Expression of Differentiated Functions in Hepatoma Cell Hybrids: Reappearance of Tyrosine Aminotransferase Inducibility after the Loss of Chromosomes*

(dexamethasone/actinomycin D/chromosome numbers/logarithmic growth phase)

MARY C. WEISS AND MICHÈLE CHAPLAIN

Centre de Génétique Moléculaire, C.N.R.S., 91, Gif-sur-Yvette, France

Communicated by Boris Ephrussi, September 9, 1971

ABSTRACT Hybrids from a cross of rat hepatoma cells with diploid epithelial cells from rat liver have been studied with respect to karyotype and expression of two functions limited to the hepatoma parent: high level of the enzyme tyrosine aminotransferase (EC 2.6.1.5; L-tyrosine:2-oxoglutarate aminotransferase) and its inducibility with steroid hormones. The hybrids that contain the complete chromosomal complements from both parents show low enzyme activity and no inducibility. One hybrid clone, and all of its derivatives, which have lost 30-40% of the chromosomes initially present, show enzyme inducibility. Induction of tyrosine aminotransferase in the hepatoma and hybrid cells responds similarly to inhibition by cycloheximide and actinomycin D, and to steroid concentration. The enzymes from induced and noninduced hepatoma cells and from induced hybrid cells are similar in heat sensitivity and intracellular distribution; those from non-induced hybrid and diploid rat epithelial cells are different.

In the first paper of this series (1), it was shown that somatic hybrids between mouse fibroblasts and well differentiated rat hepatoma cells fail to exhibit high baseline levels and steroid-mediated induction of the enzyme tyrosine aminotransferase (EC 2.6.1.5; L-tyrosine:2-oxoglutarate aminotransferase; TAT). Both a high baseline and inducibility of TAT are characteristics of hepatic differentiation and are unique to the hepatoma parent. These results are similar to those reported for hybrids from several other crosses between cells exhibiting one or more specialized functions and fibroblasts (2, 3).

The study of the expression of TAT in somatic hybrids has been extended to hybrid cells from a new cross: rat hepatoma cells x epithelial cells derived from rat liver. Although these hybrid cells have properties similar to those previously reported, the use of selective conditions has permitted the isolation of numerous hybrid subclones that have undergone extensive chromosome losses, and several of these, in spite of a low basal level of TAT, have been found to show enzyme induction.

MATERIALS AND METHODS

Cultures were maintained as described (1). Chemicals were purchased from Sigma or Calbiochem; actinomycin D was obtained from Merck Co.

Fu5-5 is a subclone of the line Fu5 (1), derived from the Reuber H35 (4) rat hepatoma line H4IIEC3 of Pitot *et al.*

Abbreviation: TAT, tyrosine aminotransferase (L-tyrosine:2-oxoglutarate aminotransferase).

*This is the third paper of a series: "Expression of Differentiated Functions in Hepatoma Cell Hybrids". The first paper is ref. 1.

(5). BRL-1 is a subclone of line BRL-1D1 (generously supplied by Dr. Hayden Coon), one of the many diploid epithelial lines established by direct cloning from the liver of a young Buffalo rat (6). Although several of these lines have been reported to express some differentiated liver functions (6), none has been detected in BRL-1; these include aldolase B (EC 4.1.2.7) and alcohol dehydrogenase (EC 1.1.1.1) (22) as well as TAT. Whether BRL-1 cells are "dedifferentiated" hepatocytes or from some other epithelial component of the liver is unknown.

Normal chromosomes of the rat can be divided into several groups: large submetacentric, small metacentric, and telocentric; 2 long satellited chromosomes have been grouped among the telocentrics. One marker, a large metacentric chromosome, is found in all cells of the parental hepatoma line.

Cell fusion was obtained by treatment of a mixture of equal numbers of parental cells with 300 hemagglutinating units of UV-inactivated Sendai virus. The cell suspension was plated at low density in normal medium. Colonies of large cells were isolated and subcultured; karyotypic analysis showed that one of these colonies was hybrid.

TABLE 1. Chromosome numbers of parental and hybrid cells*

Cell type	Total	I	II	III	IV
BRL-1	42	2	0	20	20
Fu5-5	52 (51-53)	2.0	1.0	22.6	26.1
Expected in hybrid	94 (93-95)	4.0	1.0	42.6	46.1
Observed†:					
BF5 (22)	91.9 (91-93)	4.0	1.0	41.8	45.1
BF5 (35)	92.0 (91-93)	4.0	1.0	41.8	45.2
BF5 (75)	91.4 (89-93)	4.0	1.0	38.6	47.8
BF5-A (45)	90.4 (88-91)‡	4.0	1.0	41.0	44.2
BF5-B (46)	75.0 (73-76)‡	3.0	1.0	34.0	37.5
BF5-C (51)	92.0 (91-93)‡	4.0	1.0	42.8	44.2
BF5-β (55)	58.9 (58-59)	2.0	1.0	27.9	28.0
BF5-1 (65)	63.0 (61-68)‡	2.8	0.8	27.8	32.0
BF5-1-1 (80)	63.1 (60-66)	3.1	1.0	27.7	31.3
BF5-1-1-b (110)	55.1 (52-57)	2.0	1.0	24.4	27.3

I, II, III and IV are large submetacentric, large metacentric, small metacentric, and telocentric chromosomes, respectively.

* Mean values are given, as well as the range (in parentheses) of the total number of chromosomes. For chromosome classifications, see *Methods*.

† The calculated total number of cell generations at the time of karyotypic analysis is given in parentheses.

‡ Based upon the analyses of 5 metaphases; all of the other numbers are based on 10-20 metaphases.

TABLE 2. Activity and inducibility of tyrosine aminotransferase during the growth cycle

Cell type	No. of cells per 10-cm dish ($\times 10^6$)			Specific activity of tyrosine aminotransferase*							
				Assay I†				Assay II‡			
				5 hr		24 hr		5 hr		24 hr	
	Inoculum	Assay I†	Assay II‡	C§	I§	C	I	C	I	C	I
BRL-1	0.4	1.8	2.1	0.2	0.3	—¶	0.2	—	—	0.7	0.6
BF5-C	0.2	1.2	2.7	0.4	0.4	0.4	0.4	0.4	0.6	0.4	0.5
BF5- β	0.1	0.75	2.3	0.6	0.7	0.2	0.3	0.3	0.9	0.2	0.3
BF5-1-1	0.2	4.1	17.2	0.5	4.3	0.6	3.9	0.4	5.7	0.4	1.3
BF5-1-1-b	0.2	4.0	12.0	1.1	1.6	—	9.2	0.2	3.0	—	8.4
Fu5-5	0.2	2.9	10.4	16.9	144.5	21.0	583.0	41.6	171.0	38.5	224.0

* One unit of TAT activity equals 1 μ mol of *p*-hydroxyphenylpyruvate formed per min at 37°C. Specific activity is expressed as milli-units per mg of protein.

† Assay I: cultures in early logarithmic growth phase, 3–4 days after inoculation.

‡ Assay II: cultures in late logarithmic or stationary phase, 6–7 days after inoculation.

§ C: control or noninduced; I: induced (1 μ M dexamethasone).

¶ —, Not assayed.

Cells were induced with 1 μ M dexamethasone, added to fresh growth medium, containing the standard 5% fetal calf serum. At the start of each experiment, cultures were renewed with fresh growth or induction medium; in most experiments, control and induced cultures were harvested simultaneously in order to correct for possible effects of medium renewal on TAT activity.

Cultures were collected, enzyme extracts were prepared, protein was determined, and TAT specific activity was defined as described (1). For the separation of soluble and mitochondrial TAT activity, cells were homogenized in the standard TAT buffer (7), centrifuged, and washed twice ($12,000 \times g$ for 20 min); all supernatants were retained and pooled to be used as the soluble extract. Mitochondria were broken by freezing and thawing three times in alcohol-dry ice; both mitochondrial and crude soluble extracts were then centrifuged for 1 hr at $20,000 \times g$.

TAT was assayed as described (1), in duplicate, with duplicate blanks. Control assays have shown that the simultaneous presence of all three components of the reaction (tyrosine, 2-oxoglutarate and pyridoxal-5'-phosphate) is required to obtain activity in this assay, and that for extracts containing very little enzyme, activity is a linear function of protein concentration, up to 0.4 mg/ml of protein.

Inhibition of protein synthesis by cycloheximide was determined by treating a series of cultures with 0.02 μ Ci/ml of L-[14 C]leucine (C.E.A., Saclay; sp act 127.2 Ci/mol), both in the presence and absence of 0.1 mM cycloheximide. At various times, the cells were collected and washed (as for the TAT assay), the pellets were treated as described by McCormick and Penman (8) and counted in a Packard TriCarb scintillation counter as described by Attardi *et al.* (9).

RESULTS

A single hybrid clone, designated BF5, was isolated from the cross of BRL-1 $\times$ Fu5-5. It can be seen (Table 1) that the hybrid cells retained, during 4 months (75 generations) of continuous culture, almost exactly the expected number of chromosomes. Assays of TAT in the hybrid cells showed that the enzyme behaves in these hybrids as in those reported (1): a low basal level (<1.0) and no inducibility. The TAT activity

in the hybrids is similar to that in BRL-1 cells, and less than 1/20th of that of noninduced hepatoma (Fu5-5) cells.

It is known that modifications in the morphology and growth properties of hybrid cells may occur as the result of loss of chromosomes (10, 11). The BF5 cells, like BRL-1, show contact inhibition of growth. By contrast, the hepatoma cells grow in multiple layers. In order to select for variant hybrids that show hepatoma-like growth, confluent cultures of BF5 were maintained for 1–3 months with frequent renewals of medium. After several weeks, foci (colonies) of multilayered growth were observed. 14 colonies of variant morphology, from five different culture bottles, were isolated, and in some cases, recloned. All had reduced chromosome numbers: instead of the 91–93 chromosomes of BF5, these variants contained 60–80 chromosomes.

Simple cloning of BF5 was undertaken in parallel, in an attempt to isolate similar but spontaneously occurring variants with reduced chromosome numbers; among three subclones (BF5-A, -B, and -C) thus isolated, none showed clearly multilayered growth, and only one showed a reduction in chromosome number (Table 1). By contrast, two of the variants, BF5- β and BF5-1, contained chromosome numbers only slightly higher than in Fu5-5; Table 1 shows that the distributions of numbers in each of the chromosome groups is similar to that of Fu5-5. Also included in Table 1 are two subclones (BF5-1-1 and BF5-1-1-b) isolated from BF5-1; each number or letter in the strain designation indicates a successive clonal isolation.

All variants and subclones were tested for TAT activity and inducibility. All had only very small amounts of activity, but one of them, variant BF5-1 (and all of its descendents), showed increased activity in the presence of dexamethasone. This observation suggested the interesting possibility that BF5-1 re-expresses hepatoma genes involved in TAT induction as a consequence of loss of certain BRL-1 chromosomes. However, preliminary experiments showed that TAT induction in BF5-1 is transitory: a significant increase occurs after 5 hr of incubation with dexamethasone, but is followed by a rapid decline, whereas hepatoma cells retain high activity after at least 24 hr in dexamethasone. Moreover, the maximum "induced" TAT activity in these hybrid cells is less

FIG. 1. Time course of tyrosine aminotransferase induction in Fu5-5 (left) and BF5-1-1 cells (right). For both cell types, two curves are shown, representing early logarithmic cultures (open circles) and late logarithmic or stationary cultures (closed circles).

than 50% of that of noninduced hepatoma and less than 5% of that of induced hepatoma cells. In view of these differences between hepatoma and BF5-1 cells, a systematic comparison of the parameters of TAT induction in both cell types was undertaken.

Table 2 shows TAT activity and inducibility during the early and late phases of the growth cycle of the parental and hybrid cells. BRL-1 cells and the hybrid clones BF5-C (with the complete parental chromosome sets) and BF5- β (segregated) showed no significant increases in TAT activity during any stage of the growth cycle. By contrast, BF5-1-1 cells showed a significant increase in TAT after 5 hr in dexamethasone; cultures in the early logarithmic phase maintained high TAT activity even after 24 hr. Moreover, in BF5-1-1-b cells, prolonged induction is maintained even in late log or stationary growth phase (Table 1 shows that this subclone has undergone further chromosome loss). Finally, Table 2 shows that in Fu5-5 cells there are differences both in basal and induced levels of TAT during the growth cycle.

These data show that the increase in TAT activity upon exposure to dexamethasone is limited to the hepatoma and the group of hybrids (clone BF5-1 and its descendants), which have lost large numbers of chromosomes. The fact that an independent hybrid clone (BF5- β), which has lost as many chromosomes as BF5-1, is not inducible suggests

FIG. 2. Inhibition of tyrosine aminotransferase induction by cycloheximide. The concentrations of dexamethasone and cycloheximide used were 1 μ M and 0.1 mM, respectively. A series of cultures of Fu5-5 (left) and BF5-1-1 cells (right) were treated with the combinations of reagents indicated below, and collected for assay every 2 hr for the next 8 hr. The points at time 0 (baseline) are from untreated cultures. Notice that the scales (ordinates) for the specific activity of the enzyme are different for the two cell types. ●—●, dexamethasone; ○—○, cycloheximide; ■—■, dexamethasone plus cycloheximide; □—□, dexamethasone for 4 hr, then cycloheximide was added.

TABLE 3. Inhibition of tyrosine aminotransferase induction by actinomycin D*

Dexamethasone (1 μ M)	Actinomycin D (μ g/ml)	Fu5-5		BF5-1-1	
		Specific activity	% Inhibition of induction	Specific activity	% Inhibition of induction
—	0	27.5	—†	0.4	—
+	0	121.0	0	5.1	0
+	0.1	61.0	64	2.0	66
+	0.5	43.1	83	0.9	89
+	1.0	34.8	92	0.7	94
—	1.0	36.1	—	0.4	—

* Cells were collected and assayed after exposure for 5 hr to the agents indicated.

† —, Not induced.

that the expression of this property requires the loss of specific chromosomes. Moreover, the inducibility of clone BF5-1 proved to be an extremely stable property: 12 subclones of BF5-1 have been isolated and assayed, and all possessed this property; none had an elevated TAT baseline.

The time course of TAT induction in BF5-1-1 and Fu5-5 cells is shown in Fig. 1. In all experiments, a lag of 1.5–2 hr has been observed in the hybrid cells before the enzyme activity begins to increase; in the hepatoma cells, the lag is 0.5–1 hr. The curves in Fig. 1 show the differences in induction kinetics of early and late log cultures of each of the two cell types and the similarities between hepatoma cells in the late log phase and hybrid cells in the early log phase.

It has been shown (12, 13) that TAT induction in liver and in hepatoma cells is the result of *de novo* synthesis of the enzyme. Experiments involving the use of inhibitors (described below) show indirectly that synthesis of both RNA and protein is required for the induction of TAT in hepatoma and hybrid cells.

Cycloheximide (0.1 mM) inhibits protein synthesis in Fu5-5 and BF5-1-1 cells by 96 and 99%, respectively. A

TABLE 4. Intracellular distribution of tyrosine aminotransferase activity in control and induced cells

Cell type	Percent of total enzyme activity		Corrected total specific activity*
	Soluble	Mitochondrial	
BRL-1 control	27	73	0.6
BF5-1-1 control	52	48	0.7
BF5-1-1 induced†	83	17	4.0
Fu5-5 control	83	17	21.0
Fu5-5 induced†	89	11	68.3

* These values were determined by division of the sum of the activities in the two fractions by the sum of the protein content of the two fractions. The comparison of data shown in Fig. 1 with this column shows that full enzyme activity is recovered in these preparations.

† 5 hr exposure to 1 μ M dexamethasone.

series of parallel cultures were treated with combinations of dexamethasone and cycloheximide, as indicated in Fig. 2. It can be seen that for both cell types, cycloheximide completely inhibits enzyme induction. Moreover, the addition of cycloheximide to induced cultures blocks further induction; the rate of decline, a rough measure of the rate of enzyme degradation, is similar for both Fu5-5 and BF5-1-1 (the calculated half life of TAT is about 4-6 hr in both cases). Note, however, that cycloheximide causes a rapid decline of the noninduced enzyme only in the hepatoma cells.

Table 3 shows the effect of the simultaneous addition of dexamethasone and actinomycin D on TAT induction in Fu5-5 and BF5-1-1 cells. All concentrations of actinomycin D tested inhibited induction; the degree of inhibition caused by each concentration was similar for the two cell types.

The spectrum of dexamethasone concentrations eliciting TAT induction in Fu5-5 and BF5-1-1 cells was also examined. Fig. 3 shows that significant induction of the enzyme in the two cell types was obtained with 0.01-1 μ M dexamethasone. However, the hepatoma cells are slightly more sensitive than the hybrids, for only in the former was detectable induction obtained with 1 nM dexamethasone. It should be mentioned that for both cell types, optimal induction is obtained with concentrations of dexamethasone as low as 10 nM, but the rate of increase and the stability of the maximum induced activity vary with the concentration of the inducer.

It is known that rat liver TAT is present in both the soluble and mitochondrial fractions, and that induction leads to an increase in the enzyme activity in both fractions (14). As can be seen in Table 4, the distributions of TAT activity in

FIG. 3. Induction of tyrosine aminotransferase in Fu5-5 (closed circles) and BF5-1-1 cells (open circles) by different concentrations of dexamethasone. The indicated concentrations of dexamethasone were added to the cultures 5 hr before harvesting the cells for enzyme assays. The maximum specific activity of the enzyme was found in both hepatoma and hybrid cells that were treated with 1 μ M dexamethasone; activities found at all other concentrations are plotted as percent of this maximum. It will be noticed that the baseline activities represent 18 and 8% of the maximum induced activity for Fu5-5 and BF5-1-1, respectively (see points on ordinate).

FIG. 4. Heat inactivation of tyrosine aminotransferase from induced and noninduced parental and hybrid cells. Cell extracts were subjected to heating at 68°C; at the times indicated, the extracts were agitated, aliquots were removed and rapidly cooled in ice. After a brief centrifugation, all samples were assayed. No activity was detected in the BRL-1 extract that was heated for 5 min; the amount of activity remaining in the noninduced (control) BF5-1-1 extracts was barely measurable. ●, Fu5-5 control; ○, Fu5-5 induced; □, BF5-1-1 induced; ■, BF5-1-1 control; ×, BRL-1 control.

(induced and noninduced) hepatoma cells, and in induced BF5-1-1 cells are very similar. The activity of TAT of BRL-1 is primarily in the mitochondrial fraction, and that of noninduced BF5-1-1 is equally divided between the two fractions. These results show a similarity between hepatoma and induced hybrid cells, and a difference in noninduced hybrid cells.

These studies leave little doubt that the TAT induction observed in the hybrid cells is real, in spite of the enormous quantitative differences between the enzyme activities of these cells and hepatoma cells. However, the intracellular distribution of TAT in the noninduced BF5-1-1 cells, as well as the apparent absence of rapid degradation of noninduced BF5-1-1 enzyme in the presence of cycloheximide (see Fig. 2), suggest that there may be significant differences between the induced and noninduced enzymes in hybrid cells. By contrast, the enzymes from induced and noninduced hepatoma cells show identical physical (15) and serological (13) properties.

TAT of liver and hepatoma cells is known to be resistant to high temperature (15, 1). Enzyme extracts from parental and hybrid cells, both induced and noninduced, were subjected to heating at 68°C for various periods. As can be seen in Fig. 4, this treatment results in very little loss of TAT activity from extracts of Fu5-5 and induced BF5-1-1 cells. However, enzymes from noninduced BF5-1-1 and BRL-1 cells are rapidly degraded. These results show that in the hybrid cells, the baseline enzyme is different from the induced one. Whether this indicates the existence of a second and independent enzyme possessing TAT activity (16), or modification of the TAT molecule in noninduced hybrid cells, is not clear at present. Nevertheless, the measurements of baseline activity in the hybrid cells clearly lead to an over-

estimation of the activity of heat-stable TAT, which is inducible with dexamethasone.

DISCUSSION

In most studies of intraspecific somatic hybrids, chromosome loss has been found to be slight (3), except in the cases of very prolonged cultivation of L-cell hybrids (17), and selection *in vivo* of malignant segregants of A9-Ehrlich hybrids (11). In the present study, the use of selective conditions has permitted the isolation of hybrid subclones containing only 4–10 more chromosomes than the hepatoma parent.

The properties of TAT in the BF5 hybrid with complete sets of parental chromosomes are similar to those of the enzyme in rat hepatoma (Fu5) x mouse fibroblast (3T3) hybrids (1), and show that extinction of TAT and its inducibility in the interspecific hybrids is not merely a consequence of species differences. However, unlike 3T3, the BRL-1 parental cells used in these studies are: (a) diploid (rather than sub-tetraploid like cells of most permanent mouse lines) and contributed to the hybrid a balanced diploid set of chromosomes; (b) epithelial liver cells and are therefore ontogenetically less far removed from the hepatoma partner than fibroblasts. Nevertheless, the BRL-1 component suppresses hepatoma TAT as effectively as a fibroblast. In a study of heterokaryons formed by fusion of TAT inducible hepatoma cells with cells of a line similar in origin to BRL-1, Thompson and Gelehrter (18) have reported similar findings. Moreover, analysis of aldolase B activity in both 3T3 x Fu5 and BRL-1 x Fu5-5 hybrids has shown that this hepatoma function is similarly abolished in the two kinds of hybrids (22).

The properties of the segregated hybrid subclones that are inducible show a clear separation between two properties: high baseline of TAT and its inducibility. The model of Tomkins *et al.* of TAT induction proposes the existence of two genes controlling these properties (19); our study of TAT in hepatoma x fibroblast hybrids supported this suggestion (1). The observation that TAT induction can take place in the absence of appreciable "baseline" activity further supports the hypothesis that a specific gene mediates enzyme induction, and that its action is independent of the high TAT baseline.

Perhaps the most interesting conclusion to be drawn from our studies is that the extinction of a differentiated function requires the *continuous presence* of specific chromosomes, and when these are lost, the function reappears. This leads to the inference that the potentiality to perform this function can be inherited in the absence of its expression. Earlier, Klebe *et al.* (20) arrived at similar conclusions in their study showing re-expression of kidney esterase-2 in somatic hybrids after the loss of specific chromosomes of the nondifferentiated parent. These two observations suggest that re-expression of differentiated functions after loss of specific chromosomes may be a general phenomenon; if so, it should be possible to obtain as many classes of hybrids as there are (unlinked) genes that affect, directly or indirectly, the expression of differentiated functions within a given cell type.

The inducible hybrids possess little or no baseline activity of (heat stable) TAT; this is most unusual in mammalian systems. In spite of the described similarity of the parameters of TAT induction in hepatoma and segregated hybrid cells, there exist some differences. In particular, the phenomenon of actinomycin D "super-induction", well established for HTC (Hepatoma Tissue Culture) cells (15, 21) and observed also in Fu5 (1) and Fu5-5 cells, has never been observed in the hybrid cells. One interpretation of this difference is that in the hepatoma and hybrid cells, the factors that limit the complex series of molecular events culminating in induced TAT synthesis, are present in different proportions.

We thank Melle Anne Debon for excellent technical assistance, Drs. Drew Schwartz and Boris Ephrussi for stimulating discussions, and Dr. Ephrussi for critical reading of the manuscript. It is a pleasure to acknowledge the participation of Dr. Jerry Schneider in the early stages of this work. This work was conducted with the aid of a grant to Dr. Ephrussi from the Délégation Générale à la Recherche Scientifique et Technique.

1. Schneider, J. A., and M. C. Weiss, *Proc. Nat. Acad. Sci. USA*, **68**, 127 (1971).
2. Davidson, R. L., B. Ephrussi, and K. Yamamoto, *Proc. Nat. Acad. Sci. USA*, **56**, 1437 (1966).
3. Ephrussi, B., *Hybridization of Somatic Cells* (Princeton University Press, in press).
4. Reuber, M. D., *J. Nat. Cancer Inst.*, **26**, 891 (1961).
5. Pitot, H. C., C. Peraino, P. A. Morse, and V. R. Potter, *Nat. Cancer Inst., Monogr.*, **13**, 229 (1964).
6. Coon, H. G., *J. Cell Biol.*, **39**, 29a (1968); Coon, H. G., *Carnegie Inst. Washington, Yearb.*, **67**, 419 (1969).
7. Gelehrter, T. D., and G. M. Tomkins, *Proc. Nat. Acad. Sci. USA*, **64**, 723 (1969).
8. McCormick, W., and S. Penman, *J. Mol. Biol.*, **39**, 315 (1969).
9. Attardi, G., H. Parnas, M.-I. Hwang, and B. Attardi, *J. Mol. Biol.*, **20**, 145 (1966).
10. Weiss, M. C., G. J. Todaro, and H. Green, *J. Cell. Physiol.*, **71**, 105 (1968).
11. Harris, H., O. J. Miller, G. Klein, P. Worst, and T. Tachibana, *Nature*, **223**, 363 (1969).
12. Kenney, F. T., *J. Biol. Chem.*, **237**, 3495 (1962).
13. Granner, D. K., S. Hayashi, E. B. Thompson, and G. M. Tomkins, *J. Mol. Biol.*, **35**, 291 (1968).
14. Fellman, J. H., P. J. Vanbellighen, R. T. Jones, and R. D. Koler, *Biochemistry*, **8**, 615 (1969).
15. Thompson, E. B., G. M. Tomkins, and J. F. Curran, *Proc. Nat. Acad. Sci. USA*, **56**, 296 (1966).
16. Miller, J. E., and G. Litwack, *J. Biol. Chem.*, **246**, 3234 (1971).
17. Engel, E., B. J. McGee, and H. Harris, *Nature*, **223**, 152 (1969).
18. Thompson, E. B., and T. D. Gelehrter, *Proc. Nat. Acad. Sci. USA*, **68**, 2589 (1971).
19. Tomkins, G. M., T. D. Gelehrter, D. Granner, D. Martin Jr., H. Samuels, and E. B. Thompson, *Science*, **166**, 1474 (1969).
20. Klebe, R. J., T. Chen, and F. R. Ruddle, *Proc. Nat. Acad. Sci. USA*, **66**, 1220 (1970).
21. Thompson, E. B., D. K. Granner, and G. M. Tomkins, *J. Mol. Biol.*, **54**, 159 (1970).
22. Bertolotti, R., and M. C. Weiss, *J. Cell Physiol.*, in press.