tional changes involving only minor electron relocalization. This is not so with H atoms as the reducing agent (*7*, *16*). Hydrogen atoms react faster than e^-_{aq} with some amino acid components of protein—for example, aromatic amino acids—but e^-_{aq} react faster than H atoms with CIII. The competition by the protein moiety should be more effective in the case of H atoms. Competition experiments involving steady state irradiation (*5*) or H atoms as such introduced into the solution from the gas phase (*6*) showed that under our conditions all H atoms react with CIII, $k = 1 \times 10M^{-1}$ sec^{-1}. This rate constant was also obtained in the pulse radiolysis experiments, where 15 to 20 percent of the H atoms reacted with CIII in a very fast second order process, yielding CII (*7*). An additional 35 to 40 percent of CII is then formed in intramolecular reactions over the time scale of 10^{-5} to 10^{-1} second (Fig. 1). The total reduction efficiency by H atoms is thus some 50 percent of all H atoms taken up by CIII. Figure 1 shows the kinetics of CII formation with malate and with H atoms. When all the CIII has been reduced to CII by H atoms, the H atoms continue to react with CII, leading—as the steady state chemical experiments showed (*6*)—to reactions with the protein and heme.

Our results thus indicate specific complete reduction of CIII by malate in a second order reaction which is about 30 times slower than the diffusion controlled rate, has a low activation energy, and is thus consistent with selective interaction with part of the enzyme surface. Hydrogen atoms react with CIII at close to the diffusion controlled rate, but only part of H so taken up leads directly to the appearance of CII. The results are consistent with uptake of H by several sites on the enzyme; intermediate enzyme radicals are formed, and the added electron equivalent not residing on the iron part of these radicals transfers intramolecularly to yield CII.

Fast, consecutive intramolecular radical transfer processes have been established in the ribonuclease molecule, and spectroscopic evidence indicated that the sites of the intermediate radicals include divalent sulfur and aromatic amino acids (*17*). For cytochrome c we do not yet know what the sites are.

AVIGDOR SHAFFERMAN
GABRIEL STEIN
Department of Physical Chemistry, Hebrew University, Jerusalem, Israel

References and Notes

1. R. E. Dickerson, T. Takano, D. Eisenberg, O. B. Kallai, L. Samson, A. Cooper, E. Margoliash, *J. Biol. Chem.* **246**, 1511 (1971); R. E. Dickerson, *Sci. Am.* **226** (No. 4), 58 (1972); E. Margoliash and A. Schejter, *Adv. Protein Chem.* **21**, 113 (1966).
2. N. Sutin, *Chem. Br.* **8**, 148 (1972); ——— and J. K. Yandell, *J. Biol. Chem.* **247**, 6932 (1972); J. Jordan, paper presented at the Symposium on Bioelectrochemistry, Princeton, New Jersey, 11 to 15 October 1971; D. DeVault, J. H. Parkes, B. Chance, *Nature (Lond.)* **215**, 642 (1967); M. E. Winfield, *J. Mol. Biol.* **12**, 600 (1965); R. E. Dickerson, T. Takano, O. B. Kallai, L. Samson, in *Structure and Function of Oxidation Reduction Enzymes*, A. Akeson and A. Ehrenberg, Eds. (Pergamon, Oxford, 1972), p. 69.
3. G. Stein and A. J. Swallow, *Nature (Lond.)* **173**, 937 (1954); L. K. Mee and G. Stein, *Biochem. J.* **62**, 377 (1956).
4. S. Munk and G. Stein, in *Proceedings of the Second Conference on Atomic Energy*, Geneva (1958); vol. 22, p. 535.
5. J. Rabani and G. Stein, *Radiat. Res.* **17**, 327 (1962).
6. G. Czapski, N. Frohwirth, G. Stein, *Nature (Lond.)* **207**, 1191 (1965).
7. N. N. Lichtin, J. Ogdan, G. Stein, *Isr. J. Chem.* **9**, 579 (1971); N. N. Lichtin, A. Shafferman, G. Stein, in preparation.
8. E. J. Land and A. J. Swallow, *Arch. Biochem. Biophys.* **145**, 365 (1971); A. J. Swallow, *Isr. J. Chem.* **10**, 999 1972; I. Pecht and M. Faraggi, *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **13**, 221 (1971); K. Nilsson, *Isr. J. Chem.* **10**, 1011 (1972); J. Wilting, R. Braams, H. Nanta, K. J. H. von Buuren, *Biochim. Biophys. Acta* **283**, 543 (1972).
9. N. N. Lichtin, A. Shafferman, G. Stein, *Science* **179**, 680 (1973); *Biochim. Biophys. Acta* **314**, 117 (1973).
10. M. J. Day and G. Stein, *Nature (Lond.)* **168**, 644 (1951); *Nucleonics* **8** (No. 2), 34 1951); *Radiat. Res.* **6**, 666 (1957); G. Stein, *Discuss. Faraday Soc.* **12**, 227 (1952).
11. A. J. Swallow, *Radiation Chemistry* (Longman, London, 1973), p. 151.
12. A. Shafferman and G. Stein, in preparation.
13. G. Scholes and M. Simic, *J. Phys. Chem.* **68**, 1738 (1964).
14. M. Simic, P. Neta, E. Hayon, *ibid.* **73**, 3794 (1969).
15. R. Braams, *Radiat. Res.* **27**, 319 (1966); P. Neta and R. H. Schuler, *ibid.* **47**, 612 (1971).
16. N. N. Lichtin, A. Shafferman, G. Stein, in preparation.
17. N. N. Lichtin, J. Ogdan, G. Stein, *Biochim. Biophys. Acta* **263**, 14 (1972); *ibid.* **276**, 124 (1972); L. K. Mee, S. J. Adelstein, G. Stein, *Radiat. Res.*, **52**, 588 (1972).
18. Supported by the Division of Biology and Medicine, U.S. Atomic Energy Commission, under contract AT(11-1)-3009. We thank N. N. Lichtin for valuable discussion.

5 July 1973; revised 13 September 1973

Protein Kinase Translocation as an Early Event in the Hormonal Control of Uterine Contraction

Abstract. *β-Adrenergic stimulation with isoproterenol inhibits contractility, increases cyclic adenosine monophosphate (AMP) concentration, decreases the concentration of unsaturated cyclic AMP receptor sites, and increases cyclic AMP–independent kinase in the uterus of ovariectomized rats. The total soluble kinase activity is reduced. The protein kinase activity lost from the cytosol was translocated to the microsomal fraction mostly in a cyclic AMP–independent form, suggesting a particulate substrate for the activated enzyme.*

Regulation of protein kinase activity by hormones has been reported for diaphragm (*1*), adrenal (*2*), and adipose tissue (*3*), but the substrate for activated kinase was not studied; however, the phosphorylase-glycogen synthetase (*4*) and triglyceride lipase (*5*) systems may be assumed to have been involved. Our studies of hormonal regulation of adenylate cyclase in the uterus have led us to investigate the relation of elevation of cyclic adenosine monophos-

Table 1. Effects of isoproterenol on the adenylate cyclase responsive system of rat uterus. Each value is the mean of triplicate determinations and indicates nanomoles of ^{32}P incorporated per milligram of protein.

Protein kinase activity (nmole ^{32}P/mg protein)				Cyclic AMP binding sites (pmole/mg protein)		Cyclic AMP (pmole/mg protein)	
Control		Treated					
−Cyclic AMP	+Cyclic AMP	−Cyclic AMP	+Cyclic AMP	Control	Treated	Control	Treated
			10^{-5}M Isoproterenol, 10 minutes				
0.31	2.66	0.86	1.45	3.5	1.7	30.9	490
0.25	1.91	0.41	0.65	2.7	1.0	18	70
0.11	0.51	0.32	0.44	3.6	0.9	9	250
			10^{-5}M Isoproterenol, 30 minutes				
0.44	1.19	0.61	0.77	11.4	3.4	3.5	40
0.31	1.48	0.50	0.51	8.0	6.7	6.4	34
			5×10^{-6}M Isoproterenol, 20 minutes				
0.24	1.31	0.28	0.81	14.5	11.9	5.4	15
			5×10^{-6}M Isoproterenol, 30 minutes				
0.30	1.30	0.50	1.14	12.6	10.2	1.3	1.7
0.35	0.77	0.66	0.77	20.0	12.8	9.4	28
0.28	0.69	0.36	0.41	5.4	4.4	5.7	22

phate (AMP) to protein kinase activation (*6, 7*). We now report that adenylate cyclase stimulation by isoproterenol reduced the number of unsaturated cyclic AMP binding sites and increased the concentration of cyclic AMP–independent protein kinase in a manner roughly proportional to the degree of the stimulation. The apparent concentration of protein kinase in the supernatant fraction of homogenates of uterus tissue decreased significantly after adenylate cyclase activation, and the lost kinase activity could be recovered in the (operationally defined) microsomal fraction by extraction with Triton X-100.

Uteri from 15 to 25 ovariectomized (5 to 8 day) rats were pooled, and incubated for 20 minutes in Eagle's minimal essential medium at 37°C. They were then placed in control or isoproterenol-containing medium. At the intervals indicated (Table 1), the tissues were placed in liquid nitrogen, and a sample was homogenized directly in trichloroacetic acid for cyclic AMP assay (*6*). The frozen tissues were pulverized in liquid nitrogen with a mortar and pestle and then homogenized for 30 seconds in a small glass motor-driven homogenizer in 3 ml of 0.05*M* sodium acetate buffer, *p*H 6.5, containing 1 m*M* *β*-mercaptoethanol and 5 m*M* theophylline. After filtration through two layers of cheese cloth, the homogenate was centrifuged at 20,000*g* for 15 minutes, and the supernatant was used for kinase assay (*8, 9*). The number of unsaturated receptor sites was determined by the method of Chambaut *et al.* (*10*).

Since the response of the uterus in vitro to isoproterenol was dose and time dependent, we studied the course of the cyclic AMP increment, receptor saturation, and kinase activation (Table 1). The stimulation of cyclic AMP followed the expected relation to dose and time. The increase of cyclic AMP corresponded to a decrease in unsaturated cyclic AMP binding sites.

As we expected, there was a substantial increase of cyclic AMP–independent protein kinase activity (Table 1). Correlation of the increment in cyclic AMP and that in cyclic AMP–independent kinase in 14 experiments in which isoproterenol doses and times were varied, resulted in a value of $r = .82$, suggesting that activated kinase was closely regulated by the concentration of cyclic AMP.

Unexpectedly, a substantial fall in total kinase activity was noted to occur as a result of treatment with isoproterenol (Table 1, + cyclic AMP). This could have been due either to degradation or to transfer to another cellular compartment. To test the second possibility, we extracted the subcellular fraction of the myometrium with 0.7 percent Triton X-100 and determined the kinase activity (*11, 12*).

When the 20,000*g* pellet was extracted with 0.7 percent Triton X-100, no consistent pattern of response to isoproterenol was found. The degree of kinase extraction by successive treatments with Triton was unpredictable.

The supernatant from the 20,000*g* centrifugation was then centrifuged at 145,000*g* and the kinase activity in the 20,000 to 145,000*g* pellet was determined by Triton extraction (Table 2). The usual increment in cyclic AMP–independent and decrement in cyclic AMP–dependent kinase was seen in the cytosol. The 20,000 to 145,000*g* pellet showed a substantial increment in the specific activity of the cyclic AMP–independent kinase and a small increase in cyclic AMP–dependent kinase. When calculated on the basis of the weight of the tissue, the increment in microsomal kinase was slightly more impressive, but losses due to Triton-induced foaming were significant.

When the 20,000*g* supernatants of control and treated uteri, exhibiting the usual pattern of decline of total kinase activity with isoproterenol treatment, were subjected to Triton extraction, there was a substantial increase of kinase activity and full recovery of the loss due to hormone treatment (Table 3). The kinase activity in the cytosol and that in the purified protein were not affected by Triton.

Our studies suggest that translocation of cyclic AMP–dependent protein kinase, mostly of the catalytic subunit to a particulate component of the 20,000*g* to 145,000*g* pellet (microsomal fraction), is an intrinsic feature of the response of the cell to *β*-adrenergic stimulation. Previous reports of kinase activation (*1–3*) did not demonstrate this phenomenon.

The nature of the substrate for protein kinase was not determined. However, calcium accumulation by microsomes has been shown to accompany isoproterenol administration in smooth muscle (*13*), cardiac muscle (*14*), and skeletal muscle (*15*). A phosphorylated component constituted approximately 50 percent of the sarcoplasmic vesicle membrane protein and acted as the calcium-binding intermediate of the calcium pump. This reaction requires adenosine triphosphate and Mg^{2+}, the cofactors for protein kinase. In sarcoplasmic vesicles of dog cardiac muscle, cyclic AMP–dependent protein kinase activity was found to phosphorylate an intermediate believed to be responsible for calcium uptake (*16*). A reasonable conclusion, then, would be that translocation of kinase activity to a substrate in the sarcoplasmic vesicle membrane may be re-

Table 2. Extraction of protein kinase activity, expressed as nanomoles of ^{32}P incorporated per milligram of protein. The 20,000*g* to 145,000*g* pellet (microsomal fraction) was taken up in assay buffer containing 0.7 percent Triton X-100, and the protein kinase was determined in the solubilized pellet and in the cytosol.

Fraction	Protein kinase activity			
	Control		Treated	
	−Cyclic AMP	+Cyclic AMP	−Cyclic AMP	+Cyclic AMP
Cytosol	0.39	2.1	0.73	1.5
Microsomal	0.57	1.1	1.1	1.2
Cytosol	0.16	0.91	0.17	0.66
Microsomal	0.33	0.62	0.63	1.3
Cytosol	0.24	0.96	0.34	0.62
Microsomal	0.41	1.6	0.61	1.8

Table 3. Recovery of particulate kinase activity (nanomoles of ^{32}P incorporated per milligram of protein) by incubation in the presence of Triton X-100. Each value represents triplicate estimates from a pool of 15 to 25 uteri.

	Protein kinase activity			
	Control		Treated	
	−Cyclic AMP	+Cyclic AMP	−Cyclic AMP	+Cyclic AMP
Supernatant	0.86	2.25	0.95	1.37
+Triton	1.14	3.53	2.41	3.55
Supernatant	0.13	2.37	0.94	1.50
+Triton	0.38	4.0	2.07	3.63
Supernatant	0.39	1.65	0.63	1.49
+Triton	0.52	2.12	0.70	2.02
		Means		
Supernatant	0.46 ± .21	2.09 ± .22	0.84 ± .11	1.45 ± .04
+Triton	0.68 ± .23	3.22 ± .47	1.73 ± .52	3.07 ± .53

sponsible for isoproterenol action. However, the evidence for the presence of sarcoplasmic vesicles in uterus is not great (*17*), and a recent report suggests that perhaps the microsomal calicum uptake system of smooth muscle is derived from the cell membrane (*18*). Definition of the cellular locus and chemical nature of the particulate kinase substrate will be of great importance.

STANLEY G. KORENMAN
Department of Internal Medicine, University of Iowa, and *Veterans Administration Hospital, Iowa City 52240*
RAMESH C. BHALLA
Department of Anatomy, University of Iowa College of Medicine, Iowa City
BARBARA M. SANBORN
Department of Reproductive Medicine, University of Texas School of Medicine, Houston
REGGIE H. STEVENS
Department of Internal Medicine, University of Iowa, and *Veterans Administration Hospital, Iowa City*

Reference

1. O. Walaas, E. Walaas, O. Gronnerod, *Israel J. Med. Sci.* **8**, 353 (1972).
2. S. Ichii, *Endocrinol. Japan* **19**, 229 (1972).
3. T. R. Soderling, J. D. Corbin, C. R. Park, *J. Biol. Chem.* **248**, 1822 (1973).
4. T. R. Soderling, J. P. Hickenbottom, E. M. Reimann, F. L. Hunkeler, D. A. Walsh, E. G. Krebs, *ibid.* **245**, 6317 (1970).
5. J. K. Huttunen, D. Steinberg, S. E. Mayer, *Proc. Natl. Acad. Sci. U.S.A.* **67**, 290 (1970).
6. R. C. Bhalla, B. M. Sanborn, S. G. Korenman, *ibid.* **69**, 3761 (1972).
7. B. M. Sanborn, R. C. Bhalla, S. G. Korenman, *Endocrinology* **92**, 494 (1973).
8. ———, *J. Biol. Chem.* **248**, 3593 (1973).
9. J. F. Kuo and P. Greengard, *ibid.* **245**, 4067 (1970).
10. A. M. Chambaut, F. Leray, J. Hanoune, *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **15**, 328 (1971).
11. H. Maeno, E. M. Johnson, P. Greengard, *J. Biol. Chem.* **246**, 134 (1971).
12. E. M. Johnson, H. Maeno, P. Greengard, *ibid.*, p. 773.
13. R. Andersson, L. Lundholm, E. Mohme-Lundholm, K. Nilsson, in *Advances in Cyclic Nucleotide Research*, P. Greengard, R. Paoletti, G. A. Robison, Eds. (Raven, New York, 1972), vol. 1.
14. M. L. Entman, G. S. Levey, S. E. Epstein, *Circ. Res.* **25**, 429 (1969).
15. G. Meissner and S. Fleischer, *Biochim. Biophys. Acta* **241**, 356 (1971).
16. H. Wray, R. R. Gray, R. A. Olsson, *J. Biol. Chem.* **248**, 1496 (1973).
17. M. E. Carsten, *J. Gen. Physiol.* **53**, 414 (1969).
18. L. Hurwitz, D. F. Fitzpatrick, G. Debbas, E. J. Landon, *Science* **179**, 384 (1973).

30 July 1973

Carbonic Anhydrase and Osteoclasts: Localization by Labeled Inhibitor Autoradiography

Abstract. *Autoradiography with tritiated acetazolamide indicates that osteoclasts of the hen and chick contain concentrations of carbonic anhydrase which are similar to those in pancreatic acinar cells. Grain counts of osteoblasts and osteocytes were not different from background. Thus, a sufficient quantity of carbonic anhydrase seems to be present in osteoclasts to be of physiological importance in bone resorption.*

Several studies (*1*, *2*) indicate that carbonic anhydrase (E.C. 4.2.1.1), an enzyme which is widespread in biological systems, may play a role in bone demineralization, possibly by decreasing *p*H in areas of bone resorption. A major deficiency in proving this hypothesis has been the lack of an unequivocal localization of carbonic anhydrase (CA) in osteoclasts or osteocytes, the cells which occur in areas of osteoclastic bone resorption and osteocytic osteolysis, respectively. Although several authors (*1*, *3*) have found significant amounts of CA in bone by chemical analysis, it is uncertain if the enzyme activity is associated with bone cells or the preerythrocytes of bone marrow, which are known to be rich in CA (*4*). Since bone mineral and marrow form an interlocking system, which cannot be separated, the best approach for determining if the enzyme is present in bone cells is cellular localization. The only localization of CA in bone cells (*5*) is based on a method which has been found to be invalid since staining is caused by CA-independent alkalinization of the medium (*6*).

We have developed an alternate procedure for localizing CA. The method, which has been described in detail elsewhere (*7*), involves labeling of CA in vivo with tritiated acetazolamide, a specific inhibitor (*8*, *9*). Tissues are then prepared for autoradiography.

A 1-year-old White Leghorn laying hen, two 10-day-old male commercial broiler chicks, both *Gallus domesticus*, and two 3-month-old laying quail, *Coturnix coturnix japonica*, were injected intravenously with sodium [acetyl-^{3}H]acetazolamide (160 mc/mmole, New England Nuclear, Boston, Massachusetts). The quail and chicks received 16.4 to 28.1 mc and the hen 2.3 mc per kilogram of body weight. This corresponded to an acetazolamide concentration of 22.8 to 39.1 mg per kilogram of body weight for quail and chicks. The injection received by the hen was supplemented with 10 mg of unlabeled sodium acetazolamide per kilogram of body weight so that the total drug concentration was 13.2 mg/kg. These dosages have been reported to cause maximum inhibition of CA in all organs of various species tested (*9*) and of egg shell formation in laying hens (*10*). Twenty-four hours later, tissues were removed from the hen and chicks. At this time the concentration of acetazolamide in plasma of the laying hen was about 100 times lower than that in CA-rich red blood cells (*7*), a finding which indicated that free and loosely bound acetazolamide was rapidly cleared from the plasma and most tissues. The quail were killed 3 hours after injection of the drug since experiments in our laboratory had shown that there is little inhibition of quail shell gland CA by acetazolamide after longer time intervals. Pieces of tissue about 2 mm^3 in volume were removed from birds which were under sodium pentobarbital anesthesia (25 to 100 mg per kilogram of body weight). The tissues were frozen immediately in isopentane at −160°C, freeze-dried, fixed with OsO_4 vapor, and embedded in Epon. Thus, the only liquid which came in contact with the tissues was a nonaqueous plastic resin.

Table 1. Average grain counts over osteoclasts, pancreas, and muscle. The values are corrected for background; S.E.M., standard error of the mean.

Animal	Grains per 1000 $\mu m^2 \pm$ S.E.M. over		
	Labeled osteoclasts	Pancreas	Muscle
Hen	20.5 ± 2.4*	24.8 ± 2.7*	0.2 ± 0.7
Quail 1	16.5 ± 1.8*	52.3 ± 2.1*	3.5 ± 0.8
Quail 2	21.9 ± 2.6*	65.5 ± 5.0*	6.2 ± 1.1
Chick 1	7.7 ± 1.1	11.8 ± 0.9*	2.3 ± 0.7
Chick 2	15.4 ± 1.9*	15.5 ± 1.5*	2.6 ± 0.6

* Significantly different from background at $P < .05$ by Duncan's new multiple range test (*17*).