Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 6913–6916, September 1989
Biochemistry

Expression in *Xenopus* oocytes of rat liver mRNA coding for a bile salt-dependent cholesteryl ester hydrolase

(pancreatic cholesteryl esterase/neutral cholesteryl esterase/sodium cholate)

REZA ZOLFAGHARI, EARL H. HARRISON, A. CATHARINE ROSS, AND EDWARD A. FISHER*

Division of Nutrition, Department of Physiology and Biochemistry, The Medical College of Pennsylvania, Philadelphia, PA 19129

Communicated by Nevin S. Scrimshaw, June 19, 1989

ABSTRACT **A catalytically active bile salt-dependent cholesteryl ester hydrolase (CEH) was expressed when *Xenopus* oocytes were injected with rat liver mRNA. The expressed CEH activity was highly dependent on the presence of trihydroxy bile salts (cholate or one of its conjugates); maximum hydrolytic activity was observed in the presence of 10 mM sodium cholate. The expressed CEH was not activated by dihydroxy bile salts (deoxycholate and its conjugates). In the presence of 10 mM sodium cholate, the CEH activity was maximal near pH 7 but was significant between pH 6 and 8. Monospecific immune IgG raised against rat pancreatic CEH completely inhibited the CEH expressed in *Xenopus* oocytes. Phenylmethylsulfonyl fluoride, a serine enzyme inhibitor, was inhibitory to the expressed CEH activity, whereas *p*-chloromercuribenzoate (up to 5 mM), a potent thiol-blocking agent, did not significantly inhibit the expressed activity. These experiments clearly demonstrate that the liver contains an mRNA encoding a bile salt-dependent CEH activity and suggest that the uptake of pancreatic enzyme is not necessarily the source of liver CEH as has been speculated.**

The liver plays a central role in the metabolism of lipid esters. For example, following the absorption of dietary fats, chylomicron remnants deliver cholesteryl esters and triacylglycerols to the hepatocyte. After hepatic uptake, the esters are, at least in part, hydrolyzed in lysosomes by the well-characterized lysosomal acid lipase (1). However, liver homogenates also contain a number of enzymes with neutral pH optima that can hydrolyze lipid esters. In terms of those with cholesteryl ester hydrolase (CEH) activity, such enzymes have been reported to occur in both cytosolic and membrane fractions of cell homogenates (2–4). None of these CEHs with neutral pH optima have been purified to homogeneity, and uncertainty exists regarding their physiological roles.

Previous studies of rat liver homogenates demonstrated that they contain a neutral CEH activity with several unusual properties (5–8). First, the CEH activity requires millimolar concentrations of trihydroxy bile salts for activity; second, the activity shows an unusual distribution among subcellular fractions of liver homogenates, being active in both the nuclear and soluble fractions of rat liver; third, the absolute enzyme activity varies markedly among individual rats of both outbred and inbred strains. The enzymatic properties of this bile salt-dependent CEH activity of liver homogenates are very similar to those of purified rat pancreatic CEH (cholesteryl esterase, EC 3.1.1.13). Indeed, antibodies prepared against the pancreatic enzyme can specifically inhibit the bile salt-dependent CEH activity in liver homogenates. These and other considerations led us to speculate recently that the bile salt-dependent CEH of rat liver homogenates might represent the uptake of pancreatic enzyme by the liver (8).

We were interested in exploring further the possible origin(s) of the bile salt-dependent CEH of rat liver homogenates and, in particular, we wished to determine whether the enzyme could be synthesized in the liver *per se*. As an initial question, we wanted to ask if the liver produces an mRNA for this enzyme. However, because neither the liver enzyme nor cDNA clones for the pancreatic enzyme were available, we could not assay directly for liver mRNAs specific for CEH. Therefore, the approach adopted was to attempt to express the bile salt-dependent CEH activity in *Xenopus* oocytes after microinjection of rat liver mRNA. As detailed in this report, the oocytes do not express an endogenous enzyme activity, but, following injection of liver mRNA, they express a neutral, bile salt-dependent CEH activity with many of the properties of the activity observed in rat liver homogenates. The results thus suggest that the liver can synthesize the enzyme and focus attention on the liver *per se* for future studies on the regulation of the enzyme activity.

MATERIALS AND METHODS

Isolation of mRNA. Adult Lewis female rats (Charles River Breeding Laboratories) were euthanized, and the livers were removed immediately and washed with cold phosphate-buffered saline solution (0.15 M NaCl, 8 mM sodium phosphate, and 2 mM potassium phosphate at pH 7.5, with 3 mM KCl, 1 mM $CaCl_2$, and 0.5 mM $MgSO_4$). The livers were minced, and total RNA was extracted by homogenization in 5 M guanidinium isothiocyanate followed by precipitation in the presence of 6 M guanidine hydrochloride (9). The total RNA so obtained was precipitated by 2.5 volumes of cold ethanol after addition of 0.1 volume of 2.5 M sodium acetate (pH 5.0). The RNA was stored at −20°C until further use.

To isolate poly(A)-containing mRNA (hereafter referred to simply as mRNA), the precipitated total RNA was first centrifuged at 15,000 × *g* for 10 min at 4°C, and the pellet was dissolved in 0.01 M Tris·HCl, pH 7.5/1 mM EDTA. This solution was then subjected to affinity chromatography on oligo(dT)-cellulose (Boehringer Mannheim) as described elsewhere (10), and the mRNA was precipitated as described above. The precipitate was then washed with cold 70% (vol/vol) ethanol, dried under reduced pressure, dissolved in autoclaved water, and stored at −70°C until the oocyte microinjection experiments. Enrichment for and integrity of mRNA were verified by Northern blotting (11, 12) of total RNA and mRNA with an actin cDNA probe (kindly provided by Lawrence Kedes, Stanford University).

Preparation and Microinjection of *Xenopus laevis* Oocytes. These procedures were performed essentially as described by White *et al.* (13). Ovary lobes were collected from adult

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: CEH, cholesteryl ester hydrolase; PMSF, phenylmethylsulfonyl fluoride.

*To whom reprint requests should be addressed.

Xenopus laevis (Xenopus I, Ann Arbor, MI) and washed with ND-96 [96 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/1 mM $MgCl_2$/5 mM Hepes (adjusted to pH 7.6 with NaOH) supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml)], followed by incubation of oocytes with calcium-free OR-2 solution (82 mM NaCl/2.5 mM KCl/1 mM $MgCl_2$/5 mM Hepes (adjusted to pH 7.6 with NaOH) containing collagenase (2 mg/ml, type IA, Sigma) at 19°C for 2 hr. The oocytes were kept in ND-96 solution at 4°C overnight, after which individual oocytes were manually peeled and then injected with about 50 nl of a solution of mRNA (2 mg/ml in water). The control oocytes were injected with an equal volume of water. Oocytes were incubated in ND-96 solution at 19°C for 1–7 days, with changes of ND-96 solution done twice daily.

At the end of the incubations, the oocytes were washed several times with 0.01 M Tris maleate, pH 7.5, containing 0.25 M sucrose and then homogenized with a glass homogenizer in groups of 50 oocytes per ml of the same buffer. The homogenate was centrifuged at 1000 × *g* for 15 min at 4°C to remove large granules of yolk protein. The supernatant was then collected for enzyme measurements. For simplicity, we considered 20 μl of supernatant to be equivalent to one oocyte since the supernatant from 50 oocytes was 1 ml.

Assay of CEH Activity. CEH activity was determined radiometrically, based on procedures previously described (5, 6). Typically, the reaction mixtures contained 0.05 M Tris maleate, pH 7.0, 10 mM sodium cholate, and appropriately diluted enzyme (up to 15 μl of original supernatant) in a final volume of 0.2 ml. The reaction was initiated by addition of 2 nmol (0.05 μCi; 1 Ci = 37 GBq) of cholesteryl [1-^{14}C]oleate (Amersham; the specific activity of the compound as supplied was 52 Ci/mol. This was diluted to 25 Ci/mol by the addition of unlabeled cholesteryl oleate) in 0.01 ml of ethanol, and the tubes were capped and incubated in a water bath at 37°C for various times up to 24 hr. The released [^{14}C]oleate was assayed as described (8). Each experiment included controls containing the inactivated oocyte supernatant (mRNA-injected oocyte supernatant placed in a boiling water bath for 5 min) before addition of substrate. All experiments were carried out at least twice. A unit of enzyme activity is defined as 1 pmol of [^{14}C]oleate released per 24 hr.

Antibody Neutralization Experiments. Portions of oocyte supernatant containing the expressed CEH activity were added to screw-capped test tubes along with various amounts of either rabbit anti-rat pancreatic IgG (a gift of Linda L. Gallo) or normal rabbit IgG, dissolved in 0.154 M NaCl, in a final volume of 0.07 ml. Control tubes contained the supernatant alone. After incubation at 4°C overnight (16–17 hr), the samples were assayed for CEH activity for 24 hr at 37°C in the presence of 10 mM sodium cholate.

RESULTS

When mRNA purified from rat liver was injected into *X. laevis* oocytes, a neutral bile salt-dependent CEH activity was expressed. As presented in Table 1, enzymatic activity was not expressed in oocyte homogenates immediately after injection but was detectable after 1 day and continued to increase during 6 days of incubation. CEH activity was not present in uninjected oocytes, oocytes injected with water, or mRNA-injected oocyte homogenates incubated at 100°C for 5 min. After 3 days, the average activity for oocytes injected with rat liver mRNA equaled 27 ± 9 activity units (pmol of [^{14}C]oleate released per 24 hr; mean ± SD) per oocyte for three different preparations of mRNA. This amount of activity was clearly detectable, since it corresponded to more than 1000 cpm above background, which averaged 20 ± 1 cpm.

Table 1. Time course for expression of CEH in *Xenopus* oocytes injected with rat liver mRNA

Incubation period after injection, days	CEH activity, units per oocyte
0	0
1	4
2	10
3	27
4	59
5	105
6	159

Xenopus oocytes were injected with either rat liver mRNA or water (control) and then incubated at 19°C for different periods of time up to 7 days. The expressed CEH activity was then determined in the 1000 × *g* supernatant fraction of oocyte homogenate incubated with cholesteryl [^{14}C]oleate at 37°C for 23 hr. One CEH activity unit represents 1 pmol of [^{14}C]oleate released per 24 hr. The values given are the means of three determinations on each day for the oocytes injected with rat liver mRNA. The control values were <0.3 activity unit per oocyte on each day.

The expressed CEH activity in the oocyte supernatant was highly dependent on the presence of trihydroxy bile salts (cholic acid or one of its conjugates). In the absence of sodium cholate, CEH activity was almost undetectable (Fig. 1*A*). However, addition of 0.5–50 mM sodium cholate resulted in a marked increase in activity in a concentration-dependent manner. Maximum hydrolytic activity was observed in the presence of 10 mM (about 0.5%) sodium cholate, a concentration well above the critical micellar

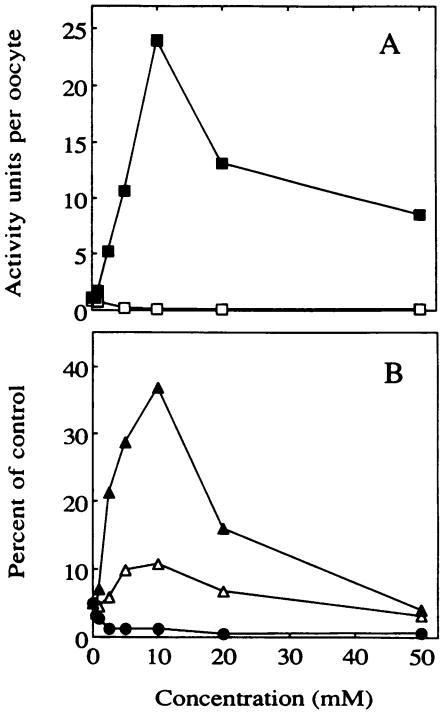

FIG. 1. Effect of sodium cholate (*A*) or other bile salts (*B*) on the activity of rat liver CEH expressed in *Xenopus* oocytes. (*A*) Supernatant fractions of oocytes were prepared 3 days after injection with either rat liver mRNA (■) or water (□) and were then incubated with 10 μM cholesteryl [^{14}C]oleate at 37°C for 24 hr in the presence of 0–50 mM sodium cholate. (*B*) Supernatants of mRNA-injected oocytes were incubated in the same manner with 0–50 mM sodium taurocholate (▲), sodium glycocholate (△), or sodium deoxycholate (●), and the CEH activity was then determined and related to the percent of control activity in samples containing 10 mM sodium cholate. Points represent the means of four determinations from two separate microinjections.

concentration [approximately 5 mM under the conditions employed in this experiment (14)]. Fig. 1*B* illustrates that, for two conjugated forms of cholate tested, activity was also maximal at 10 mM; however, the extent of stimulation of CEH activity by these conjugates was significantly lower than that for sodium cholate itself. The expressed CEH activity was not stimulated at all by addition of sodium deoxycholate (Fig. 1*B*), and other dihydroxy bile salts, including glycochenodeoxycholate, glycodeoxycholate, taurochenodeoxycholate, and taurodeoxycholate, did not activate the expressed enzyme (data not shown). Furthermore, the detergents Triton X-100 (0.2%) and 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (known as CHAPS) (0.5–50 mM) did not stimulate CEH activity.

The dependence of the expressed CEH activity on pH was determined. Similar to the bile salt-dependent CEH from rat liver (7, 8), hydrolytic activity was maximal near pH 7 (Fig. 2) but was significant between pH 6 and 8, with almost no activity in the acidic pH range, wherein precipitation of cholate occurred. At pH 7 and in the presence of 10 mM sodium cholate, the rate of hydrolysis of cholesteryl oleate increased proportionately with the amount of oocyte supernatant (up to 0.75 oocyte equivalent, Fig. 3*A*) and was curvilinear with incubation time up to 24 hr (Fig. 3*B*). Thus, considerable stability of the CEH activity is demonstrated both by the accumulation of reaction product throughout 24 hr of supernatant incubation at 37°C and by the increasing CEH activity expressed during incubation of oocytes at 19°C for up to 6 days before homogenization (Table 1).

Because previous studies have shown that the activity of neutral, bile salt-dependent CEH activity in rat liver can be completely inhibited by addition of specific IgG prepared against rat pancreatic CEH (8), it was of interest to determine whether the CEH activity expressed in *Xenopus* oocytes is also inhibited by this antibody. Increasing amounts of rabbit anti-rat pancreatic CEH IgG or equivalent amounts of nonimmune IgG were incubated at 4°C for 16–17 hr with the supernatant of the mRNA-injected oocyte homogenate, and then CEH activity was measured. As illustrated in Fig. 4, the expressed CEH activity was inhibited in a dose-dependent manner by anti-pancreatic CEH IgG but was unaffected by nonimmune IgG. As little as 0.1 μg of the specific anti-pancreatic CEH IgG inhibited the activity by >40%, and 5 μg produced >90% inhibition.

Pancreatic neutral CEH has been reported to behave differently against some specific inhibitors when the enzyme

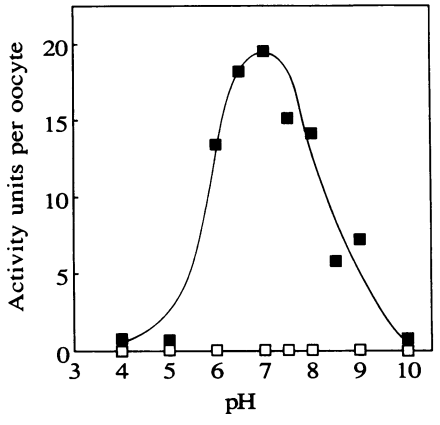

FIG. 2. Dependence on pH of CEH activity expressed in *Xenopus* oocytes. All incubations included 10 mM sodium cholate. Supernatants were from oocytes injected with mRNA (■) or water (□). Buffers used included sodium acetate for pH 4 and 5, Tris maleate for pH values between 6 and 8.5, and glycine adjusted to the desired pH with NaOH for pH 9 and 10. Other conditions were as described in Fig. 1.

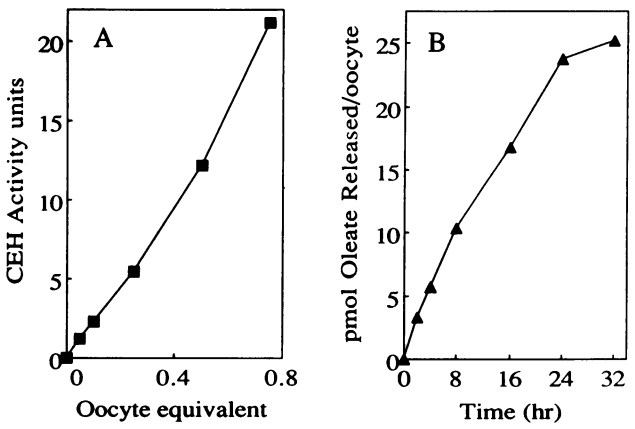

FIG. 3. CEH activity as a function of the amount of *Xenopus* oocyte equivalent (*A*) and incubation time (*B*). All incubations contained 10 mM sodium cholate; other conditions were as described in Fig. 1. Each point represents the mean of two determinations.

is first treated with trihydroxy bile salts; therefore, some further characteristics of the expressed CEH were investigated in oocyte supernatants. For example, taurocholate has been shown to increase the sensitivity of pancreatic neutral CEH enzyme to phenylmethylsulfonyl fluoride (PMSF), an inhibitor of enzymes requiring free serine hydroxyl groups for activity (15). When oocyte supernatants were incubated with PMSF prior to addition of cholate and incubation with cholesteryl oleate substrate, 1 and 2 mM PMSF caused 25% and 66% inhibition of CEH activity, respectively. However, when cholate was added to the supernatants prior to incubation with the same concentrations of PMSF, CEH activity was inhibited by 65 and 91%, respectively. It has also been reported that cholate can protect pancreatic CEH against inactivation by *p*-chloromercuribenzoate, an inhibitor of enzymes requiring free sulfhydryl group for activity (16). In contrast to the observations with pancreatic CEH, addition of *p*-chloromercuribenzoate at concentrations up to 5 mM did not significantly inhibit the CEH activity, whether or not cholate was present in the preincubation.

DISCUSSION

Xenopus oocytes injected with mRNA from rat liver expressed a catalytically active bile salt-dependent CEH with

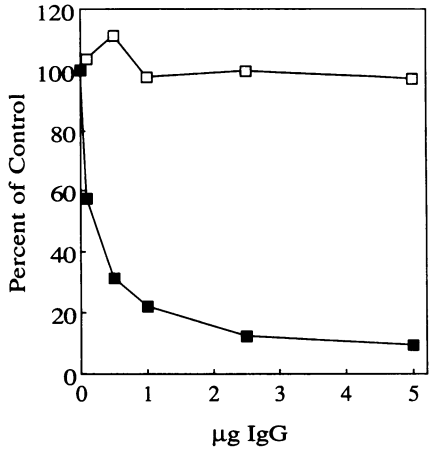

FIG. 4. Inhibition of rat liver CEH activity in *Xenopus* oocytes by immune IgG to rat pancreatic CEH. A fixed amount of the oocyte supernatant fraction (about 18 CEH activity units) was incubated with various amounts of rabbit anti-rat pancreatic CEH IgG (■) or normal rabbit IgG (□). Points represent the mean of two determinations.

many of the properties of a neutral CEH activity, requiring millimolar concentrations of bile salts (hereafter referred to simply as CEH) that has been described in a variety of rat tissues, especially the pancreas, liver, and intestine (17, 18). The expressed CEH in oocyte supernatants had a broad pH optimum centered at pH 7 and a requirement for cholate, a trihydroxy bile salt, at millimolar concentrations for maximal activity. Dihydroxy bile salts, including deoxycholate and its conjugates, had no stimulatory effects on the expressed CEH activity, similar to results with CEH from rat liver homogenates and pancreas (7, 16, 19), despite the fact that these bile salts are as effective as the trihydroxy species in solubilizing cholesteryl oleate. That cholate may serve another purpose in addition to the solubilization of substrate is suggested by its augmentation of the inhibition of the expressed CEH and pancreatic CEH activities by PMSF (15). This inhibition also implies that the expressed enzyme requires serine for catalytic activity. The lack of inhibition by *p*-chloromercuribenzoate, however, suggests that sulfhydryl groups are not required for the expressed CEH activity, in contrast with what has been reported for pancreatic CEH (16).

The expressed CEH activity was inhibited by monospecific anti-rat pancreatic CEH antibodies, similar to the results we have reported previously for CEH in liver homogenates (8). This does not necessarily imply identity of the expressed enzyme with the pancreatic CEH, since it has been reported that this antibody, in immunodiffusion assays, reacts with pancreatic and intestinal homogenates but not with liver homogenates (20). Thus, the particular environment of the enzyme may affect its reaction with the antibody. Definitive studies that could determine the relationship of liver CEH to CEH activities of other organs will be discussed below.

Based on various lines of evidence, it is currently thought that intestinal CEH is derived from pancreatic secretions (20–22). It has been proposed that the hepatic enzyme could also be derived from pancreatic secretions absorbed in the intestine since (*i*) CEH that is immunoreactive with antibodies to pancreatic CEH is found in intestinal cells and lymph (20), (*ii*) CEH activity is present in plasma (8), and (*iii*) specific binding of purified pancreatic CEH to liver cells has been observed (Linda L. Gallo, personal communication). Our results clearly demonstrate, however, that the liver contains an mRNA that encodes an enzyme with CEH activity and with many of the key properties reported for the CEH activity in both liver homogenates and pancreatic secretions. Not only may the intestinal route not be needed to explain the source of the liver enzyme, but, in fact, the intestinal CEH could be derived from both the liver and pancreas, because bile contains CEH activity (8) and bile and pancreatic secretions are delivered to the duodenum through the ampulla of Vater.

We and others have previously noted (7, 8) a marked quantitative variability in CEH activity in liver homogenates of both inbred and outbred strains of rats. Because we obtained relatively uniform levels of expressed CEH activity in oocytes, in spite of using three separate preparations of mRNA, we suggest that the source of variability in liver may be related to posttranslational events. Once cDNA probes are available for liver CEH, definitive experiments to correlate mRNA levels with enzyme activity in rat livers could be performed. Additionally, the cloning of the cDNAs for hepatic CEH and pancreatic CEH would facilitate determination of the exact relationship between the enzymes in these tissues.

Our results suggest that, for rat liver CEH, the oocyte expression system could be used to isolate the specific mRNA encoding the CEH activity in a manner similar to what has been done for a number of membrane receptors and channels (see ref. 23 for a recent example). Given the reported instability of liver CEH during purification procedures (7), the possibility of deriving its amino acid sequence by this strategy is particularly attractive.

In conclusion, we have shown that *Xenopus* oocytes injected with rat liver mRNA express an enzyme activity with remarkably similar properties to those described for liver CEH. These results imply that the liver itself is capable of synthesizing CEH and, hence, that uptake of pancreatic enzyme need not be invoked as the source of the liver enzyme.

We wish to thank Dr. Michael M. White of the University of Pennsylvania for his generous help in setting up the oocyte microinjection techniques and Dr. Linda L. Gallo of The George Washington University Medical Center for kindly supplying antibodies to rat pancreatic CEH. This work was supported by grants from the Howard Heinz Endowment, the National Institutes of Health (HL-22633), and the W. W. Smith Foundation. A.C.R. is the recipient of National Institutes of Health Research Career Development Award HD-00691.

1. Fowler, S. D. & Brown, W. J. (1984) in *Lipases*, eds. Borgström, B. & Brockman, H. L. (Elsevier, Amsterdam), pp. 329–364.
2. Deykin, D. & Goodman, D. S. (1962) *J. Biol. Chem.* **237**, 3649–3656.
3. Goller, H. J. & Sgoutas, D. S. (1970) *Biochemistry* **9**, 4801–4806.
4. Goller, H. J., Sgoutas, D. S., Ismail, D. A. & Gunstone, F. D. (1970) *Biochemistry* **9**, 3072–3076.
5. Harrison, E. H., Smith, J. E. & Goodman, D. S. (1979) *J. Lipid Res.* **20**, 760–771.
6. Prystowsky, J. H., Smith, J. E. & Goodman, D. S. (1981) *J. Biol. Chem.* **256**, 4498–4503.
7. Blaner, W. S., Prystowsky, J. H., Smith, J. E. & Goodman, D. S. (1984) *Biochim. Biophys. Acta* **794**, 419–427.
8. Harrison, E. H. (1988) *Biochim. Biophys. Acta* **963**, 28–34.
9. Han, J. H., Stratowa, C. & Rutter, W. J. (1987) *Biochemistry* **26**, 1617–1625.
10. Jacobson, A. (1987) *Methods Enzymol.* **152**, 254–261.
11. Fourney, R. M., Miyakoshi, J., Day, R. S., III, & Paterson, M. C. (1988) *Focus* **10**, 5–7.
12. Wahl, G. M., Ong, E., Meinkoth, J., Franco, R. & Barinaga, M. (1981) *Transfer and Immobilization of Nucleic Acids to S & S Solid Supports* (Schleicher & Schuell, Keene, NH), p. 16.
13. White, M. M., Mayne, K. M., Lester, H. A. & Davidson, N. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 4852–4856.
14. Carey, M. C. & Small, D. M. (1972) *Arch. Intern. Med.* **130**, 506–527.
15. Hyun, J., Treadwell, C. R. & Vahouny, G. V. (1972) *Arch. Biochem. Biophys.* **152**, 233–242.
16. Hyun, J., Kothari, H., Herm, E., Mortenson, J., Treadwell, C. R. & Vahouny, G. V. (1969) *J. Biol. Chem.* **244**, 1937–1945.
17. Brockerhoff, H. & Jensen, R. G. (1974) *Lipolytic Enzymes* (Academic, New York), pp. 176–193.
18. Rudd, E. A. & Brockman, H. L. (1984) in *Lipases*, eds. Borgström, B. & Brockman, H. L. (Elsevier, Amsterdam), pp. 185–204.
19. Vahouny, G. V., Weersing, S. & Treadwell, C. R. (1965) *Biochim. Biophys. Acta* **98**, 607–616.
20. Gallo, L. L., Cheriathundam, E., Vahouny, G. V. & Treadwell, C. R. (1978) *Arch. Biochem. Biophys.* **191**, 42–48.
21. Gallo, L. L., Newbill, T., Hyun, J., Vahouny, G. V. & Treadwell, C. R. (1977) *Proc. Soc. Exp. Biol. Med.* **156**, 277–281.
22. Gallo, L. L., Chiang, Y., Vahouny, G. V. & Treadwell, C. R. (1980) *J. Lipid Res.* **21**, 537–545.
23. Julius, D., MacDermott, A. B., Axel, R. & Jessel, T. M. (1988) *Science* **241**, 558–564.