*Proc. Natl. Acad. Sci. USA*
Vol. 79, pp. 3134–3137, May 1982
Biochemistry

# Heat-stable inhibitor of translation in reticulocyte lysates

**(polypeptide chain initiation/inhibition of translation/protein phosphorylation/eukaryotic initiation factor 2 kinase)**

CESAR DE HARO*, VEERASWAMY MANNE†, ANTONIO G. DE HERREROS*, AND SEVERO OCHOA†*

†Roche Institute of Molecular Biology, Nutley, New Jersey 07110; and *Centro de Biologia Molecular, CSIC-Universidad Autonoma, Madrid 34, Spain

*Contributed by Severo Ochoa, February 19, 1982*

**ABSTRACT** **Inhibition of translation in hemin-containing reticulocyte lysates by catalytic subunit (cS) preparations of cAMP-dependent protein kinase from bovine heart, reported earlier by our group, is due to a highly active heat-stable protein contaminant (HS). The specific activity for translational inhibition goes up by a factor of 10 when cS is heated for 10 min at 80°C, which completely destroys histone phosphorylation activity. HS has been purified to homogeneity from bovine heart. It consists of a single polypeptide chain ($M_r \approx 68{,}000$). HS inhibits translation with biphasic kinetics similar to those of hemin deficiency and induces pronounced phosphorylation of the $\alpha$ subunit of the eukaryotic initiation factor eIF-2. The inhibition is relieved by eIF-2 or GTP but not by high concentrations of double-stranded RNA, thus ruling out involvement of the double-stranded RNA-activated inhibitor. Judged by poly(U) translation, HS has no effect on chain elongation. When added to crude preparations of the proinhibitor form (proHCI) of the heme-controlled translational inhibitor (HCI), HS appears to produce an increase of the HCI-to-proHCI ratio. The mode of action of HS is as yet unknown.**

Reticulocyte lysates contain two potential inhibitors of polypeptide chain initiation. One, the heme-controlled inhibitor (HCI), is activated in the absence of heme, the other, the double-stranded RNA (ds RNA)-activated inhibitor (DAI), is activated by low concentrations of ds RNA in the presence of ATP. Both HCI and DAI are cAMP-independent protein kinases that phosphorylate the $\alpha$ (38-kilodalton) subunit of the eukaryotic chain initiation factor eIF-2, causing translational inhibition. Once activated, HCI and DAI inhibit translation in reticulocyte lysates in the presence of heme, but the mechanism of activation remains obscure (for a review see refs. 1 and 2). When looking whether HCI might be activated through phosphorylation catalyzed by a cAMP-dependent protein kinase, we observed that catalytic subunit (cS) preparations of cAMP-dependent protein kinase from bovine heart (BH kinase) inhibited translation in hemin-containing reticulocyte lysates and appeared to promote formation of active HCI (3). Because other investigators (1, 4, 5) reported weak or no translational inhibition by various cS preparations we compared the activities of preparations of different degrees of purity on (*i*) histone phosphorylation and (*ii*) translational inhibition and found an inverse relationship between the two—i.e., preparations of lower specific activity for (*i*) had higher specific activity for (*ii*) and conversely (unpublished data). This clearly indicated that the translational effect was due to a contaminant. We now find that this contaminant is a heat-stable protein (HS). It has been purified to homogeneity from bovine heart and we report here some of its properties. HS promotes increased phosphorylation of the eIF-2 $\alpha$ subunit in reticulocyte lysates and may be similar to the heat-stable reticulocyte factor of Henderson *et al.* (6).

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.

## MATERIALS AND METHODS

**Assays.** HS was assayed by its inhibitory effect on translation in hemin-containing reticulocyte lysates. Translation was assayed in 30-$\mu$l samples essentially as described by Hunt *et al.* (7) with 18 $\mu$l of 1:1.5 lysate and [$^{14}$C]leucine as the labeled amino acid, with or without 20 $\mu$M hemin and HS as indicated. Incubation was for 45–60 min at 30°C. One unit is defined as the amount of HS causing 50% inhibition of hemin-dependent translation, and specific activity is expressed as units/mg of protein. Inhibition is proportional to HS concentration up to about 70%. Protein was determined by the Lowry (8) or the Bradford (9) procedure with bovine serum albumin as the standard. "Activation" of the proinhibitor (proHCI) was assayed by inhibition of either translation in hemin-containing lysates or ternary complex formation. The latter was measured by assay B, as described (10), with use of ≈25% pure eIF-2 CM350 (≈2 pmol of eIF-2) and crude eIF-2-stimulating protein (ESP) CM200 (≈6 $\mu$g). The amounts of proHCI and HS used in either assay are specified in the legends. Histone phosphorylation was assayed as described (3). One unit of kinase activity was taken as the amount of enzyme catalyzing the transfer of 1 pmol of $^{32}$P from [$\gamma$-$^{32}$P]ATP to histone per min, under standard assay conditions, and the specific activity is expressed as units/mg of protein. The conditions for assay of phosphorylation of the eIF-2 $\alpha$ subunit are described in the legend to Fig. 1*D*. Dephosphorylation of eIF-2($\alpha$-$^{32}P$) in reticulocyte lysates was assayed under conditions optimal for translation as described in the legend to Fig. 2.

**Preparations.** Rabbit reticulocyte lysates were prepared as described (7). eIF-2 CM350 and ESP CM200 were prepared as described (10). cS was prepared from Sigma BH kinase by the method of Rubin *et al.* (11). It is referred to as cS-BHK. cS prepared from highly purified BH kinase (11) was a gift of R. Rangel-Aldao and O. M. Rosen (Albert Einstein College of Medicine). It is referred to as cS-OMR. Homogeneous cS from BH kinase (12) was kindly provided by E. H. Fischer (University of Washington, Seattle). It is referred to as cS-EHF. Crude proHCI was prepared by the Gross and Rabinovitz procedure (13) as described (3), except that the lysate was directly chromatographed on CM-Sephadex to avoid pressure activation by high-speed centrifugation (1). For preparation of eIF-2($\alpha$-$^{32}P$), 360 $\mu$g of (≈90% pure) eIF-2 (2,200 pmol), prepared by the

Abbreviations: eIF-2, eukaryotic initiation factor 2; ESP, crude form of the eIF-2-stimulating protein (see ref. 10); Met-tRNA$_i$, eukaryotic initiation methionyl-tRNA; BH kinase, cAMP-dependent bovine heart protein kinase; cS and rS, catalytic and regulatory subunits (or subunit-containing fractions) of BH kinase; HS, heat-stable translational inhibitor from bovine heart; HCI, heme-controlled translational inhibitor (an eIF-2 $\alpha$ kinase); ds RNA, double-stranded RNA; DAI, ds RNA-activated translational inhibitor (another eIF-2 $\alpha$ kinase); proHCI, the proinhibitor (inactive) form of HCI; eIF-2($\alpha$-*P*), $\alpha$-phosphorylated eIF-2; HCI(*P*), phosphorylated (active) form of HCI.

Table 1. Presence of heat-stable translational inhibitor in cAMP-dependent BH kinase

| Fraction assayed | Protein, mg | Histone phosphorylation Units | Histone phosphorylation Specific activity | Translational inhibition Units | Translational inhibition Specific activity |
|---|---|---|---|---|---|
| 1. BH kinase (Sigma) | 9.1 | 178,400* | 19,600 | 2,200 | 240 |
| 2. cS | 0.3 | 87,600 | 292,000 | 300 | 1,000 |
| 3. cS heated | 0.02 | | | 253 | 12,800 |
| 4. rS | 5.6 | 18,500 | 3,300 | 1,700 | 300 |
| 5. rS heated | 3.0 | | | 1,700 | 570 |
| 6. cS-OMR | ND | 7,800 | | 21 | |
| 7. cS-OMR heated | ND | 6 | | 28 | |
| 8. cS-EHF | ND | 112,000 | | 40 | |
| 9. cS-EHF heated | ND | 1 | | 39 | |

Heating was for 10 min at 80°C; cS and rS fractions in lines 2 and 4 were derived from Sigma BH kinase. ND, not detectable.
* Assay in the presence of cAMP (10 μM).

procedure of Benne *et al.* (14) through the phosphocellulose step, was incubated for 30 min at 30°C in a solution (0.4 ml) containing 20 mM Tris·HCl at pH 7.6, 5 mM $Mg(OAc)_2$, 5 mM

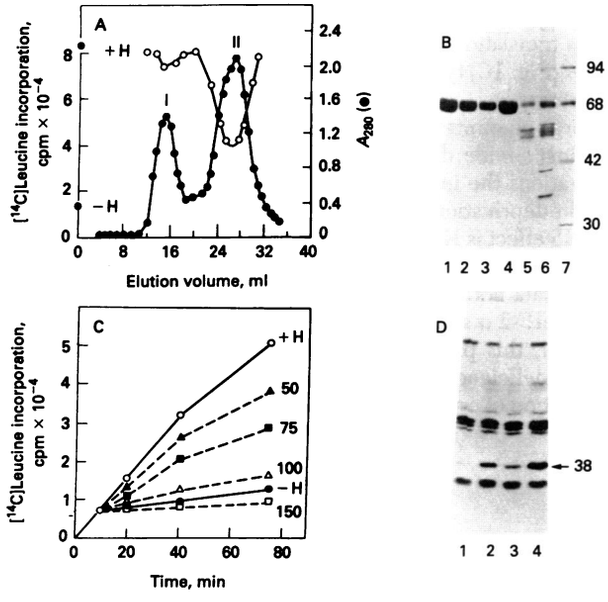


FIG. 1. (*A*) Chromatography of step 4 HS on Sephadex G-200. ●, $A_{280}$; ○, [$^{14}$C]leucine incorporation (incubation, 60 min). Control translation values, without hemin (−H) and with 20 μM hemin (+H) are shown by ● on the ordinate. (*B*) $NaDodSO_4$/polyacrylamide gel electrophoresis of HS at various steps of purification. Tracks: 1, step 5 (24 μg); 2, step 5 (15 μg); 3, step 5 (9 μg); 4, step 4 (26 μg); 5, step 3 (30 μg); 6, step 2 (48 μg); 7, markers (kilodaltons), phosphorylase *b* (94), bovine serum albumin (68), ovalbumin (43), carbonic anhydrase (30). (*C*) Kinetics of translational inhibition by HS. Without (●) and with (○) 20 μM hemin controls. With 20 μM hemin and increasing amounts of step 5 HS: ▲, 50; ■, 75; △, 100; and □, 150 ng. (*D*) Effect of step 5 HS on translation and on phosphorylation of the eIF-2 α subunit. Translation reaction samples were incubated for 60 min at 30°C when aliquots were taken for assay of (*i*) [$^{14}$C]leucine incorporation (6 μl) and (*ii*) eIF-2 phosphorylation. For the latter purpose 10-μl aliquots were supplemented with various components to give the following concentrations or amounts: Hepes buffer, pH 7.6, 20 mM; dithiothreitol, 1 mM; $Mg(OAc)_2$, 0.5 mM; eIF-2 CM350, ≈3.5 pmol of eIF-2; [γ-$^{32}$P]ATP, 30–40 × $10^6$ cpm. After incubation for 4 min at 30°C, the aliquots were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography (10). The conditions were: track 1, with 20 μM hemin; track 2, without hemin; track 3, with hemin and 70 ng of HS; track 4, with hemin and 200 ng of HS. The corresponding 60-min translation values were 49,500, 14,100, 28,700, and 12,200 cpm.

Table 2. Purification of HS from 160 g of bovine heart muscle

| Step | Volume, ml | Protein, mg | Translational inhibition Units | Translational inhibition Specific activity |
|---|---|---|---|---|
| 2. Ammonium sulfate | 75 | 1,200 | 192,000 | 160 |
| 3. Heating | 100 | 190 | 93,000 | 490 |
| 4. DEAE-cellulose | 5 | 5.5 | 22,600 | 4,110 |
| 5. Sephadex G-200 | 0.6 | 1.1 | 14,500 | 13,180 |

2-mercaptoethanol, 0.1 mM [γ-$^{32}$P]ATP (3,360 cpm/pmol), and 70 μg of partially purified HCI prepared as described (15). eIF-2(α-$^{32}$P) was isolated by heparin-Sepharose chromatography (16). The pooled peak fractions (2.1 ml) contained 872 pmol of eIF-2 and 815.5 pmol of $^{32}$P, a $^{32}$P/eIF-2 ratio of 0.93. GDP-free GTP (used throughout) was prepared as described (10). Ubiquitin was a gift of Irwin W. Rose (Cancer Research Institute, Philadelphia). Reovirus ds RNA was kindly provided by A. J. Shatkin of this Institute. Other preparations were obtained commercially.

## RESULTS

**Presence of Heat-Stable Translational Inhibitor in BH Kinase.** As seen in Table 1, translation inhibitory activity in BH kinase is quantitatively recovered in cS and the regulatory subunit (rS), although the bulk of the units (85%) is in the latter fraction (column 5, compare lines 1, 2, and 4). Heating cS (10 min at 80°C) abolishes histone phosphorylation (column 3, lines 6–9) but leaves the translation inhibitory activity unchanged (column 5, lines 2–5 and 6–9). In fact, heating precipitates 90% of the protein (column 2, lines 2 and 3), resulting in greater than 10-fold increase of the specific activity of the supernatant for translational inhibition (column 6, lines 2 and 3). Ninety nanograms of protein inhibited translation ≈50%. Even homogeneous cS (12) contains HS (column 5, lines 8 and 9), although much less than cS preparations of lower purity. The ratio of translational inhibition to histone phosphorylation (units × $10^3$) was 3.4, 2.7, and 0.4 for cS-BHK, cS-OMR, and cS-EHF, respectively. Evidently a heat-stable contaminant of BH kinase was responsible for our earlier (3) results.

**Purification of HS.** HS was purified to apparent homogeneity from bovine heart. The first two steps (homogenization and ammonium sulfate precipitation) were carried out essentially as described by Rubin *et al.* (11) for BH kinase. The precipitate was dissolved in 50 mM Tris·HCl at pH 7.6 (buffer A) containing 10 mM KCl and dialyzed overnight against three 1.5-liter vol of the same buffer, and the resulting precipitate was removed by centrifugation (step 2). The step 2 solution was heated for

Table 3. Translational inhibition by HS is reduced by GTP or eIF-2

| Additions | Hemin-dependent translation, cpm | Inhibition, % |
|---|---|---|
| None | 67,100* | |
| HS (100 ng) | 9,900 | 85 |
| HS + GTP (1 mM) | 51,900 | 23 |
| HS + eIF-2 (9 pmol) | 43,100 | 35 |

Step 5 HS was used.
* Incorporations with and without hemin were 80,600 and 13,500 cpm, respectively.

Table 4. Effect of HS on HCI-to-proHCI ratio (translation assay)

| Supplements to basic reaction mixture | Hemin-dependent [$^{14}$C]leucine incorporation, cpm | Inhibition, % |
|---|---|---|
| None | 47,700 | |
| proHCI (1.5 μg protein) | 47,700 | 0 |
| HS (25 ng) | 45,000 | 6 |
| proHCI + HS | 15,700 | 67 |

Samples (12 μl) containing 20 mM Hepes buffer at pH 7.6, 0.8 mM $MgCl_2$, and 80 μM ATP (basic reaction mixture) with or without proHCI (9.0 μg of protein) and with or without HS (150 ng) were incubated for 10 min at 30°C and 2-μl aliquots were assayed. The amounts of supplements given in the table are per 2 μl. Two more experiments gave equivalent results: 51% inhibition with 2.5 mg of proHCI protein and 15 ng of HS, and 46% with 1.5 μg of proHCI protein and 15 ng of HS (3–6% inhibition with either alone). Step 5 HS was used.

Table 5. Effect of HS on HCI-to-proHCI ratio (ternary complex formation assay)

| Supplements to basic reaction mixture | [$^3$H]Met-tRNA$_i$ bound, pmol | Inhibition, % |
|---|---|---|
| None | 0.50 | |
| ProHCI (0.3 μg protein) | 0.46 | 8 |
| HS (20 ng) | 0.48 | 4 |
| ProHCI + HS | 0.27 | 46 |

Basic reaction mixtures (30 μl) containing 33 mM Hepes buffer at pH 7.6, 20 mM KCl, 0.83 mM $Mg(OAc)_2$, 1.37 mM dithiothreitol, 83 μM ATP, eIF-2 CM350 (≈2 pmol of eIF-2), ESP CM200 (≈6 μg), with or without supplements as indicated, were incubated for 6 min at 30°C. They were then supplemented with KCl (final concentration 100 mM), 1.5 pmol of [$^3$H]Met-tRNA$_i$, and 32 μM GTP (final volume 50 μl), incubated for a further 6 min at 30°C, and assayed for ternary complex formation. Step 5 HS was used.

15 min at 80°C with continuous mechanical stirring and the copious precipitate was removed by centrifugation (step 3). The supernatant was applied to a column (0.9 × 58 cm) of DEAE-cellulose (Whatman DE 52), previously equilibrated with buffer A containing 10 mM KCl, and washed with the same buffer until $A_{280}$ was negligible. The column was then eluted stepwise with buffer A containing successively 80, 130, and 300 mM KCl. The translation inhibitory activity was eluted at 130 mM KCl. This fraction was dialyzed against buffer A containing 10 mM KCl (step 4). The step 4 solution was concentrated by filtration through an Amicon membrane, applied to a column (1.5 × 28 cm) of Sephadex G-200 (Pharmacia), previously equilibrated with buffer A containing 10 mM KCl, and eluted with the same buffer. Fractions, 0.5 ml, were collected. As seen in Fig. 1*A*, protein was eluted in two major peaks. The bulk of the translation inhibitory activity was in peak II and the corresponding fractions were pooled (step 5). A summary of the purification procedure is given in Table 2.

**Properties of HS.** The $A_{280}/A_{260}$ ratio of step 5 HS is 1.6. The protein nature of HS is also supported by its sensitivity to trypsin (not shown). Its apparent molecular weight from gel filtration data is about 70,000. $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 1*B*) showed a single band ($M_r \approx 68{,}000$) not only for step 5 but also for step 4 HS. This suggests that step 5 HS is monomeric and that peak I (Fig. 1*A*) contains an inactive (or poorly active) polymeric form or aggregate of HS. HS inhibits translation in hemin-containing lysates with biphasic kinetics (Fig. 1*C*) typical of hemin lack or HCI addition to hemin-containing lysates (1, 2), and the inhibition is similarly relieved by large amounts of GTP or eIF-2 (Table 3). Reaction with *N*-ethylmaleimide does not affect HS activity. At high HS concentrations the inhibition may be greater than that caused by hemin deprivation alone (Fig. 1*C*). These results suggest that the HS effect is HCI-mediated, and this suggestion is borne out by the fact that, whereas HS does not phosphorylate eIF-2 by itself (data not shown), it produces increased phosphorylation of the eIF-2 α subunit in lysates (Fig. 1*D*). At high HS concentrations, this phosphorylation is greater than that caused by hemin deficiency (Fig. 1*D*, compare tracks 2 and 4). Moreover, incubation of crude proHCI with HS and ATP results in an ap-

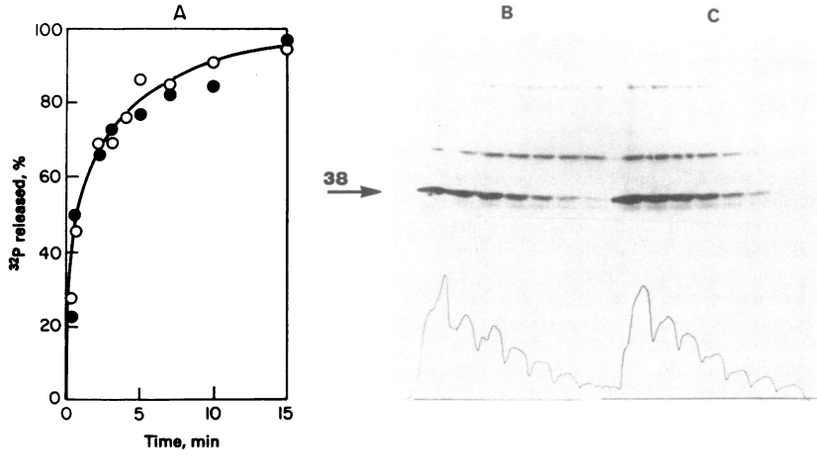


FIG. 2. Dephosphorylation of eIF-2(α-$^{32}$P) in reticulocyte lysate in the absence or presence of HS. Except for the presence of eIF-2(α-$^{32}$P), incubations were conducted under conditions optimal for translation. (*A*) Two reaction samples, each 300 μl, were prepared; they contained 10 mM Tris·HCl at pH 7.6, 100 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM ATP, 0.2 mM GTP, 10 mM creatine phosphate, 37.5 μg of creatine kinase, a mixture of 20 amino acids in the proportions found in rabbit globin, 20 μM hemin, 135 μl of 1:1.5 lysate, and 4.08 μg (28 pmol) of eIF-2(α-$^{32}$P) (2,700 cpm/pmol). One of the samples received 540 ng of step 5 HS, the other an equal volume of water. Both samples were incubated at 30°C and aliquots (25 μl) were taken for assay of acid-soluble radioactivity at the indicated times. ○, No HS; ●, HS addition. (*B* and *C*) Identical reaction samples were prepared, one without (*B*), the other with (*C*) HS addition. They were incubated at 30°C and suitable aliquots were taken at 0, 0.5, 1, 3, 5, 7, and 10 min for analysis by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography (*B* and *C Upper*). The 38-kilodalton bands of the autoradiograms were scanned at 595 nm with an Ortec model 4310 densitometer (*B* and *C Lower*). Time increase is from left to right. Assays without eIF-2(α-$^{32}$P) showed that, under the conditions of these experiments, translation was almost completely inhibited by the concentration of HS used. Dephosphorylation of eIF-2(α-$^{32}$P) was negligible in controls without lysate.

parent increase of active HCI as judged by (*i*) inhibition of translation in hemin-containing lysates (Table 4) or (*ii*) inhibition of ternary complex formation (Table 5) and is also accompanied by significantly increased phosphorylation of the eIF-2 $\alpha$ subunit (not shown).

## DISCUSSION

A homogeneous heat-stable protein (HS) isolated from bovine heart is a potent inhibitor of translation in reticulocyte lysates. We have not examined reticulocytes for a similar protein, but HS may well be the counterpart of the heat-stable reticulocyte factor of Henderson *et al.* (6). Like the Henderson factor, HS has no effect on chain elongation [poly(U) translation]. Both factors inhibit chain initiation by promoting phosphorylation of the $\alpha$ subunit of the initiation factor eIF-2, and this effect appears to be mediated by HCI. HS could either activate HCI (or DAI) or inhibit protein phosphatase(s) acting on eIF-2($\alpha$-*P*), HCI(*P*), or both. Protein phosphatase inhibition was conceivable after the discovery of low molecular weight heat-stable protein inhibitors of phosphorylase *a* phosphatase (17, 18). DAI activation and inhibition of eIF-2($\alpha$-*P*) dephosphorylation can be ruled out, the former because high concentrations of ds RNA (which block DAI activation) are without effect on HS activity (not shown), the latter because eIF-2($\alpha$-$^{32}$*P*) is rapidly dephosphorylated in lysates whether HS is present or not (Fig. 2). The possibility that HS inhibits HCI(*P*) dephosphorylation has also been ruled out. As for activation, HS could activate HCI in various ways (direct or indirect, physiological or not)—e.g., conformational change, phosphorylation, limited proteolysis.‡ Proteolysis may probably be ruled out for protease inhibitors (phenylmethylsulfonyl fluoride, soybean trypsin inhibitor) did not affect the translation inhibitory activity of HS, and ubiquitin (22, 23) had no effect on translation with or without HS. It may be tentatively concluded that HS inhibits translation in hemin-supplemented reticulocyte lysates by augmenting the HCI-to-proHCI ratio, resulting in increased phosphorylation of the eIF-2 $\alpha$ subunit, but it is not known whether HS is part of a physiological system of modulation of HCI activity or has no physiological role.

‡ Non-cAMP-dependent protein kinases can be activated by proteolysis (e.g., by trypsin). Among others are phosphorylase kinase (19) and histone kinase from reticulocytes (20) and other animal tissues (21).


1. Hunt, T. (1979) in *Miami Winter Symposium: From Gene to Protein*, eds. Russell, T. R., Brew, K., Schultz, J. & Haber, H. (Academic, New York), Vol. 16, pp. 229–243.
2. Ochoa, S. & de Haro, C. (1979) *Annu. Rev. Biochem.* **48**, 549–580.
3. Datta, A., de Haro, C., Sierra, J. M. & Ochoa, S. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1463–1467.
4. Grankowski, N., Kramer, G. & Hardesty, B. (1979) *J. Biol. Chem.* **254**, 3145–3147.
5. Levin, D., Ernst, V. & London, I. M. (1979) *J. Biol. Chem.* **254**, 7935–7941.
6. Henderson, A. B., Miller, A. H. & Hardesty, B. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 2605–2609.
7. Hunt, T., Vanderhoff, G. & London, I. M. (1972) *J. Mol. Biol.* **66**, 471–481.
8. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
9. Bradford, N. M. (1976) *Anal. Biochem.* **72**, 248–254.
10. de Haro, C. & Ochoa, S. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1741–1745.
11. Rubin, C. E., Erlichman, J. & Rosen, O. M. (1972) *J. Biol. Chem.* **247**, 36–44.
12. Peters, K. A., Demaille, J. G. & Fischer, E. H. (1977) *Biochemistry* **16**, 5691–5697.
13. Gross, M. & Rabinovitz, M. (1972) *Biochim. Biophys. Acta* **287**, 340–352.
14. Benne, R., Wong, C., Luedi, M. & Hershey, J. W. B. (1976) *J. Biol. Chem.* **251**, 7675–7681.
15. de Haro, C. & Ochoa, S. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2713–2716.
16. Goldstein, J. & Safer, B. (1979) *Methods Enzymol.* **60**, 165–181.
17. Nimmo, A. & Cohen, P. (1978) *Eur. J. Biochem.* **87**, 341–351.
18. Foulkes, J. G. & Cohen, P. (1980) *Eur. J. Biochem.* **105**, 195–203.
19. Cohen, P. (1973) *Eur. J. Biochem.* **34**, 1–44.
20. Tahara, S. M. & Traugh, J. A. (1981) *J. Biol. Chem.* **256**, 11558–11564.
21. Inoue, M., Kishimoto, A., Takai, Y. & Nishizuka, Y. (1977) *J. Biol. Chem.* **252**, 7610–7616.
22. Wilkinson, K. D., Urban, M. K. & Haas, A. L. (1980) *J. Biol. Chem.* **255**, 7529–7532.
23. Haas, A. L. & Rose, I. A. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 6845–6848.