lated kinase activity and ^{3}H-labeled cyclic AMP binding (Fig. 1).

Thus, the dose-response relationships for all four types of clones determined in vitro by assay of catalytic or binding activities of cyclic AMP–dependent protein kinase accurately predict the response of the corresponding intact cells to exogenous dibutyryl cyclic AMP. The differing properties of the variant enzymes are also reflected by the responses of the corresponding clones to endogenous cyclic AMP, elevated within the cell in response to stimulators of adenylate cyclase, such as cholera enterotoxin (results not shown).

In addition to establishing the role of cyclic AMP–dependent protein kinase in S49 cells, these results offer insight into the quantitative relationship between cyclic AMP concentrations and biologic effects. In several tissues, hormones can stimulate the accumulation of more cyclic AMP than necessary for a maximum biologic response (*10*). It is not known which cellular components limit maximum responses to cyclic AMP. Clone kin B contains reduced kinase activity and exhibits a proportional decrease in biologic response to cyclic AMP. Thus, at least in the S49 cell, the cellular content of cyclic AMP–dependent kinase determines responsiveness to the cyclic nucleotide.

Kuo and Greengard (*2*) have proposed that in animal cells all cyclic AMP effects are mediated by protein kinase. Our results are consistent with this hypothesis. However, we cannot formally exclude the possibility that these actions of cyclic AMP are mediated by the enzyme's cyclic AMP–binding subunit alone, in an analogous fashion with the cyclic AMP receptor protein of *Escherichia coli* (*11*), rather than by phosphorylation of specific protein substrates.

The precise biochemical lesions of the clones which we describe remain to be defined. Mixing experiments, with cytosol extracts of the wild type and the three variants, provide no evidence for an aberrant activator or inhibitor of cyclic AMP–dependent kinase in any of the clones (data not shown). The cyclic AMP–stimulated enzymes of all three cyclic AMP–responsive cell lines show similar requirements for adenosine triphosphate (ATP), histone, and magnesium (*12*).

The phenotype of each cyclic AMP–resistant clone has been stable in the absence of dibutyryl cyclic AMP for more than 200 generations in culture. A working hypothesis, supported by genetic evidence (*13*), is that cyclic AMP resistance represents a mutation. Further studies that indicate that the kin A lesion can be specifically assigned to a structural alteration in one of the subunit peptides for the cyclic AMP–dependent kinase (*14*) provide more direct evidence for the mutational origin of at least some of these variants. The kin B and kin C lesions have not been defined in detail; either or both could represent alterations in cellular concentration of the kinase, because of regulatory rather than structural mutations.

PAUL A. INSEL, HENRY R. BOURNE
PHILIP COFFINO, GORDON M. TOMKINS*
Departments of Medicine, Pharmacology, Microbiology, and *Biochemistry and Biophysics*, and *Cardiovascular Research Institute, University of California School of Medicine, San Francisco 94143*

References and Notes

1. T. R. Soderling, J. P. Hickenbottom, E. M. Reimann, F. L. Hunkeler, D. A. Walsh, E. G. Krebs, *J. Biol. Chem.* **245**, 6317 (1970); H. L. Segal, *Science* **180**, 25 (1973); D. K. Huttunen and D. Steinberg, *Biochim. Biophys. Acta* **239**, 911 (1971).
2. J. F. Kuo and P. Greengard, *Proc. Natl. Acad. Sci. U.S.A.* **64**, 1349 (1969); T. A. Langan, in *Advances in Cyclic Nucleotide Research*, G. I. Drummond, P. Greengard, G. A. Robison, Eds. (Raven, New York, 1973), vol. 3, pp. 99–153; W. O. Wicks, J. Koontz, K. Wagner, *J. Cyclic Nucleotide Res.* **1**, 49 (1975).
3. K. Horibata and A. W. Harris, *Exp. Cell Res.* **60**, 61 (1970).
4. H. R. Bourne, P. Coffino, Y. Weinstein, K. Melmon, G. M. Tomkins, in *Advances in Cyclic Nucleotide Research*, G. I. Drummond, P. Greengard, G. A. Robison, Eds. (Raven, New York, 1975), vol. 5, pp. 771–786.
5. P. Coffino, J. Gray, G. M. Tomkins, *Proc. Natl. Acad. Sci. U.S.A.* **22**, 878 (1975).
6. H. R. Bourne, G. M. Tomkins, S. Dion, *Science* **181**, 952 (1973).
7. P. Coffino, H. R. Bourne, G. M. Tomkins, *J. Cell Physiol.* **85**, 603 (1975); H. R. Bourne, P. Coffino, G. M. Tomkins, *ibid.*, p. 611; clone kin C in the present report was identified as cAR.1 in the earlier studies.
8. V. Daniel, G. Litwack, G. M. Tomkins, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 751 (1973).
9. Of 14 clones obtained in the presence of 0.5 m*M* dibutyryl cyclic AMP and 0.1 m*M* theophylline, two clones were kin C, one was kin A, and the remainder were kin B. A clone representative of each phenotype was selected for further analysis.
10. G. A. Robison, R. W. Butcher, E. W. Sutherland, *Cyclic AMP* (Academic Press, New York, 1971), p. 31.
11. M. Emmer, E. de Crombrugghe, I. Pastan, R. Perlman, *Proc. Natl. Acad. Sci. U.S.A.* **66**, 480 (1970).
12. Suspensions of sonicated wild-type S49 cells yield apparent K_m's (Michaelis constants) for ATP of approximately $1 \times 10^{-5}M$, and for Mg^{2+} of $1.3 \times 10^{-3}M$. Histone phosphorylation increases linearly up to 1.6 mg/ml. Similar responses for all three substrates and cofactors are noted in kin A, kin B, and wild-type clones.
13. As reported previously (*7*), fluctuation analysis demonstrated that the emergence of cyclic AMP–resistant clones was a random event unrelated to the selection process. Chemical mutagens increased the number of such clones.
14. J. Hochman, P. Insel, H. R. Bourne, P. Coffino, G. M. Tomkins, in *Proc. Natl. Acad. Sci. U.S.A.*, in press.
15. The binding activity of kin B relative to the wild type cells, as shown here, differs slightly from the relationship for histone phosphorylation in those clones. The maximum values for kin B cells are always lower than for wild-type cells for both activities. However, minor variation between experiments in both activities in crude cell lysates can easily account for the difference between the figures. The concentration of cyclic AMP producing half maximum binding activity is approximately tenfold less than that producing half maximum kinase activation. Such a difference has also been noted in studies with purified protein kinase when cyclic AMP binding is examined in the absence of ATP and magnesium [J. A. Beavo, P. J. Bechtel, E. G. Krebs, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3580 (1974)].
16. A. G. Gilman, *Proc. Natl. Acad. Sci. U.S.A.* **67**, 305 (1970).
17. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
18. J. A. Beavo, J. G. Hardman, E. W. Sutherland, *ibid.* **245**, 5649 (1970); substrate (^{3}H-labeled cyclic AMP) concentration in the assays was 1.0 μM. The increase in phosphodiesterase activity was prevented by incubation with $2 \times 10^{-4}M$ cycloheximide, as previously reported (*6*, *7*).
19. We thank V. Hill, S. Dion, J. Fenno, and H. Dovey for technical assistance, K. L. Melmon for advice and support, and J. Gray for help with flow microfluorimetric analysis. Work supported by PHS grants GM 16496, HL 15851, GM 17239-06, and American Cancer Society California Division grant 738. H.R.B. is an Established Investigator of the American Heart Association.
* Deceased 22 July 1975.

11 June 1975; revised 18 July 1975

Collagen Biosynthesis in Blood Vessels of Brain and Other Tissues of the Hypertensive Rat

Abstract. *It was previously shown that hypertension elevates collagen biosynthesis and increases collagen deposition in peripheral arteries and that antihypertensive agents prevent and reverse the increased synthesis. These findings have now been extended to the microvessels of the central nervous system and to other small vessels.*

In a previous report we demonstrated that collagen biosynthesis is increased in a large artery (aorta) and in arteries of medium caliber (mesenteric arteries) of rats made hypertensive by treatment with deoxycorticosterone acetate (DOCA) and in spontaneously hypertensive rats (*1*). We further showed that treatment with the antihypertensive drugs chlorothiazide or reserpine can prevent or reverse this hypertension-induced biosynthesis of vascular collagen.

Recently Brendel *et al.* (*2*) reported a simple procedure for the isolation of metabolically active brain microvessels. We have isolated brain microvessels (arterioles, capillaries, and venules) from DOCA-salt hypertensive rats and have shown elevations in their prolyl hydroxylase activity and in their ability to synthesize collagen in vitro. These markers of collagen biosynthesis were also found to be elevated by hypertension in the pial arteries, in the circle of Willis, and in testicular arteries. Treatment of the DOCA-salt rats with reserpine prevented the increase in blood pressure as well as the increase in vascular prolyl hydroxylase.

Hypertension (*3*) was produced in uninephrectomized, 8-week-old, male Wistar

Table 1. Final blood pressures and body weights of DOCA-salt treated rats with and without reserpine and of normotensive controls. Uninephrectomized, 8-week-old, male Wistar rats were given DOCA (5 mg per rat) twice weekly for 4 to 6 weeks and maintained on 1 percent saline. Control animals were uninephrectomized and maintained on 1 percent saline. Concomitant with DOCA treatment, reserpine (0.75 mg/kg) was administered daily by intraperitoneal injection. Each value represents the mean and standard error of the mean. The values in parentheses represent the number of rats examined.

Treatment	Blood pressure (mm-Hg)		Body weight (g)	
Control	120 ± 2	(23)	343 ± 8	(23)
DOCA-salt	210 ± 3**	(24)	339 ± 6	(24)
DOCA-salt + reserpine	139 ± 3**	(8)	289 ± 6**	(8)

**Statistically significant compared to normotensive control, $P < .01$.

rats by subcutaneous injection of DOCA (5 mg per rat) twice weekly and permitting free access to 1 percent saline as a source of drinking water. Reserpine (0.75 mg per kilogram of body weight) was administered to some of the DOCA-salt rats by daily intraperitoneal injection. The blood pressure was monitored, without anesthesia, by the tail cuff and photoelectric method (*4*). After 4 or 6 weeks of DOCA-salt treatment, the rats were killed by decapitation. Brain and testes were immediately excised and kept in cold oxygenated Earle's balanced salt solution buffered with 28 m*M* HEPES (*5*) (*p*H 7.4). The pial membrane was removed from each brain for further isolation of pial arteries and of the arteries at the base of the brain (basilar and circle of Willis). Then the brain was cut sagittally at the midline to remove remaining pieces of pial membranes and arachnoidal plexuses in the ventricles.

Brain microvessels were isolated by homogenizing the brain tissue, after removal of cerebellum and spinal cord, and sieving three times according to the method of Brendel *et al.* (*2*). More strokes were included in the homogenization step than were used in the original method because the brain tissues used in this study included not only gray matter but also white matter, which is harder to disrupt. The purity of the microvessels was checked with a binocular dissecting microscope (× 15 to 45). For assay of prolyl hydroxylase it was necessary to pool the microvessels dissected from five to nine rat brains. These were suspended in 0.5 to 0.7 ml of 0.25*M* sucrose containing 10 m*M* tris(hydroxymethyl)aminomethane (tris)–HCl (*p*H 7.4), 100 μM dithiothreitol, 10 μM ethylenediaminetetraacetic acid, and 0.1 percent (by volume) Triton X-100 (tris-sucrose buffer) and centrifuged at 1500*g*. The sedimented pellet of microvessels was homogenized in a Teflon-glass homogenizer. After centrifugation of the homogenate (1500*g*), the pellet was rehomogenized in the same supernatant fluid and sonicated for 30 seconds at 20 watts on a Biosonik II instrument equipped with a needle probe 3 mm in diameter.

Pial arteries were dissected from pial membranes under a binocular dissecting microscope. The arteries can be easily differentiated from veins in this manner. The basilar artery and the circle of Willis were dissected from the pial membrane in the same way and combined for assay. Testicular arteries were dissected from seminiferous tubules after removing the fibrous capsule of the testes (tunica albuginea). All the procedures described above were performed in cold Earle's balanced salt solution buffered with HEPES as described above. For enzyme assay, blood vessels from four to nine rats were pooled and homogenized in 0.5 ml of the tris-sucrose buffer with a small glass homogenizer chilled with ice. Rehomogenization and sonication were not necessary. The mesenteric artery and the entire aorta free of perivascular adipose tissue were homogenized in the tris-sucrose buffer with a ground glass homogenizer or Polytron ST-10 system, respectively (*1*).

The homogenates and sonicates prepared as described above were centrifuged at 15,000*g* for 20 minutes. Portions of the supernatant containing 70 to 200 μg of protein were assayed for prolyl hydroxylase by the tritium release method of Hutton *et al.* (*6*) modified as described in (*7*). Protein and DNA were determined on portions of the 15,000*g* supernatants and in whole homogenates by the methods of Lowry *et al.* (*8*) and Burton (*9*), respectively. Three rats in each experimental group were used for histopathological studies.

As shown in Table 1, the blood pressures of DOCA-salt animals were markedly elevated over those of the normotensive controls and of those treated with reserpine. The prolyl hydroxylase activities reported in Table 2 are given in terms of enzyme activity per milligram of vessel DNA. The values calculated per milligram of protein were proportionately the same. The yields of blood vessels and their protein and DNA content were not significantly altered by the experimental procedures. As shown in Table 2, DOCA-salt treatment led to an elevation in prolyl hydroxylase in all the blood vessels examined, but the increase occurred only in the cardiovascular system; tissues such as lung and liver showed no increase. In DOCA-salt animals in which reserpine was used to prevent hypertension, prolyl hydroxylase activity was even lower than in the control animals (*10*). In another experiment we

Table 2. Prolyl hydroxylase activity of various blood vessels in DOCA-salt treated rats with and without reserpine and normotensive controls. In the case of the smaller arteries, the values in parentheses represent the number of pools of tissue, each pool comprising four to ten rats. For the larger arteries the values in brackets signify the number of rats used. Enzyme activity is expressed as counts per minute per milligram of DNA in each homogenate and is presented ± the standard error of the mean. Boiled blanks showed no increase over the nonenzyme blank and were never more than 5 percent of the actual assay value. Treated groups were compared to normotensive control.

Treatment	Prolyl hydroxylase activity	
Brain microvessels		
Control	10,448 ± 490	(4)
DOCA-salt	14,437 ± 901**	(4)
DOCA-salt + reserpine	9,228	(1)
Pial artery		
Control	33,973 ± 2,218	(3)
DOCA-salt	45,795 ± 2,959*	(3)
DOCA-salt + reserpine	21,714	(1)
Basilar artery and circle of Willis		
Control	33,307 ± 2,116	(3)
DOCA-salt	44,573 ± 8,019	(3)
DOCA-salt + reserpine	26,607	(1)
Testicular artery		
Control	14,917 ± 997	(5)
DOCA-salt	30,556 ± 1,767**	(5)
DOCA-salt + reserpine	13,226	(1)
Mesenteric artery		
Control	27,775 ± 3,123	[6]
DOCA-salt	54,269 ± 7,074**	[6]
DOCA-salt + reserpine		
Aorta		
Control	10,912 ± 976	[10]
DOCA-salt	30,476 ± 3,507**	[10]
DOCA-salt + reserpine	5,768 ± 877**	[5]

*$P < .05$. **$P < .01$.

measured in vitro collagen synthesis by blood vessels from control and DOCA-salt animals as indicated by incorporation of [^{14}C]proline into collagenase digestible material. The methods used were those reported previously (*1*). The values obtained, in counts per minute per milligram of DNA, were: for brain microvessels, control 44,000 and DOCA-salt 131,000; for pial artery, control 216,000 and DOCA-salt 696,000; and for mesenteric artery, control 50,000 and DOCA-salt 248,000. In these experiments also, administration of reserpine abolished the increase produced by DOCA-salt treatment.

In our previous study (*1*) we showed that in the peripheral vessels two additional markers, including net collagen deposition in vivo, were affected in the same way by hypertension. We may therefore conclude with some degree of certainty that DOCA-salt–induced hypertension increases the

biosynthesis and deposition of collagen in rat brain vessels and in testicular arteries as it does in other peripheral large blood vessels. In preliminary studies it has been shown that epinephrine-thyroxine treated rabbits, which exhibit increased collagen synthesis in peripheral blood vessels (*7*), also show increases in prolyl hydroxylase and in the in vitro synthesis of collagen in brain microvessels.

Pathologic examination of the rats showed that after 6 weeks of treatment with DOCA-salt, there was only occasional and mild fibrinoid necrosis in arteries and arterioles of the brain, pial membrane, and testes, which correspond to the changes observed by Gardner and Matthews (*11*). The large artery (aorta) and arteries of medium caliber (basilar artery, mesenteric artery, and circle of Willis) showed no pathological changes except for a slight thickening of the vascular wall.

The findings reported here indicate that there is increased collagen biosynthesis in arterioles and arteries in the periphery and in the central nervous system of DOCA-salt hypertensive rats and provide an early biochemical indication that fibrogenesis is involved in the production of hypertensive vascular damage (arteriosclerosis). It was previously shown (*1*) that the increase in prolyl hydroxylase activity is the result of increased production of the enzyme. We have calculated that DOCA-salt treatment increases synthesis of vascular collagen and prolyl hydroxylase to a far greater extent than it does total protein. These observations lend further biochemical support to the findings of Freis and co-workers (*12*) and of Hollander (*13*) that hypertension shortens life and produces pathologic lesions and that these changes are prevented by antihypertensive drugs. It is also likely that the structural hypertrophy of the resistance vessels induced by hypertension (*14*) is, at least in part, due to collagen deposition. As far as the brain microvessels are concerned, it is interesting to consider the possibility that in untreated hypertension, deposition of small amounts of collagen, in quantities insufficient to be observed by standard pathologic techniques as frank arteriosclerosis, could nevertheless alter vascular permeability and transport mechanisms and thereby diminish mental function.

A. OOSHIMA
Roche Institute of Molecular Biology, Nutley, New Jersey 07110

G. FULLER
University of Rhode Island, Kingston 02881

G. CARDINALE
S. SPECTOR
S. UDENFRIEND
Roche Institute of Molecular Biology

References and Notes

1. A. Ooshima, G. C. Fuller, G. J. Cardinale, S. Spector, S. Udenfriend, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3019 (1974).
2. K. Brendel, E. Meezan, E. C. Carlson, *Science* **185**, 953 (1974).
3. J. DeChamplain, L. R. Krakoff, J. Axelrod, *Circ. Res.* **23**, 479 (1968); J. DeChamplain and M. R. van Ameringen, *ibid.* **31**, 617 (1972).
4. J. R. Williams, T. R. Harrison, A. Grollman, *J. Clin. Invest.* **18**, 373 (1939).
5. *N*-2-Hydroxyethylpiperazine-*N*-2-ethanesulfonic acid.
6. J. J. Hutton, Jr., A. L. Tappel, S. Udenfriend, *Anal. Biochem.* **16**, 384 (1966).
7. G. C. Fuller and R. O. Langer, *Science* **168**, 987 (1970).
8. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
9. K. Burton, *Biochem. J.* **62**, 315 (1956).
10. Reserpine, in the doses given here, also lowers prolyl hydroxylase in tissues other than blood vessels, as might be expected from its effect on rat growth (Table 1). However, reserpine lowers vascular prolyl hydroxylase far more than it does prolyl hydroxylase in other tissues. Both chlorothiazide and reserpine were found to reverse the effects of hypertension on vascular collagen synthesis (*1*). It was noted that the response treated animals yielded values that were even lower than the normotensive controls, indicating that this drug has an effect over and above its effect on blood pressure.
11. D. L. Gardner and M. A. Matthews, *J. Pathol.* **97**, 51 (1969).
12. E. D. Freis, D. Ragan, H. Pillsbury, III, M. Matthews, *Circ. Res.* **31**, 1 (1972); E. D. Freis, *Circ. Res. Suppl. 2* **29**, 70 (1971).
13. W. Hollander, *Circulation* **48**, 1112 (1973).
14. B. Folkow, M. Hallbäck, Y. Lundgren, R. Sivertsson, L. Weiss, *Circ. Res. Suppl. 1* **32**, **33**, 2 (1973).

23 June 1975; revised 13 August 1975

Orientational Anisotropy in Infant Vision

Abstract. *Infants prefer to look at horizontal and vertical gratings rather than at oblique gratings only when they are at or near threshold spatial frequencies, as would be expected if acuity for oblique edges is lower than that for horizontal and vertical edges. That such a bias exists as early as 6 weeks of age suggests that the orientational asymmetry of the visual system depends on endogenous maturation rather than exposure to a carpentered world.*

Under a wide variety of conditions, the visual acuity of adult human observers is greater for horizontal and vertical edges than for oblique edges (*1, 2*). This "oblique effect" has been attributed to early visual experience in our carpentered environment, with its preponderance of vertical and horizontal contours (*3*). The notion that the sensitivity of the visual system to contours of various orientations is shaped at an early stage of development by the prevalence and clarity of the edges to which the eye is exposed implies a form of neural plasticity. This viewpoint is supported by claims that the distribution of orientation-sensitive single units in the visual cortices of kittens is altered by rearing the kittens in orientationally biased environments (*4*) and by reports that astigmatic human observers suffer a residual loss of acuity for edges along the blurred axis even when optical factors are eliminated (*5*).

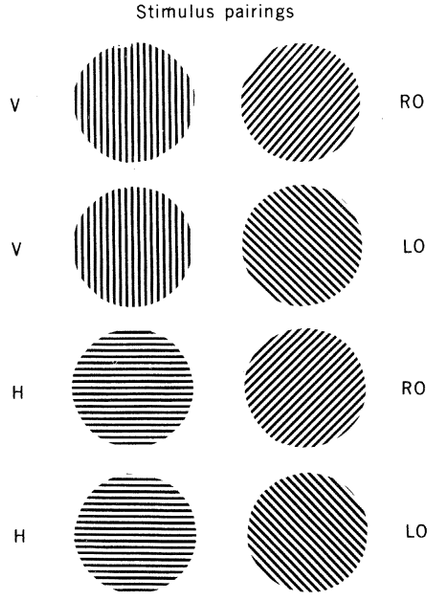

Fig. 1. Schematic representation of stimulus pairs. In all cases, either a vertical or a horizontal grating was paired with a left- or a right-oblique grating of the same spatial frequency.

If environmental exposure were responsible for the orientational asymmetries in the visual system, then these asymmetries should not be evident prior to an appropriate period of exposure. Evidence for an oblique effect has been described in 2-year-old children (*6*), but in the only study of infant acuity as a function of edge orientation, no meridional differences in sensitivity were observed (*7*). However, using a similar but more sensitive technique, we have found that the oblique effect is present in human infants as young as 6 weeks of age. Our result casts doubt upon explanations of the oblique effect solely in terms of environmental biasing.

We investigated the development of orientational differences in the acuity of infants using a modification of Teller *et al.*'s two-alternative preferential looking technique (*7*). It has been demonstrated that infants preferentially fixate patterned over homogeneous stimuli (*8*). Teller *et al.* paired a grating of a given orientation with a homogeneous gray target of equal luminance. We simultaneously presented two gratings of the same spatial frequency (one cycle consists of one bar and one space of equal width) but of different orientations. Our technique is based on the premise that an infant will preferentially fixate the more