

A HISTOCHEMICAL METHOD FOR THE DEMONSTRATION OF TYROSINE AMINOTRANSFERASE IN TISSUE CULTURE CELLS, AND STUDIES OF THIS ENZYME IN HEPATOMA TISSUE CULTURE CELLS

E. BRAD THOMPSON and GORDON M. TOMKINS. From the Laboratory of Biochemistry, National Cancer Institute, and the Laboratory of Molecular Biology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014. Dr. Tomkins' present address is the Department of Biochemistry, University of California, School of Medicine at San Francisco, San Francisco, California 94122

Previous studies have shown that certain steroid hormones induce about a 10-fold increase in tyrosine aminotransferase (TAT)¹ in hepatoma tissue culture cells (HTC cells) derived from Morris' rat hepatoma 7288c (1, 2). These studies had been carried out in the uncloned line and in relatively few clones. Since direct biochemical assay of individual cells is impossible, and of large numbers of clones is impracticable, we previously had been unable to determine whether all cells in the HTC line are inducible or even whether they all contain TAT. A tetrazolium salt technique, formerly utilized for qualitative assay of TAT in acrylamide gels (3), has been adapted for use as a histochemical reaction for the enzyme. Two TAT-containing cell lines and seven lines in which TAT is absent have been examined by the technique, and except for a peculiar, lone exception the histochemical reaction correlated with the biochemical presence or absence of the enzyme. The details of this technique and its use to investigate the presence of TAT in individual HTC cells are reported herein.

MATERIALS AND METHODS

Cell Lines

HTC cells were grown in monolayer culture on glass or plastic surfaces in Swim's medium S77 supplemented with 20% bovine and 5% fetal bovine serum, by the techniques previously described (1). The diploid liver cell lines RL 13 and BRL 62 were the generous gift of Dr. Hayden Coon, Department of Zoology, Indiana University. They were grown in medium WO/5 (4, 5). Chang liver, HeLa, and HEp

¹ *Abbreviations used in this paper:* HTC cells, hepatoma tissue culture cells; NAD, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide, reduced form; NADP, nicotinamide adenine dinucleotide phosphate; PBS, phosphate-buffered saline; TAT, tyrosine aminotransferase.

2 cells were obtained from Flow Labs., Inc., Rockville, Md. Chinese hamster cells were donated by Dr. W. McBride, National Cancer Institute, Bethesda, Md. Walker carcinoma cells were obtained from the American Type Culture Collection, Rockville, Md. All these latter lines were raised on Eagle's Basal Medium supplemented with serum. NIH 3T3 cells were obtained as a gift from Dr. Stuart Aaronson, National Cancer Institute. They were grown in Dulbecco-Vogt Medium supplemented with 10% fetal calf serum.

Protein content was assayed by the method of Lowry et al. (1951) (7). TAT was assayed biochemically by the method of Diamondstone, slightly modified (6).

Reagents

Sera used in the tissue culture media were obtained from Microbiological Associates, Inc., Bethesda, Md. Nitro blue tetrazolium was bought from Dajac Laboratories, Philadelphia, Pa.; moniodotyrosine (3-iodo-L-tyrosine) from Aldrich Chemical Co., Inc., Milwaukee, Wis.; and phenazine methosulfate from Calbiochem, Los Angeles, Calif. Swim's medium S77 was obtained in premixed powder form from Grand Island Biological Co., Grand Island, N. Y. The final Swim's growth medium, Eagle's medium, and Dulbecco's phosphate-buffered saline (PBS) were prepared by the National Institutes of Health Media Preparation Section. Coon's medium WO/5 was obtained from Laboratory Cell Suppliers, Frederick, Md.

The details of the tetrazolium staining procedure will be given below in the section on Results.

RESULTS

Histochemical Assay for TAT

The reaction catalyzed by the enzyme TAT is: L-tyrosine + α -ketoglutarate \rightleftharpoons *p*-hydroxyphenylpyruvate + L-glutamate.

Using an adaptation of the tetrazolium salt reduction method previously employed for detection of TAT in acrylamide gels and in eluates

from column chromatography (3), we sought optimal conditions for demonstrating the stain in tissue culture cells. The final combinations of reagents and their proportions are given in Table I.

It will be noted from Table I that reagents for the usual pathway to tetrazolium salt reduction, via nicotinamide adenine dinucleotide (NADH), are here lacking. In our original attempts to develop this assay we intended to link the glutamate formed by the TAT-catalyzed reaction to NADH production by adding NAD and glutamic dehydrogenase. The NADH so formed, we thought, should then reduce tetrazolium salt via phenazine methosulfate in the usual way. However, during a series of controls we discovered that the NADH generating system was redundant, and that the products of the TAT reaction alone were needed. Direct examination in test tubes in the absence of enzymes or any cellular material showed, in fact, that *p*-hydroxyphenylpyruvate reduces a tetrazolium when phenazine methosulfate is present. In the TAT-containing HTC cell line, a strong positive reaction occurred when all the reagents in Table I were included. The color intensity was slightly less when phenazine methosulfate was omitted, and no reaction occurred when the substrate moniodotyrosine was omitted. We interpret these results to mean that a direct electron-transfer reaction can occur between *p*-hydroxyphenylpyruvate and tetrazolium, via phenazine methosulfate, or in the cell, via endogenous electron-transfer systems as well.

While the staining quality of the formazans resulting from the reduction of nitro blue tetra-

zolium and iodinitrotetrazolium was approximately equal, the former dye was selected for routine use. Moniodotyrosine was used instead of L-tyrosine, the natural substrate, because the iodine-substituted analog had nearly as great an affinity for TAT as L-tyrosine, and had considerably greater solubility in the staining solution.

Various ways of preparing HTC cells for reaction were tried. Living intact cells, either in their serum-containing growth medium or in complete medium except with serum omitted, were found to react poorly during the several-hour time period they were exposed to the dye. Cells fixed with formaldehyde, glutaraldehyde, or methanol did not react. Heating the cells to 60°C for a few minutes in medium enriched with excess pyridoxal phosphate yielded only poor results, due to leakage of enzyme from the heated cells. This also occurred when the cell monolayers were quick frozen in liquid nitrogen and then flooded with reactant solution. Acetone fixation resulted in preparations with very poor morphological preservation but allowed clear-cut positive results, and therefore might be useful for certain applications. The procedure employed was to remove the growth medium from the monolayer, to rinse gently with phosphate-buffered saline (PBS) several times, and then to fix immediately with acetone for 5–10 min. The acetone was then removed, and the cell layer was rinsed once with PBS and then incubated in reactant solution for several hours at 37°C in a humidified incubator.

The best method of cell preparation, however, was found to be an air-drying technique similar

TABLE I
Reagents for Histochemical Assay of Tyrosine Aminotransferase

Dye mix	Relative volume	Substrate mix	Relative volume
Nitro blue tetrazolium 10 mg/ml water*	1.0	Moniodotyrosine 0.02 M in PBS†	5.0
Phenazine methosulfate 0.4 mg/ml PBS†	1.0	α -ketoglutaric acid 0.3 M in PBS, pH adjusted to 7 with 10 N NaOH	0.05
(Ethylenedinitrilo) tetracetic acid, disodium salt 0.1 M	0.375	Pyridoxal phosphate 1.3×10^{-3} M in PBS	0.2
Dulbecco's phosphate-buffered saline (PBS)	2.25		

* Made fresh. Dissolved by heating and protected from light.

† Stock solution, stored at 4°C in brown bottle. At time of use, 1 part dye mix is combined with 4 parts substrate mix.

to that which Wajntal and DeMars used for demonstration of glucose-6-phosphate dehydrogenase in cultured human fibroblasts with tetrazolium salts (8). The procedure was as follows: Monolayer cultures of cells were grown on glass cover slips or in plastic vessels in a humidified incubator gassed with 3% CO₂-97% air. Growth medium was removed from the cultures and the latter were rinsed two or three times by flooding gently with 0.1 M potassium phosphate buffer, at 4°C and pH 7.6, containing 2 × 10⁻⁴ M pyridoxal phosphate and 5 × 10⁻⁴ M α-ketoglutarate. (The cofactor and substrate were added because they stabilize TAT in cell-free solutions [9].) The final rinse was allowed to remain on the cells for 1-2 min and then was removed by suction, after which the monolayers were tipped so as to drain dry at room temperature for at least 1-2 hr. After drying, the cellular TAT activity was found to be rather stable, so that after several days' storage at 4°C in an ordinary refrigerator, the air-dried cell layers still gave positive stains, although cell morphology suffered. Histochemical reaction was carried out by flooding the preparations with the substrate-dye solution given in Table I and by incubating in the dark in the 37°C humidified air-CO₂ incubator for approximately 4 hr. Usually the blue reaction product could be seen in TAT-positive cells after 2 hr.

The distinction between TAT-positive and TAT-negative cells was greatest between 3 and 6 hr, after which a slow, gradual coloring of all cells, not dependent on TAT substrate, occurred. By 18 hr, even biochemically TAT-negative cells contained sufficient nonspecifically reduced tetrazolium to show a blue color. However, for at least the first 6 hr of incubation, color was almost entirely dependent on substrate, and omission of moniodotyrosine from the incubation solution resulted in a failure of cells to stain, as is shown in Fig. 2.

Several tissue culture cell lines have been examined, comparing the TAT activity found biochemically in cell extracts with the histochemical assay. The results of these examinations are given in Table II, and some examples of the histochemically positive and negative cells from Table II are shown in Figs. 1 and 3. As the data in the table show, the two cell lines positive for TAT biochemically (HTC and RL 13) were histochemically positive, and all but one of the biochemically negative cell lines were histochemically negative. Walker carcinoma cells, obtained from American Type Culture Collection, showed no TAT when extracts were assayed chemically, but by histochemical examination the cells showed a peculiar pattern of staining, with about half the cells showing no staining and the other half dem-

TABLE II
Comparison in Several Cell Lines of Tyrosine Aminotransferase Activity as Determined Biochemically and Histochemically

Cell line	TAT (Specific activity)	TAT stain
	<i>μmoles product/min per mg protein</i>	
HTC		
basal	7.8	+
steroid-treated	50	++
RL 13	4	+
BRL 62	1.5*	0
Chang liver	1‡	0
HeLa	1‡	0
HEp. 2	1‡	0
Chinese hamster	2‡	0
Walker	0	0 (ca 50%) + (ca 50%)
NIH 3T3	0	0

*BRL 62 extracts show a low level of activity which is not blocked by anti-TAT antiserum known to inhibit the enzyme in HTC cells.

‡ These low activities are probably not due to TAT; they represent very low optical density measurements. In some experiments extracts of these cell lines were completely without TAT activity.

onstrating blue perinuclear staining. We do not know the reason for this artifact; it does make clear that, since such "false positives" can occur in at least one cell line, it would be important to confirm the histochemical findings with direct biochemical assay of any new cell line examined. The results with the other cell lines examined, however, were clear, with only the cells known to contain TAT developing a deep blue cytoplasmic stain. Usually this occurred as a uniform blue color. Occasionally, the formazan was deposited in a more granular form as well. The TAT-negative cells in Figs. 1 and 3, and the HTC cells from which the substrate, moniodotyrosine, was omitted in Fig. 2, all showed a pale cytoplasm, ranging from completely unstained to light sky blue. Co-cultivation and reaction of TAT-positive and negative cell lines produced no cross-staining whatsoever, as is shown, for example, in Fig. 1.

The sensitivity of the method has not been systematically examined; however, some estimate of its lower limit of sensitivity can be derived from the fact that BRL 62 cells, with an apparent specific TAT activity of at most 1-2 $\mu\text{moles } p\text{-hydroxyphenylpyruvate formed/min per mg protein}$, become faintly blue, while uninduced HTC cells (specific activity of about 8 in this experiment) exhibit a definite color reaction (compare Figs. 1 and 4). Also, the RL 13 cell line, with a specific activity of 4, showed clear-cut staining.

Examination of TAT in HTC Cells by the Tetrazolium Salt Method

Populations of HTC cells were examined histochemically. Uncloned cells were grown in monolayers, exposed to TAT-inducing steroids or not, dried, and reacted. Figs. 1, 2, and 4 show an example of the results obtained. Several things should be noted in these plates. First, it is clear that the induced cells of Fig. 1 (TAT specific activity 50-100 when cultured alone) are more intensely colored than are the uninduced cells of Fig. 4 (TAT specific activity *circa* 8). Furthermore, all HTC cells contain some color, indicating that the uncloned line consists almost, if not entirely, of TAT-producing cells. On closer examination it can be seen that there is considerable variation in reaction color from cell to cell in both the induced and control cultures. This can be seen better in Fig. 5, showing induced HTC cells at a greater magnification. This could be due to inherent TAT content in the cells, to differential losses of enzyme during drying and incubation, or to a combination of these effects. In the course of several years' experiments with HTC cells, we have isolated and tested biochemically for TAT over 100 clones and have never found a clone completely negative for the enzyme, although we have seen up to 10-fold variation in enzyme content from clone to clone (Thompson, unpublished results). Part of the cell-to-cell variation in histo-

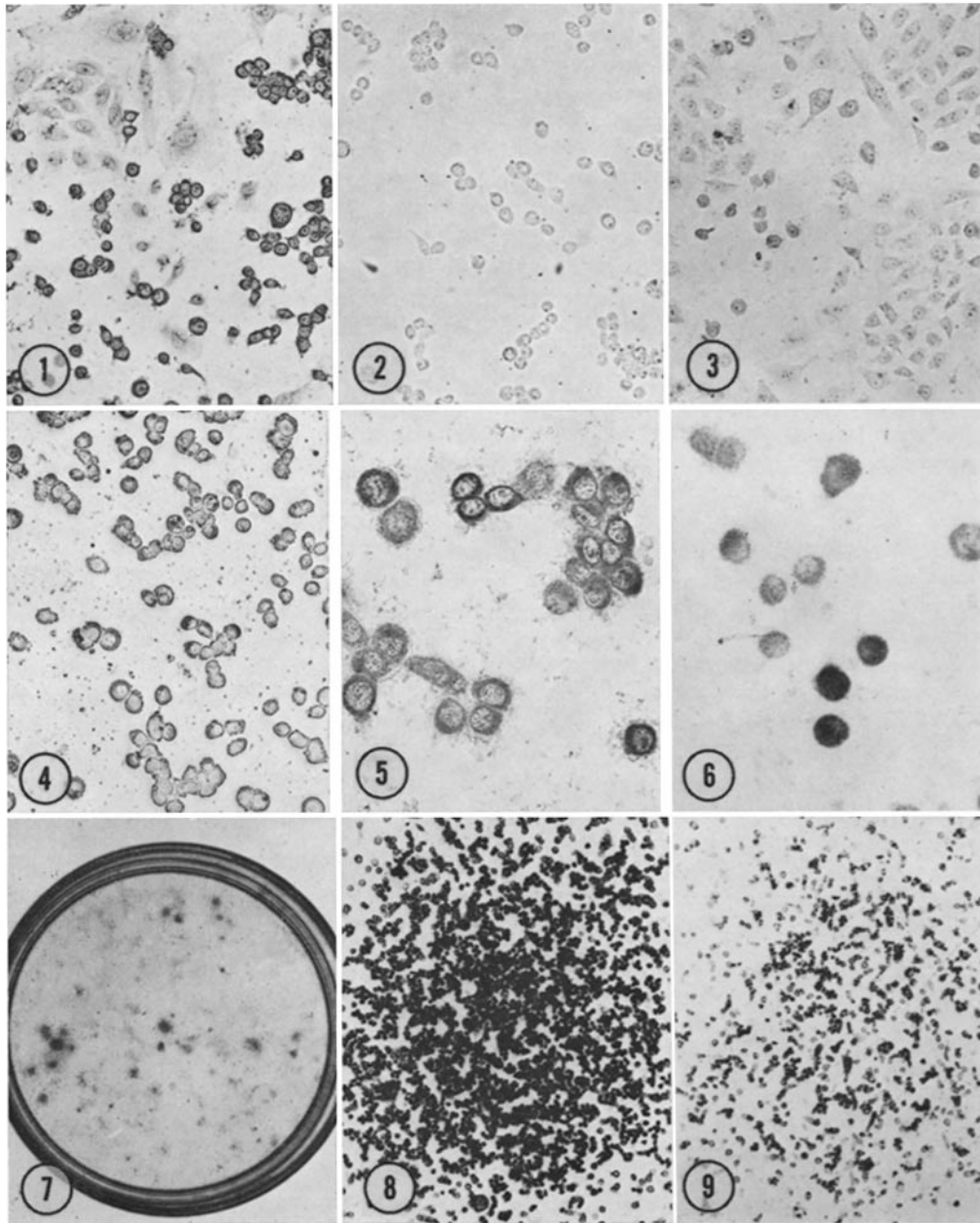
FIGURE 1 HTC and BRL-62 cell lines grown and examined together for the presence of tyrosine aminotransferase (TAT) by the procedure given in the text. Dexamethasone phosphate, 10^{-6} M, was added to the cultures 18 hr before preparation for staining, in order to achieve induced levels of enzyme in the HTC cells. These cells predominate in the lower half and right-hand side of the figure. They are mostly round, with a high nuclear/cytoplasmic ratio, and show intensely colored cytoplasm. TAT-negative BRL-62 cells, concentrated in the upper left-hand corner of the figure, are pale, polygonal, and show a relatively low nuclear/cytoplasmic ratio. Magnification, 100.

FIGURE 2 Steroid-induced HTC cells, substrate moniodotyrosine omitted during histochemical reaction. Magnification, 100.

FIGURE 3 NIH 3T3 cells, negative for TAT. Contrast heightened slightly photographically. Magnification, 100.

FIGURES 4, 5, and 6 TAT-containing HTC cells examined by the histochemical method described in the text. Fig. 4: Uninduced cells. Magnification, 100. Fig. 5: Induced cells, random culture. Magnification, 250. Fig. 6: Cells from a single induced clone. Magnification, 250.

FIGURES 7, 8, and 9 Clones of HTC cells stained for TAT. Fig. 7: Petri dish containing stained clones actual size. Figs. 8 and 9: Two clones from Fig. 7, magnified 40.



chemical reaction, therefore, might well be due to inherent variations in TAT levels. To examine this, cells were cloned by trypsinizing and plating small numbers of 90% monodisperse cells into Petri dishes. After about 2 wk of growth, macroscopic clones could be distinguished. These were induced for TAT by incubating overnight in medium containing 10^{-5} M dexamethasone phosphate and then were reacted histochemically (Fig. 7). Figs. 8 and 9 show examples of two clones, selected to exhibit the extremes of color intensity observed on the Petri plate in Fig. 7. We found that there was clone-to-clone variation in reaction but that the cell-to-cell variability within a given clone often exceeded that obtained between the clones (see Fig. 6). The differences in color intensity between clones depended not only on the average reaction of individual cells, but also on the growth pattern and number of cells per clone, as can be seen by the examples shown. We have examined over 9000 clones by the histochemical method and have found all of them positive for TAT. TAT induction in HTC cells, therefore, does not seem to be due to stimulation of a TAT-positive subpopulation nor to selection against or suppression of a TAT-negative subgroup. The data indicate that practically all HTC cells participate in induction, although the absolute level of enzyme may vary from cell to cell.

DISCUSSION

The use of tetrazolium dyes in histochemistry has permitted the study of a variety of enzymes. This paper presents a method in which *p*-hydroxyphenylpyruvate, the product of the non-NAD-requiring enzyme tyrosine aminotransferase, appears to reduce tetrazolium without the need for generating NADH. Phenazine methosulfate appears to enhance the reaction. A reaction of unknown mechanism between *p*-hydroxyphenylpyruvate and phenazine methosulfate which results in a blue color has been described and used as a basis for assaying TAT (10). We do not know whether this reaction has to do with the slight enhancement of staining in the presence of phenazine methosulfate, but it was clear that tetrazolium was the dye primarily responsible for the staining, because exposure to phenazine methosulfate without tetrazolium resulted in no staining whatsoever of HTC cells. On the other hand, HTC cells did stain when incubated in a system lacking phenazine methosulfate. This

ability to stain without the electron carrier that is necessary for reduction of tetrazolium by *p*-hydroxyphenylpyruvate in the test tube may be due to alternate electron-carrying systems available in the cells. Certainly *p*-hydroxyphenylpyruvate is a compound whose structure suggests that it could easily function as an electron donor. Other enzyme reactions have been described in which tetrazolium dyes are reduced in the absence of reduction of NAD or nicotinamide adenine dinucleotide phosphate (NADP). Such reactions include those mediated by succinic dehydrogenase, choline oxidase and *d*-amino acid oxidase, and the oxidation of tryptamine by monoamine oxidase (11). In none of these is the mechanism of transfer of electrons to formazan known.

The stain technique described herein is substrate dependent and enzyme dependent, and it showed excellent correlation, in all but one case, with the TAT content of various cell lines as estimated by biochemical assay. Since one cell line tested showed a "positive" blue product in the absence of TAT content, any new cell line found to stain blue should be checked for TAT by an independent method. The false positive stains we observed presumably were due to some reducing substance remaining in the cell despite the air-drying and subsequent dilution in reagent solution.

The usefulness of the TAT histochemical technique is shown by the studies herein of HTC cells. Induction of TAT by steroids in these cells had been clearly shown to occur in the uncloned line and in a few clones, but the possibility remained that HTC cells were a mixed population, with many cells lacking TAT. The isolation and biochemical assay of sufficient clones to rule out this possibility would have been laborious. Also, unless 100% cloning efficiency were achieved, it could never be conclusively shown that TAT-negative cells were not lost during cloning. The studies described above, on the other hand, were much simpler and clearly show that all or nearly all the uncloned HTC cells are TAT positive. The possibility remains that some cells are constitutive for TAT, but comparison of reaction color on induced and uninduced cells suggests that there is at least no very large fraction of constitutives in the uncloned line. This also fits with our biochemical studies on smaller numbers of clones of HTC cells.

We can conclude, therefore, that steroid-mediated induction of TAT in HTC cells is a nearly universal cellular event, rather than an action on or a selection of one subpopulation over another.

SUMMARY

A histochemical stain based on the reduction of tetrazolium salts has been developed for the detection of tyrosine aminotransferase in cultured cells. When tested in nine different cell lines the stain correlated well with the enzyme content of the cells, measured biochemically. HTC cells, a line of cells in which tyrosine aminotransferase is inducible by certain steroid hormones, has been studied with the stain technique. The results indicate that all HTC cells in the uncloned line contain the enzyme and that induction is not due to an effect on a subpopulation containing high enzymic activity.

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