine serum albumin as a standard.

Partial purification of tyrosine transaminase with negligible loss of activity was obtained by centrifuging cell sonicates as described above and submitting the supernatant solution to 60°C for 1 hr in the presence of 8×10^{-4} *M* pyridoxal phosphate and 1.3×10^{-2} *M* α-ketoglutarate. Denatured proteins were then removed by centrifugation at 12,000 *g* for 10 min.

Disc electrophoresis was carried out on 7% lower gel in a standard Canalco apparatus by the method of Ornstein and Davis.[12] The experiments were run for about 2 hr at a constant current of 3 ma per tube in pH 8.3 Tris-glycine buffer. To the upper tank were added 1% tracking dye, 1.56×10^{-2} *M* α-ketoglutarate and 1.2×10^{-2} *M* pyridoxal phosphate. Gels were stained either for proteins (Amido Schwartz) or for tyrosine transaminase activity. The latter stain was carried out by linking the tyrosine transaminase reaction to the reduction of a tetrazolium dye to its insoluble red formazan. The reaction mixture contained per ml: 0.05 ml of 0.33 *M* α-ketoglutarate, 0.2 ml of 0.025 *M* tyrosine, 0.005 ml tautomerase (4.3–5.0 K units/ml), 0.005 ml of 0.02 *M* pyridoxal phosphate, 0.0025 ml of a suspension of 20 mg/ml crystalline glutamate dehydrogenase, 0.25 ml of iodonitrotetrazolium 3.2 mg/ml, 0.1 ml of DPN 10 mg/ml, 0.05 ml of 0.4 mg/ml phenazine methosulfate, and 0.15 *M* sodium phosphate pH 7.9 to make 1.0 ml. This solution was added directly to the unstained disc gel which was observed while the reaction took place (10–30 min); then the reaction was stopped by the addition of 7% acetic acid.

Results and Discussion.—General characteristics of HTC cells: This cell line was originally cultured in October 1964, and then carried in an unbroken series of 59 transfers over 12 months. Since then, cells frozen at passage 33 have been used and have been carried another 35 transfers. Like other tumor lines in culture, these cells form multilayered confluent sheets on glass surfaces[13] and exhibit logarithmic growth with a doubling time of approximately 24 hr (Fig. 1). Histologically they have the characteristics of "epithelioid" cells (Fig. 2) showing irregular cytoplasmic projections when growing in contact with glass and isolated from other cells, but becoming more rounded as intercellular contact is established. No blood elements or fibroblasts were seen. Compared to normal rats, which have chromosome number of 42,[14–16] a count of 100

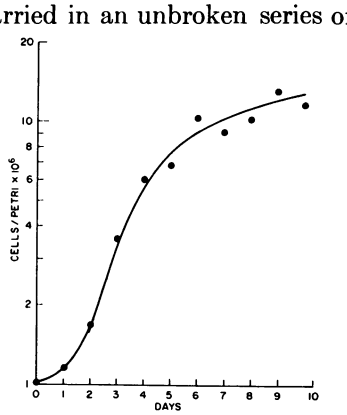

FIG. 1.—Growth curve of uncloned HTC cell line. Aliquots of 10^6 cells each were placed in a series of Petri dishes to each of which 10 ml of fresh growth medium was added. Each day the cells in one Petri were trypsinized and counted, and the medium was renewed by replacing half with fresh. Each point represents the average of at least two cell counts of at least 200 cells.

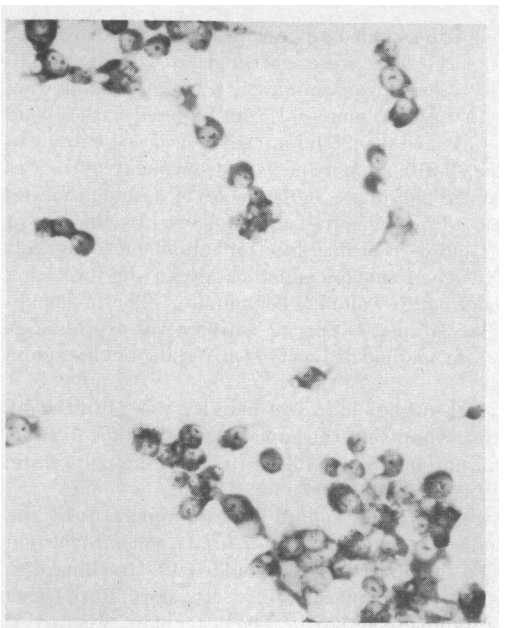

FIG. 2.—HTC cells grown on a coverslip and stained *in situ*. Giemsa stain, ×260 magnification. Area chosen to show both grouped and individually growing cells.

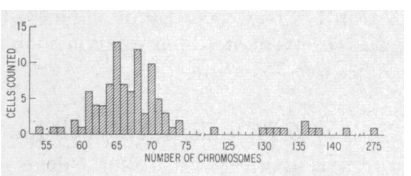

FIG. 3.—Distribution of chromosome content in uncloned HTC line, 100 cells in mitosis counted.

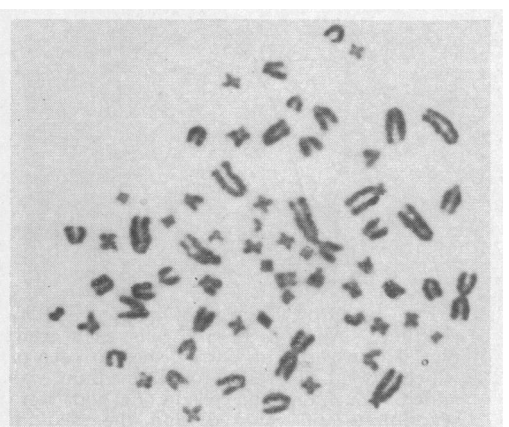

FIG. 4.—Typical set of chromosomes from cell of uncloned HTC line.

mitotic figures of HTC cells at the 46th transfer revealed a hypotetraploid number with a mean around 66, and 5% 2*s* and 1% 4*s* figures (Fig. 3). Of special interest, since they are not seen in normal rat cells, were the several metacentric chromosomes observed in all mitotic figures studied from HTC cells (Fig. 4). Tjio and Levan have described similar chromosomes in the Yoshida rat sarcoma.[14]

Cloning: Where approximately 10^5 cells/ml were used, there was a plating efficiency of 95 per cent. In contrast to the Reubner hepatoma cell line cultured by Pitot *et al.*,[1] HTC cells form clones quite easily either by serial dilution with growth on glass or in agar or by direct manual isolation of individual cells.[14–19] The cloning efficiency of dilute cell suspensions (10 cells/ml) was approximately 60 per cent.

Induction of tyrosine transaminase: Serum, although essential for growth, was not required for the induction of tyrosine transaminase. In serum-containing medium, the enzyme was inducible during either the logarithmic or the stationary phase of the growth curve. In order to provide better-defined conditions, the induction experiments described in this paper were performed in the serum-free medium, S77. In S77, cell division was not observed during a 4-day period, but when serum was added, growth promptly resumed.

After the addition of the synthetic steroid hormone, dexamethasone phosphate (Dx) 10^{-5} *M*, to a culture of HTC cells in S77 (Fig. 5), there was a 2-hr lag and then enzyme activity rose for 5–8 hr to a new plateau about 10 times the baseline level where it was maintained or slightly increased for the next 30 hr. Figure 6 shows the rate of enzyme increase at various concentrations of Dx, and in separate experi-

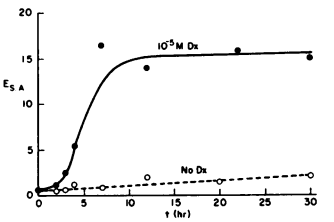

FIG. 5.—Kinetics of induction of tyrosine transaminase activity in HTC cells at 37°. At 0 time pooled cells were divided into two Petri dishes, one of which contained 10^{-5} *M* Dx. At times shown, aliquots were assayed for tyrosine transaminase as described in *Materials and Methods*. $E_{S.A.}$ refers to tyrosine transaminase specific activity.

ments, Dx concentrations higher than 10^{-5} *M* produced no further increase in induction rate. Among the hormones tested, nonsteroid as well as steroid, glucocorticoids were the most efficient inducers of tyrosine transaminase activity (Table 1). Deoxycorticosterone, with its relatively weak glucocorticoid action, induced erratically and to a lower level. Aldosterone at 3×10^{-8} *M* was not active but at 10^{-5} *M* induced to about one half the level achieved with glucocorticoids.

Steroid must be continuously present in order to maintain an induced level of enzyme, for if maximally induced cells were gently washed with induction medium containing no steroid and allowed to incubate further, the enzyme activity returned in a few hours to its uninduced level as the following experiment demonstrates: Several bottles of HTC cells were induced with 10^{-5} *M* Dx for 16 hr, the original induction medium was decanted and reserved, and the cells were pooled and washed twice with steroid-free S77. Duplicate samples were taken for enzyme activity determination and the remaining cells were divided into 5 aliquots, each of which was resuspended in one of the following media: (*A*) fresh S77 containing 10^{-5} *M* Dx, (*B*) the original induction medium, (*C*) conditioned medium containing 10^{-5} *M* Dx, (*D*) steroid-free conditioned medium, or (*E*) steroid-free fresh medium. (Conditioned medium refers to hormone-free S77 left 16 hr in contact with a fully grown layer of cells and then freed of cells by centrifugation prior to use.) As can be seen (Fig. 7), with conditions *A*, *B*, and *C* the induced enzyme level was maintained and even increased somewhat during the 10 hr of the experiment, whereas under conditions *D* and *E*, the enzyme after the first hour rapidly fell to basal levels. These data suggest that steroid was required for the maintenance of the induced level of enzyme activity. Kenney has mentioned that a very similar phenomenon occurs in cells of the line cultured from the Reuber hepatoma.[20]

Characteristics of tyrosine transaminase from HTC cells: As is found with liver tyrosine transaminase,[21] the enzyme from HTC cells was relatively heat-stable and was even more so in the presence of α-ketoglutarate. The heat stability of the steroid-induced enzyme from HTC cells was investigated further under various conditions as follows: a centrifuged cell extract containing tyrosine transaminase was passed through a column of Sephadex

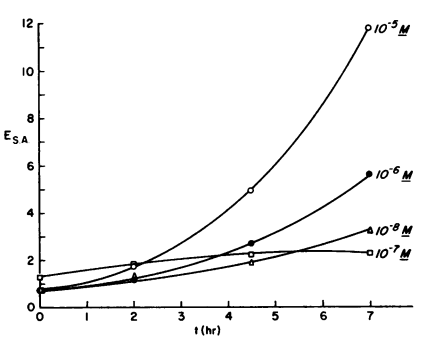

FIG. 6.—Relation of rate of tyrosine transaminase induction to concentration of Dx. A pool of cells was divided into Petri dishes in S77 at 37° with Dx at the concentrations indicated. Aliquots were assayed for enzyme at times shown. *Ordinate:* tyrosine transaminase specific activity as in Fig 5.

TABLE 1

RESPONSE OF TYROSINE TRANSMINASE ACTIVITY IN HTC CELLS TO VARIOUS STEROID HORMONES

Treatment	Tyrosine transaminase specific activity, units/mg protein
Zero time, uninduced	2.8
No additions	1.6
0.1% ethanol (95%)	2.4
Dx, 10^{-4} M	15.0
Dx, 10^{-4} M, + 0.1% ethanol	12.8
Triamcinolone, 10^{-4} M, + 0.1% ethanol	10.5
Hydrocortisone hemisuccinate, 10^{-5} M, + 0.1% ethanol	14.3
Deoxycorticosterone, 10^{-5} M, + 0.1% ethanol	7.7
Aldosterone, 10^{-5} M, + 0.1% ethanol	6.7
Aldosterone, 3×10^{-8} M, + 0.1% ethanol	1.8
Stilbesterol diphosphate, 10^{-5} M, + 0.1% ethanol	1.4
17 β-Estradiol, 10^{-5} M, + 0.1% ethanol	2.2
Testosterone, 10^{-5} M, + 0.1% ethanol	2.4
Progesterone, 10^{-5} M, + 0.1% ethanol	3.6

After sampling in duplicate for zero time, uninduced enzyme a pool of cells was divided into Petri dishes in 10 ml S77 prewarmed to 37°. To duplicate Petri dishes, steroid and/or ethanol was added to give the final concentrations indicated. After 15 hr in the CO_2 incubator, cells were harvested, washed, and assayed for enzyme as before. Tyrosine transaminase specific activity expressed as in Fig. 5. Figures given represent the average of duplicate samples.

G-25. The gel-filtered extract was then heated in a water bath at 56° and under these conditions about 50 per cent of the enzyme activity remained after 22 min. Either α-ketoglutarate, 1.3×10^{-2} M, or pyridoxal phosphate, 8.0×10^{-4} M, or both almost completely protected the activity. Interestingly, however, pyridoxal, 2.0×10^{-3} M, destabilized the enzyme so that there was complete loss of activity on heating for 20 min. Pyridoxal phosphate protected the enzyme against the loss of activity incurred by heating in the presence of pyridoxal but did not restore activity to the inactivated protein. Neither L-tyrosine at 1.4×10^{-3} M nor Dx at 1.25×10^{-3} M influenced the heat stability of the transaminase.

Using heat denaturation, the tyrosine transaminase activities from induced and uninduced cells have been compared as illustrated in Figure 8. Figure 8*a* shows that basal and induced enzymes when heated for 5 min in the presence of pyridoxal phosphate and α-ketoglutarate demonstrated identical temperature stability curves. In Figure 8*b*, the kinetics of heat denaturation at 76° are compared and again the induced and basal enzymes behave identically. Disc gel electrophoresis also suggested identity of induced and uninduced enzymes. When cell sonicates, partially purified by heating at 60° for 1 hr in the presence of pyridoxal phosphate and α-ketoglutarate, were electrophoresed and stained for tyrosine transaminase activity, basal and induced enzyme were found at a single, identical position. Therefore, the basal enzyme was indistinguishable from the steroid-induced enzyme. With a gel-filtered extract of steroid-induced HTC cells, the K_m for α-ketoglutarate was 6.65×10^{-4}; for tyrosine, 1.82×10^{-3}; and the pH optimum was 7.8.

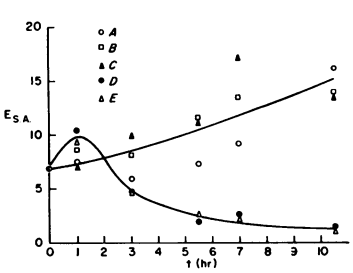

FIG. 7.—Effect of removing Dx on tyrosine transaminase activity. See text for experimental details. *Ordinate:* tyrosine transaminase specific activity as before.

Inhibition of induction: In contrast to experiments in intact animals, there was no detectable stimulation of over-all amino acid incorporation into protein during induction (Table 2).

TABLE 2

INFLUENCE OF INHIBITORS OF PROTEIN SYNTHESIS ON THE INCREASE IN TYROSINE TRANSAMINASE ACTIVITY INDUCED BY DEXAMETHASONE

0 hr			1 hr	5 hr	
Tyrosine transaminase (units/mg prot)	Leucine-C^{14} incorporation (cpm/mg prot)	Additions	Leucine-C^{14} incorporation (cpm/mg prot)	Tyrosine transaminase (units/mg prot)	Leucine-C^{14} incorporation (cpm/mg prot)
		None	257	3.2	1000
		Dexamethasone phosphate, 10^{-5} M	236	10.9	1018
3.0 (pooled cells)	12	Dexamethasone + puromycin, 4.2×10^{-4} M	48	1.7	68
		Dexamethasone + cycloheximide, 10^{-4} M	33	1.8	53
		Dexamethasone + chloramphenicol, 10^{-2} M	30	3.0	33
		Dexamethasone + progesterone, 10^{-4} M	80	2.0	273

Cells from five bottles were pooled in 100 ml of S77 at 37°. Two μc of C^{14}-leucine (Schwarz, 5 μc/μmole, 2 μmoles/ml) was added and duplicate aliquots were immediately taken for 0 hr enzyme activity and radioactivity estimation. The remaining pooled cells were then divided among prewarmed Petri dishes containing various additions and replaced in the CO_2 incubator. After 1 hr and 5 hr, aliquots were removed for estimation of radioactivity. Each aliquot was pipetted into an equal volume of 10% trichloroacetic acid (TCA), allowed to stand at 4° overnight, heated for 25 min at 85°, and chilled in an ice bath for 15 min. The precipitates were separated by slow centrifugation, washed twice in 5% TCA at 25°, drained, and dissolved in a small volume of 0.1 N NaOH. Aliquots from this were taken for protein estimation and for counting in Bray's solution in a Nuclear-Chicago scintillation counter. Five-hr aliquots were also taken for estimation of enzyme activity.

However, as is seen in liver, both perfused and *in vivo*,[22-25] induction of tyrosine transaminase in HTC cells in culture was blocked by the inhibitors of RNA synthesis, actinomycin D, and mitomycin C, as well as by compounds which more specifically prevent protein synthesis. Mitomycin C at the levels used in these experiments inhibits RNA as well as DNA synthesis. Table 2 demonstrates that puromycin, cycloheximide, and chloramphenicol, sufficient to inhibit C^{14}-leucine incorporation into TCA-precipitable material by at least 90 per cent, also completely inhibited induction. Table 3 summarizes three other experiments in which induction was followed by a longer period. In these experiments, actinomycin D, mitomycin C, or cycloheximide prevented the rise in induced enzyme activity. The variety of inhibitors of protein synthesis which also interfere with enzyme induction strongly suggests that in this case, just as in liver, enzyme induction is due to a more rapid *de novo* synthesis of enzyme molecules.[2] Many recent studies indicate that actinomycin D and mitomycin C have numerous

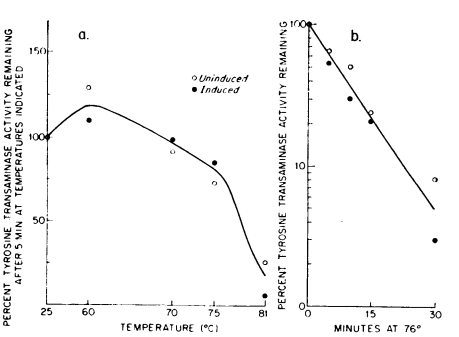

FIG. 8.—A pool of cells was divided in half and incubated in S77 overnight, one half with and one half without 10^{-5} M Dx. Cell extracts were obtained by sonication and α-ketoglutarate and pyridoxal phosphate added to 1.3×10^{-2} M and 8×10^{-4} M, respectively. Aliquots were assayed for enzyme activity, and from the remaining extracts aliquots were heated for various times and temperatures in a controlled-temperature water bath. After heating, any precipitate was removed by centrifugation at $12{,}000 \times g$ for 10 min, and the supernate assayed for transminase activity. (*a*) Each point average of duplicate assays. (*b*) Sonicates heated for various times at 76°. Each induced enzyme point was the average of three experiments in duplicate, and each basal enzyme point the average of one experiment in duplicate.

TABLE 3

INFLUENCE OF ACTINOMYCIN D, MITOMYCIN C, AND CYCLOHEXIMIDE ON DEXAMETHASONE INDUCTION OF TYROSINE TRANSAMINASE

Additions	Fraction of Initial Tyrosine Transaminase Specific Activity Time (hr) 0	2	5	6	8	12	24
(Expt. 1)							
None	1.0	0.7	0.9	—	0.8	0.7	0.6
Dexamethasone phosphate, 10^{-5} *M*	1.0	0.8	1.5	—	5.2	4.2	5.2
Actinomycin D, 5 μg/ml	1.0	1.0	1.0	—	1.7	0.9	1.0
Actinomycin D + dexamethasone phosphate, 10^{-5} *M*	1.0	0.9	1.0	—	1.1	1.1	0.8
(Expt. 2)							
None	1.0	1.5	1.1	—	1.0	—	0.7
Dexamethasone phosphate, 10^{-5} *M*	1.0	3.1	6.9	—	13.2	—	7.5
Dexamethasone + cycloheximide, 10^{-4} *M*	1.0	0.8	0.6	—	0.9	—	0.7
(Expt. 3)							
Dexamethasone phosphate, 10^{-5} *M*	1.0	—	—	6.1	—	—	7.3
Dexamethasone + mitomycin C, 15 μg/ml	1.0	—	—	3.3	—	—	1.9

Three experiments are shown. In each, cells were pooled in S77 and, after taking aliquots for basal enzyme estimation, subdivided into appropriate groups which were treated as indicated. At the times shown, aliquots were removed for enzyme determination. Experiment 3, using mitomycin C, was carried out by Dr. Beverly Peterkofsky, who kindly permitted us to include these data. All activities are expressed in fractions relative to basal tyrosine transaminase specific activity, which varied slightly, as discussed in the text.

actions in addition to their effect on DNA-directed RNA synthesis;[25-31] therefore, interference with induction by these compounds can be taken only as suggestive evidence that RNA synthesis specifically is required for induction.

In contrast to the inhibition of induction seen when actinomycin D was added simultaneously with steroid, a stimulatory action of the inhibitor was seen when 5 μg/ml was added to previously induced cells in which tyrosine transaminase had reached plateau levels. The enzyme level did not fall as might be expected, but rose instead, and the rise was inhibited by puromycin (Fig. 9). This paradoxical effect of actinomycin seems analogous to that described *in vivo* in the rat liver[25] and will be presented in detail in a separate communication.

Summary.—Tyrosine α-ketoglutarate transaminase can be induced by steroid hormones in a newly established line of tissue culture cells, derived from primary culture of the ascites form of an experimental rat hepatoma. The isolation, growth, and morphology of the tissue culture cells are described. Heat inactivation studies and disc gel electrophoresis suggest that induced and basal tyrosine transaminase are the same. The enzyme activity is stabilized to heat by pyridoxal phosphate or α-ketoglutarate, but made unstable to heat by pyridoxal. Kinetics of tyrosine transaminase induction reveal a 2-hr lag after addition of steroid, followed by a rapid

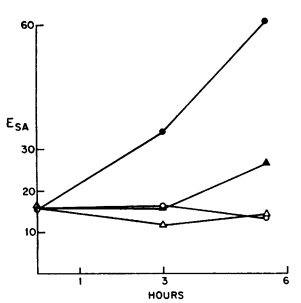

FIG. 9.—Several bottles of cells induced by incubating 40 hr in S77 containing 2×10^{-5} *M* Dx were pooled, washed twice with steroid-free S77, and suspended in S77 at 37°. After taking duplicate samples for zero time tyrosine transaminase levels, the remaining cells were divided into Petri dishes. Dx at 2×10^{-5} *M* was added to all dishes, and then groups of four received, respectively, actinomycin D 5 μg/ml (●), puromycin 8×10^{-4} *M* (○), or actinomycin plus puromycin (△). At 3 hr and $5^1/_2$ hr, aliquots were taken for estimation of enzyme activity. Each point represents the average of quadruplicate samples. Controls received only Dx (▲). $E_{S.A.}$ signifies tyrosine transaminase specific activity as in previous figures.

rise in enzyme for 5–8 hr to a plateau level about 10 times the basal activity. The induced level is maintained as long as steroid is present. The induction is blocked by puromycin, cycloheximide, chloramphenicol, progesterone, actinomycin D, and mitomycin C. After induction by steroid has taken place, actinomycin D produces a further increase in enzyme activity.

The authors would like to express their gratitude to Drs. Pitot, Morse, and Potter for very kindly supplying details of their culture methodology; to Dr. H. P. Morris for allowing cultures to be obtained from his lines of *in vivo* hepatomas; and to Drs. J. H. Tjio and J. Whang for their instruction (and friendly encouragement) in the techniques of chromosomal analysis.

Dr. Shin-ichi Hayashi in our laboratory has crystallized rat liver tyrosine transaminase and has obtained rabbit antibody to this preparation. Preliminary experiments indicate that the basal and induced enzyme are immunologically identical in liver and HTC cells. Furthermore, transaminase induction in the tissue culture cells, as well as in the liver, is accompanied by an increase in immunologically reactive protein. These experiments will be presented in detail in subsequent communications.

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