haptoglobin and hemopexin (sensitive indicators of the degree of hemolysis) are normal or only moderately reduced. Results of in vitro studies on the solubility of HbZ in whole blood and hemolyzates exposed to heat and isopropanol suggest that in vivo elevations of carboxyhemoglobin secondary to smoking may be decreasing the rate of hemolysis by stabilizing the abnormal hemoglobin.

In vitro studies have shown that the binding of CO to hemoglobin in place of O_2 prevents reactions of O_2 that give superoxide and peroxide as well as methemoglobin (*13*, *14*). Exposure of red blood cells with HbZ to certain "oxidant" drugs, especially those of the primaquine or sulfonamide class, results in the ready formation of hemoglobin precipitates (*15*). This hemoglobin denaturation-precipitation process is proposed to follow autoxidation of heme iron from Fe^{2+} to Fe^{3+} (*16*, *17*). The autoxidation can proceed via a two-electron reduction of Hb-bound O_2 to peroxide. One electron is donated by heme Fe^{2+} and one by the drug to give methemoglobin (Fe^{3+}), H_2O_2, and oxidized drug (usually a potentially destructive free radical) (*13*, *18*, *19*). Such a reaction has recently been shown to be much faster for O_2 bound to the abnormal β subunit of HbZ than for O_2 bound to normal subunits (*19*, *20*). Carboxyhemoglobin does not undergo these reactions because CO, unlike O_2, cannot serve as an electron acceptor. Therefore, replacement of O_2 by CO or the prevention of O_2 binding by bound CO can prevent the initiation of a series of destructive processes.

Further studies are necessary to determine whether carboxyhemoglobin in vivo in the range of 20 percent alters the susceptibility of HbZ subjects to the hemolytic effects of drugs or chemicals. Even if it is true, the overall risks of smoking outweigh the benefits. There appears to be no difference in the lifestyle of the smokers and nonsmokers, and one of the long-term smokers has mild chronic bronchitis. Of greater significance is the discovery that CO can modify the characteristics of an unstable hemoglobin in vivo and in vitro and that studies on the function and physicochemical properties of other unstable hemoglobins should include measurements of carboxyhemoglobin levels.

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References and Notes

1. H. F. Bunn, B. G. Forget, H. M. Ranney, *Hemoglobinopathies* (Saunders, Philadelphia, 1977), pp. 184–213.
2. W. H. Hitzig, P. G. Frick, K. Betke, T. H. J. Huisman, *Helv. Paediatr. Acta* **15**, 499 (1960).
3. R. F. Rieder, W. H. Zinkham, N. A. Holtzman, *Am. J. Med.* **39**, 4 (1965); J. D. Dickerman, N. A. Holtzman, W. H. Zinkham, *ibid.* **55**, 638 (1973).
4. W. H. Zinkham, J. S. Vangrov, S. M. Dixon, J. Hutchison, *Johns Hopkins Med. J.* **144**, 36 (1979); W. H. Zinkham, J. D. Liljestrand, S. M. Dixon, J. Hutchison, *ibid.*, p. 109.
5. R. F. Rieder, *J. Clin. Invest.* **49**, 2369 (1970).
6. P. W. Tucker, S. E. V. Phillips, M. F. Perutz, R. A. Houtchens, W. S. Caughey, in *Biochemical and Clinical Aspects of Hemoglobin Abnormalities*, W. S. Caughey, Ed. (Academic Press, New York, 1978), pp. 1–15; W. S. Caughey, R. A. Houtchens, A. Lanir, J. C. Maxwell, S. Charache, in *ibid.*, pp. 29–56.
7. W. S. Caughey, J. O. Alben, S. McCoy, S. Boyer, S. Charache, P. Hathaway, *Biochemistry* **8**, 59 (1969).
8. J. C. Maxwell, C. H. Barlow, J. E. Spallholz, W. S. Caughey, *Biochem. Biophys. Res. Commun.* **61**, 230 (1974); W. J. Wallace, J. A. Volpe, J. C. Maxwell, W. S. Caughey, S. Charache, *ibid.* **68**, 1379 (1976).
9. R. W. Carrell and R. Kay, *Br. J. Haematol.* **23**, 615 (1972).
10. R. F. Coburn, W. J. Williams, R. E. Forster, *J. Clin. Invest.* **43**, 1098 (1964); R. F. Coburn, W. J. Williams, S. B. Kahn, *ibid.* **45**, 460 (1966).
11. J. R. Smith and S. A. Landow, *N. Engl. J. Med.* **298**, 6 (1978).
12. W. M. O. Moore, F. C. Battaglia, A. E. Hellegers, *Am. J. Obst. Gynecol.* **97**, 63 (1967).
13. W. J. Wallace, R. A. Houtchens, J. M. Holt, W. S. Caughey, in *Biochemical and Clinical Aspects of Hemoglobin Abnormalities*, W. S. Caughey, Ed. (Academic Press, New York, 1978), pp. 475–493.
14. W. J. Wallace and W. S. Caughey, in *Biochemical and Clinical Aspects of Oxygen*, W. S. Caughey, Ed., (Academic Press, New York, 1979), pp. 69–86.
15. P. G. Frick, W. H. Hitzig, K. Betke, *Blood* **20**, 261 (1962).
16. J. K. French, C. C. Winterbourne, R. W. Carrell, *Biochem. J.* **173**, 19 (1978).
17. M. Brunori, E. Fioretti, G. Rotilio, *Mol. Cell. Biochem.* **19**, 43 (1978).
18. S. Kawanishi and W. S. Caughey, *Biochem. Biophys. Res. Commun.* **88**, 1203 (1979).
19. _____, in *Biochemical and Clinical Aspects of Oxygen*, W. S. Caughey, Ed. (Academic Press, New York, 1979), pp. 27–34.
20. _____, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38**, 347 (1979);
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Plasminogen Is Synthesized by Primary Cultures of Rat Hepatocytes

Abstract. *The accumulation of rat plasminogen in the medium of primary monolayer cultures of adult parenchymal hepatocytes was detected with a quantitative immunological assay. These primary cultures synthesized and secreted both circulating isozymic forms of plasminogen at rates sufficient to account for the majority of the in vivo plasminogen turnover.*

Plasminogen is the circulating glycoprotein which, upon proteolytic modification, yields the serine protease, plasmin. Circulating plasminogen is composed of two (*1*) non-interconvertible (*2*) isozymic forms that differ in their state of glycosylation (*3*). While the chemistry of the plasminogen molecule and the processes of its activation have been characterized in great detail, much less is known about its metabolism. The zymogen's in vivo site of synthesis has remained controversial and undemonstrated despite decades of investigation. The earliest effort at localizing plasminogen stores was the direct immunofluorescence study of Barnhart and Riddle (*4*). On examination of human biopsy specimens, these investigators found positive fluoresence (interpreted as plasminogen stores) associated primarily with the granules of immature eosinophilic leukocytes. This led them to postulate a role for the eosinophil in the synthesis of plasminogen, but there has been no direct demonstration of plasminogen synthesis by these cells.

Kline and Highsmith (*5*) demonstrated an apparent involvement of the kidney in the restoration of plasminogen levels after a streptokinase-induced "acute depletion." The infusion into cats of large doses of streptokinase resulted in an immediate decrease in the circulating level of plasminogen activity. After a lag period, this activity was found to return to the normal predepletion level. The return to normal could be prevented, or halted in midcourse, by ligating the kidneys of the depleted animal. This finding, coupled with the detection of a gradient of plasminogen activity across the kidney (*5*), led these investigators to conclude that the kidney was the source of plasminogen after "acute depletion." However, their data preclude the conclusion that the kidney is the site of routine plasminogen synthesis, since partially restored animals with ligated kidneys were able to maintain a constant circulating plasminogen level for more than 24 hours [see figure 3 in (*5*)]. The circulating half-life of plasminogen in the rat and rabbit is approximately 12 hours (*2*). The fact that the kidney has no role in the routine synthesis of plasminogen was clearly demonstrated by Siefring and Castellino (*6*), who showed (i) that kidney ligation of rats does not halt plasminogen synthesis and (ii) that anephric humans possess normal circulating levels of both forms of plasminogen. This

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latter demonstration effectively ruled out the possibility of a kidney-specific plasminogen isozyme.

While the liver has a well-known and well-characterized role in the synthesis of many plasma proteins, specific investigations of the liver's involvement in the synthesis of plasminogen have been conflicting and inconclusive. Mattii *et al.* (*7*) were unable to detect production of either plasminogen or plasminogen activator by their intact perfused rat liver system. They did, however, detect the appearance of antiplasmin. More recently, Losito *et al.* (*8*) found that plasminogen was present in their intact perfused rat liver system. They also were unable to detect plasminogen activators. Williams *et al.* (*9*) were unable to detect plasminogen in the medium of their primary rat hepatocyte cultures, but they did detect the production of plasminogen activators.

In order to quantitate low concentrations of rat plasminogen in the presence of an excess of fetal calf or other nonrat plasminogens, we established an enzyme-linked immunosorbent assay (ELISA) for rat plasminogen according to the procedures of Kwan *et al.* (*10, 11*).

Table 1. Plasminogen accumulation, at 24 and 48 hours of culture, for five independent hepatocyte preparations. Hepatocytes, isolated from male Sprague-Dawley rats (200 to 300 g) according to the method of Seglen (*13*), were washed in Williams medium E supplemented with dexamethasone ($10^{-8}M$), gentamycin (75 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and fetal calf serum (10 percent). The medium was deficient in arginine but contained ornithine ($10^{-4}M$). The cells were plated in 35-mm culture dishes as described (*12*) and were allowed to attach for 1 hour. After gently replacing the medium with 1.5 ml of fresh medium E, the cultures were allowed to recover for 18 hours. At this point, the cultures were rinsed with saline, and 1.5 ml of fresh medium E was again added. At 24 and 48 hours after this feeding, the medium was removed from replicate cultures of cells. The monolayers were rinsed with saline and solubilized in 0.1*N* NaOH. Rat plasminogen in the medium was quantitated with the ELISA and related to total cellular protein as determined by the method of Lowry *et al.* (*20*), with bovine serum albumin as the standard. Results are expressed as the mean ± standard deviation of the number of dishes indicated in parentheses.

Experiment	Plasminogen accumulation (micrograms of plasminogen per milligram of protein)	
	24 hours	48 hours
1	0.62 ± 0.02 (3)	0.95 ± 0.05 (2)
2	0.52 ± 0.06 (3)	1.10 ± 0.2 (3)
3	0.79 ± 0.06 (3)	1.50 ± 0.3 (6)
4	1.27 ± 0.15 (5)	2.10 ± 0.16 (4)
5	0.84 ± 0.06 (6)	1.30 ± 0.17 (5)

The standard curve of ELISA yields a linear response to plasminogen over the range of 6 to 90 ng of plasminogen per milliliter. Several nonrat plasmas and serums have been examined in the assay, and none reacted in or interfered with the assay.

The ELISA was used to quantitate the accumulation of plasminogen in the medium of primary monolayer cultures of rat parenchymal hepatocytes (*12, 13*); Table 1 shows this accumulation for five independent hepatocyte preparations. Variation in the amounts of plasminogen produced by different hepatocyte preparations has not correlated with any routinely measured parameter of the preparation (for example, cell yield, viability by trypan blue dye exclusion, or percentage of single cells). However, plasminogen accumulated in the medium of every hepatocyte preparation examined.

In order to demonstrate that this accumulation did not result from the release of previously stored protein, but rather was synthesized de novo by the cultures, flasks of hepatocytes were labeled with [^{35}S]methionine for 24 hours. The radioactive medium was removed from the cells, mixed with human plasma to provide carrier protein, and fractionated by ammonium sulfate precipitation to yield a 25 to 50 percent saturated pellet. Radioactive rat plasminogen was immunoprecipitated (*14*) from this pellet (resuspended and dialyzed against 0.15*M* NaCl) with antibodies to rat plasminogen purified by affinity chromatography on plasminogen-Sepharose. Electrophoresis (*15*) and fluorography (*16*) of the immunoprecipitated protein (Fig. 1A) yielded a single radiolabeled band that comigrated with authentic rat plasminogen.

Labeled medium was mixed with plasma and applied to a lysine-Sepharose affinity column (*17*) in order to determine which of the circulating forms of plasminogen were synthesized by these cultures. Elution of the bound plasminogens with a shallow gradient of 6-aminohexanoic acid (*1*) yielded two peaks of radioactive plasminogen corresponding precisely to the two circulating forms of the carrier plasminogen (Fig. 1B).

To gain some insight into the importance of the levels of plasminogen synthesis that we observed, calculations were made to approximate the daily in vivo amount of new plasminogen secretion. This value can be estimated from the approximations of the circulating half-life of plasminogen in the rat of about 12 hours (*2*), an estimated blood volume of 8 percent of body weight, and 0.16 mg/ml as an approximate plasma concentration of plasminogen in the rat (*18*). With these approximations, the average 250-g rat must secrete about 1.8 mg of plasminogen each day to maintain a constant plasma concentration of the protein. The amount of plasminogen that would be synthesized by a liver can be calculated from the values measured here by using data provided by Seglen (*13*). According to his figures, a gram of hepatocytes contains 230 mg of protein, and the liver is about 68 percent parenchymal hepatocytes, by weight. Therefore, a 10-g liver, producing plasminogen at the average rate measured here, could produce about 1.3 mg of plasminogen per day, or over 70 percent of the calculated need. Since the hepatocyte culture conditions were not selected to maximize either plasminogen or total protein synthesis, and because both circulating forms of the plasminogen molecule are

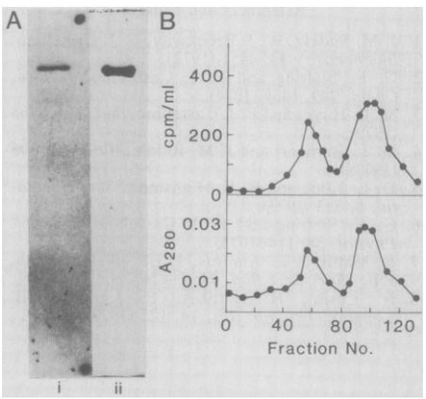

Fig. 1. (A) Polyacrylamide gel electrophoresis of immunoprecipitated radiolabeled rat hepatocyte plasminogen and purified rat plasminogen. Hepatocytes (30×10^6) were plated in 15 ml of Williams medium E in a 75-cm^2 flask. After the 18-hour recovery period, the medium was replaced with 15 ml of medium containing [^{35}S]methionine (0.1 mCi). After 24 hours, the medium was fractionated as described in the text. The immunoprecipitated protein (~ 1000 count/min) and 8 μg of rat plasminogen were electrophoresed in adjacent lanes of a 10 percent polyacrylamide gel (*15*). The lanes were separated after electrophoresis; the radioactive lane (i) was impregnated with 2,5-diphenyloxazole and fluorographed (*16*), and the protein lane (ii) was stained for protein with Coomassie R-250. (B) Gradient elution of radiolabeled hepatocyte and carrier plasminogens from a lysine-Sepharose affinity chromatography column. Labeled medium was mixed with rabbit plasma and applied to a lysine-Sepharose affinity column (*1, 17*). After thoroughly washing the resin, a gradient of 6-aminohexanoic acid (75 ml each, 0 and 0.01*M* in buffer 1) was applied to the column. Fractions of 1.2 ml were collected and analyzed for protein (absorbance at 280 nm) and for radioactivity (liquid scintillation counting). The upper curve is the elution profile of the radioactive hepatocyte plasminogen, while the lower curve is the absorbance profile of the unlabeled carrier plasminogen.

produced by these cultures, we suggest that the parenchymal hepatocyte is the major in vivo source of plasminogen.

Thus we have utilized a sensitive and specific immunological assay to demonstrate that primary cultures of rat parenchymal hepatocytes actively accumulate plasminogen in their medium. These hepatocyte cultures were further shown to synthesize and secrete both circulating forms of the plasminogen molecule. To our knowledge, this is the first unequivocal demonstration of plasminogen synthesis by any cell type. An abstract of these studies has been published (*19*).

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References and Notes

1. J. M. Sodetz, W. J. Brockway, F. J. Castellino, *Biochemistry* **11**, 4451 (1972).
2. G. E. Siefring and F. J. Castellino, *J. Biol. Chem.* **249**, 1434 (1974).
3. M. L. Hayes and F. J. Castellino, *ibid.* **254**, 8768 (1979).
4. M. L. Barnhart and J. M. Riddle, *Blood* **21**, 306 (1963).
5. D. L. Kline and R. F. Highsmith, *Ser. Haematol.* **6**, 513 (1973).
6. G. E. Siefring and F. J. Castellino, *J. Appl. Physiol.* **38**, 114 (1975).
7. R. Mattii, J. L. Ambrus, J. E. Sokal, I. Mink, *Proc. Soc. Exp. Biol. Med.* **116**, 69 (1964).
8. R. Losito, H. Gattiker, E. Lemieux, B. Longpré, *Thromb. Res.* **14**, 125 (1979).
9. G. M. Williams, E. Bermudez, R. H. San, P. J. Goldblat, M. R. Laspia, *In Vitro* **14**, 828 (1978).
10. S.-W. Kwan, G. M. Fuller, M. A. Krautter, J. H. vanBavel, R. M. Goldblum, *Anal. Biochem.* **83**, 589 (1977).
11. Rat plasminogen was purified by affinity chromatography on lysine-Sepharose (*1*, *17*) from fresh rat plasma treated with heparin (100 U/ml), *p*-nitrophenyl-*p'*-guanidinobenzoate ($10^{-5}M$), and Aprotinin (Sigma; 50 U/ml). The plasma (10 to 60 ml) was applied to a column (2 by 5 cm) of lysine-Sepharose equilibrated at 4°C in 0.1*M* sodium phosphate, *p*H 8.0 (buffer 1), and the bulk of the plasma was washed through with this buffer. A variable amount of loosely bound, nonplasminogen protein was washed from the resin with a solution of 2.0*M* NaCl and 0.1 percent Tween-20. After reequilibrating the resin in buffer 1, the bound plasminogen was eluted with 0.05*M* 6-aminohexanoic acid in buffer 1. An antiserum to this protein was produced in a rabbit, and the antibodies against plasminogen were isolated from the antiserum by affinity chromatography on rat plasminogen–Sepharose [T. G. Cooper, *The Tools of Biochemistry* (Wiley, New York, 1977)]. Both the antiserum and the antibodies (purified by affinity chromatography) were judged to be specific for rat plasminogen by immunoelectrophoresis.
12. D. K. Struck, P. B. Siuta, M. D. Lane, W. J. Lenarz, *J. Biol. Chem.* **253**, 5332 (1978).
13. P. O. Seglen, in *Methods in Cell Biology*, D. M. Prescott, Ed. (Academic Press, New York, 1976), p. 29.
14. S. Kessler, *J. Immunol.* **115**, 1617 (1975).
15. U. K. Laemmli, *Nature (London)* **227**, 680 (1970).
16. W. M. Bonner and R. A. Laskey, *Eur. J. Biochem.* **46**, 831 (1974).
17. D. G. Deustch and E. I. Mertz, *Science* **170**, 1095 (1970).
18. J. F. Bohmfalk and G. M. Fuller, unpublished data.
19. J. F. Bohmfalk, P. H. Weigel, G. M. Fuller, *J. Cell Biol.* **83**, 436a (1979).
20. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall. *J. Biol. Chem.* **193**, 265 (1951).
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Pain Responses in Nepalese Porters

Abstract. *When tested by the method of limits, Nepalese had much higher pain thresholds to electrical stimulation than Occidentals did. Discriminability was the same for both groups, however, indicating that there were no neurosensory differences. Nepalese had higher (stoical) criteria for reporting pain but were not less sensitive to noxious stimulation. The battery of sensory measurement procedures described may be applied to any modality and are particulary applicable to difficult field conditions.*

Members of non-Western ethnocultural groups are said to be less sensitive to pain (*1*). We determined that this difference was due to a culturally imposed stoicism (that is, pain was felt as intensely but was not reported) and was not due to a true hypalgesia caused by genetic or climatic differences or perhaps local botanicals. Sensory decision theory was used since this method distinguishes between sensory (d') and attitudinal (L_x) components of the report of pain (*2*, *3*). Although sensory decision theory has proved invaluable in the laboratory, it had not, to our knowledge, been tested in the field, where there is little time to gather data and where language and literacy barriers often exist. We now describe a combination of methods—the method of limits followed by forced-choice and one-interval binary decision—applicable to field conditions.

Responses to noxious transcutaneous electrical stimulation were obtained from Nepalese, East Indians, and Occidental visitors during a trek in the Himalayas (*4*). The five trekkers were English-speaking, college-educated, and of Christian or Jewish background. Their mean age was 45 years (range, 30 to 68). Although familiar with the minor discomforts encountered in hiking and camping at moderate altitudes (3000 m), they were not regularly exposed to the hardships that were routine for the Nepalese. The six Nepalese did not speak English, had little schooling, were devout Buddhists, and some were illiterate. Their mean age was 32 years (range, 23 to 42). The four porters were accustomed to carrying 77-pound packs at high altitudes wearing only light clothing, even at freezing temperatures. The cook and his assistant were more literate and accustomed to slightly better living conditions. Two additional Indian subjects from Darjeeling, whose life-style was comfortably Westernized (for example, their homes were well heated) were included in a correlational study between pain threshold and living conditions, but not in the comparisons between the Western trekkers and Nepalese.

The method of limits was followed by forced-choice and one-interval binary decision procedures of sensory decision theory to obtain measures of pain threshold and discriminability (d'). With the method of limits, the stimulus is increased stepwise until the subject rates it as "painful." In a forced-choice task the subject judges which of two stimuli has the higher intensity. In a one-interval binary decision task, the subject rates individual stimuli as "high" or "low." Sensory decision theory analysis uses the proportion of incorrect judgments to estimate the subject's sensory sensitivity.

First, with the method of limits, two sets of ascending trials were run with the stimulus intensities increasing in steps from zero until the subject's pain tolerance was reached. After each stimulus, the subject selected a response from a list of seven categories in his native language. The categories ranged from "nothing" through various degrees of "discomfort" and "pain" to "withdrawal." If the subject was illiterate, the list was rehearsed aloud. Next, each subject received four to eight trials in the forced-choice task, with stimulus intensities lying within the range of his threshold determined by the method of limits for "slightly painful" (high) and "uncomfortable" (low). The subject judged which interval contained the higher intensity stimulus and was told whether he was right or wrong. This procedure established the intensities for the subsequent binary decision task, familiarized the subject with the decision tasks, and ensured that he understood the instructions. In the final test, the stimulus intensities determined by the forced-choice procedure were used in the one-interval binary decision procedure of sensory decision theory. Ten high- and ten low-intensity stimuli were presented in random order. The subject

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