throline, a substance which inhibits kininase, an enzyme which destroys kinins (*13*). Mixtures of L-homocystine in normal plasma induced contraction of the rat uterus, while suspensions of the amino acid in plasma deficient in Hageman factor were without this effect (Table 2). Presumably, L-homocystine brought about the elaboration of kinin-like activity through its action upon Hageman factor.

Thus, at concentrations as low as $10^{-3}M$, the upper limit of its solubility, L-homocystine activates Hageman factor. It joins the lengthening list of agents which can activate this clotting factor (*14*). Hence, the unusual propensity of patients with homocystinuria to undergo thrombosis may be related to the deposition of homocystine in the intima of blood vessels. The validity of this hypothesis awaits the demonstration of homocystine in the tissues of patients with this disorder.

OSCAR D. RATNOFF
Department of Medicine, Case Western Reserve University School of Medicine, and *University Hospitals of Cleveland, Cleveland, Ohio 44106*

References and Notes

1. R. N. Schimke, V. A. McKusick, T. Huang, A. D. Pollack, *J. Amer. Med. Ass.* **190**, 711 (1965).
2. N. A. J. Carson, C. E. Dent, C. M. B. Field, G. E. Gaull, *J. Ped.* **66**, 565 (1965).
3. S. H. Mudd, J. D. Finkelstein, F. Irreverre, L. Laster, *Science* **143**, 1443 (1964).
4. T. Gerritson, J. G. Vaughn, H. A. Waisman, *Biochem. Biophys. Res. Commun.* **9**, 493 (1962); N. A. J. Carson and D. W. Neill, *Arch. Dis. Child.* **37**, 505 (1962).
5. H. A. Waisman and T. Gerritson, in *Mental Retardation*, G. A. Jervis, Ed. (Thomas, Springfield, Ill., 1967); G. M. Komrower and V. K. Wilson, *Proc. Roy. Soc. Med.* **56**, 996 (1963).
6. J. B. Gibson, N. A. J. Carlson, D. W. Neill, *J. Clin. Pathol.* **17**, 427 (1964).
7. L. McDonald, C. Bray, C. Field, F. Love, B. Davies, *Lancet* **1964-I**, 745 (1964).
8. T. L. Perry, in *Amino Acid Metabolism and Genetic Variation*, W. L. Nyhan, Ed. (McGraw-Hill, New York, 1967).
9. This was not the amount in solution, but that amount which, if it had dissolved, would have provided a concentration of $10^{-2}M$. The homocystine was finely powdered and passed through a No. 200 gauge sieve.
10. All blood was drawn through No. 19 gauge needles into silicone-coated polystyrene syringes, and plasma was separated in siliconecoated apparatus, avoiding contact with known clot-promoting surfaces. Clotting times were measured in uncoated polystyrene tubes.
11. O. D. Ratnoff and J. Colopy, *J. Clin. Invest.* **34**, 602 (1955); J. Margolis, *Nature* **178**, 805 (1956); E. Shafrir and A. de Vries, *J. Clin. Invest.* **35**, 1183 (1956); O. D. Ratnoff and J. Rosenblum, *Amer. J. Med.* **25**, 160 (1958).
12. J. Margolis, *J. Physiol.* **144**, 1 (1958); M. Webster and O. D. Ratnoff, *Nature* **192**, 180 (1961).
13. E. G. Erdös, A. G. Renfrew, E. M. Sloane, J. R. Wohler, *Ann. N.Y. Acad. Sci.* **104**, 222 (1963).
14. O. D. Ratnoff, *Progr. Hematol.* **5**, 204 (1966).
15. Supported in part by grant HE 01661 from the National Heart Institute, and in part by a grant from the American Heart Association. I am a career investigator of the American Heart Association.

1 July 1968; revised 9 August 1968

Brush Border Particulates of Renal Tissue

Abstract. *Particulates containing a large part of the alkaline phosphatase activity of renal tissue were separated from homogenates and from ribosomal preparations by zonal centrifugation. The particles had a high content of phospholipid and cholesterol that was not removed by treatment with 1 percent deoxycholate. Enzymatic activities concentrated with the particles were the alkaline phosphatase, a peptidase resistant to proteolysis, glucose-6-phosphatase, inorganic pyrophosphatase, and adenosine triphosphatase. The particles accumulated leucine with no stimulation from soluble factors and with inhibition by other amino acids; the accumulation was stimulated by adenosine triphosphate and was not inhibited by puromycin. The particles appear to be derived from the membranes of the brush borders of tubular cells.*

Particulates of renal tissue containing alkaline phosphatase and a "resistant" peptidase are found in preparations of renal ribosomes (*1*). Ribonuclease destroyed the ribosomal particles without effect upon the centrifugal behavior of the enzymatically active particles, but a complete separation from nucleic acids was not achieved in swinging-bucket rotors. The zonal rotors developed by Anderson (*2*) were found capable of separation of the particulates directly from homogenates of renal tissue (Fig. 1). In the untreated homogenate the particulates distributed with the microsomal fraction but appeared as a sharper band at 38 percent sucrose (by weight) in homogenates treated with 1 percent deoxycholate. Particles in ribosomal preparations with or without pretreatment with ribonuclease were separated sharply from ribosomal material in similar gradients and banded at 33 percent sucrose (by weight) without magnesium ion and at 38 percent with $0.005M$ magnesium ion.

Similar particles and activities are not found in the ribosomal preparations from rat liver tissue, and the similar activities in the liver tissue are solubilized by the treatment with deoxycholate.

Most of the alkaline phosphatase of the renal homogenate and all that of the microsomal fraction was found in the purified particles. The peptidase was active in the hydrolysis of leucylglycine and leucyl-β-naphthylamide but is distinct from a leucyl-β-naphthylamidase of the microsomal fraction (*3*). Other activities concentrated with the particles were glucose-6-phosphatase, inorganic pyrophosphatase (*4*), and adenosine triphosphatase (Mg^{++} activated and Na-K dependent) (*5*). In the isolation of the particulates from ribosomal preparations, the yield of these activities was

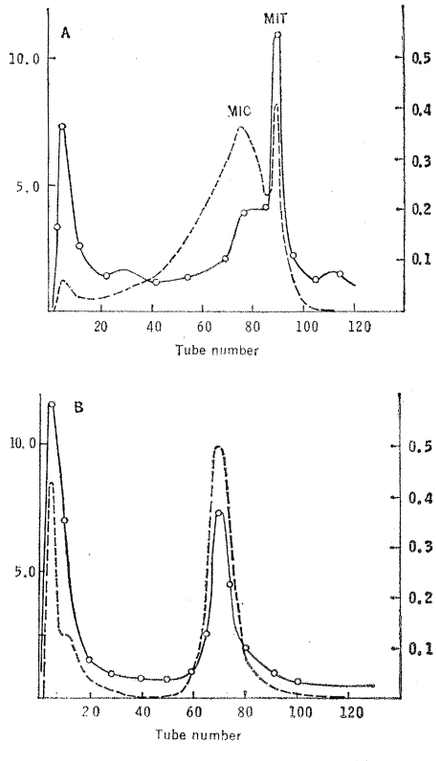

Fig. 1. Zonal centrifugation of homogenates of renal tissue. Only absorbancy at 280 mμ (○—○; read left ordinate) and alkaline phosphatase activity (- - -; read right ordinate) are plotted; units for both are micromoles per minute per milliliter. Other enzymes used for location were acid phosphatase for lysosomes, cytochrome c oxidase for mitochondria, and acylase I for the soluble fraction. Five grams of fresh rat kidney were homogenized in 15 ml of $0.25M$ sucrose containing $0.005M$ $MgCl_2$ and $0.01M$ tris buffer, *p*H 8.0, and placed on 1500 ml of 17 to 50 percent (by weight) linear sucrose gradient with a cushion of 150 ml of 55 percent sucrose and an overlay of 57 ml of $0.01M$ tris, *p*H 8.0, with $0.005M$ $MgCl_2$. Centrifugation was for 2 hours in the B-IV rotor in the Spinco L-4 centrifuge at 20,000 rev/min. Samples of 13 ml each were collected. (A) Untreated homogenate. MIC, microsomes; MIT, mitochondria. (B) Homogenate treated with 1 percent deoxycholate.

Table 1. Incorporation of ^{14}C-leucine into washed renal particles. One-half milliliter containing about 1.5 mg of washed and dialyzed particles was incubated with 0.5 μc of leucine at *p*H 8.0 in 0.01*M* tris with 0.005*M* $MgCl_2$ and 0.0025*M* glutathione in a total volume of 1 ml for 30 minutes at 38°C. Trichloroacetic acid–precipitable material was processed by the procedure of Peterson and Greenberg (*12*) prior to plating, weighing, and counting. ATP, adenosine triphosphate.

Additions	Picomoles of ^{14}C-leucine × 10^3 per milligram of precipitate
None	48
None, 10 minutes incubation	23
None, preheated to 80°C	1
None, preacidified to *p*H 5	1
ATP, 2.5 μmole	195
ATP, chloramphenicol, 100 μg	11
ATP, chloramphenicol, 10 μg	110
ATP, puromycin, 100 μg	210
ATP, amino acid mixture (*13*)	62
ATP, "*p*H 5 fraction" (*14*)	24

about half of that originally in the microsomal fraction. Particulates isolated directly from whole kidney homogenates treated with deoxycholate contained practically all of the inorganic pyrophosphatase and the glucose-6-phosphatase of the homogenate as well as all of the adenosine triphosphatase of the microsomal fraction. The adenosine triphosphatase activity of the mitochondrial fraction was solubilized by the deoxycholate.

The ribosomal preparations were found to incorporate ^{14}C-leucine without the addition of "*p*H 5 fractions" or mixtures of the other amino acids. The particulates isolated from ribosomal fractions treated with ribonuclease also incorporated leucine with stimulation by adenosine triphosphate but with inhibition by "*p*H 5 fractions" and by mixtures of the amino acids (Table 1). Maximal incorporation was dependent upon added magnesium ion (0.005*M*). The labeled particulates readily pelleted from sucrose gradients (10 to 30 percent) and the leucine was not released by treatment with 0.1*N* NaOH or HCl for 30 minutes at 100°C, nor was it soluble in warm ethanol, chloroform, or ether. Ribonucleic acid was probably not concerned with the process; none was detectable in the particles, and puromycin was ineffective as an inhibitor. Chloramphenicol inhibited at rather high levels, as did the structurally related amino acids isoleucine and valine. Thus, the process cannot be a directed synthesis of protein and should be described as a "facilitated accumulation."

The particles were found to have a composition very similar to that of the membranes of erythrocytes (*6*); a typical analysis was protein (*7*) 33.0, phospholipid (*8*) 27.3, cholesterol (*9*) 15.3, hexose (*10*) 4.2, and "sialic acids" (*11*) 1.7 percent. Pentose, hexuronic acids, and esterified cholesterol were not detected.

Although the particles are lipoprotein in nature, all the enzymes so far studied have been found to be glycoproteins. Highly purified variants of the alkaline phosphatase, released from the particles by proteolysis, differ in content of carbohydrate, ranging from a content nearly that of protein to one-fourth that of protein. The resistant peptidase and the pyrophosphatase are also glycoproteins. The glycoprotein matrix of the particles may be composed of enzymes interconnected by carbohydrate chains. Dependent upon the method of breakdown of the particle (autolysis, proteolysis, neuraminidase, and so forth), two or more activities may be found in one molecule. Some variants of the alkaline phosphatase were found to contain pyrophosphatase activity while others contained peptidase activities that were retained in constant ratios through various stages of purification and disc gel electrophoresis. Also, the apparent common identity of the various activities ascribed to the glucose-6-phosphatase, pyrophosphate-glucose transferase system of kidney (*4*) appears to be due to some similar combination of discrete enzymes; the pyrophosphatase isolated from the particles was without activity as a glucose-6-phosphatase.

The fractions from the gradients were examined by electron microscopy. The appearance of the particulates was that of vesicles formed by the closing of membrane fragments (Fig. 2). There was no recognizable brush border structure in any fraction. Apparently, the brush borders were fragmented by the homogenization, and the free fragments of the membranes formed vesicles much as do the endoplasmic membranes in the formation of the microsomes. Vesicles formed from the endoplasmic membranes were destroyed by deoxycholate, whereas deoxycholate was without effect upon those formed from the brush border membranes.

The high content of alkaline phosphatase and the occurence of leucyl-β-naphthylamidase in the particles would identify them as derived from the brush

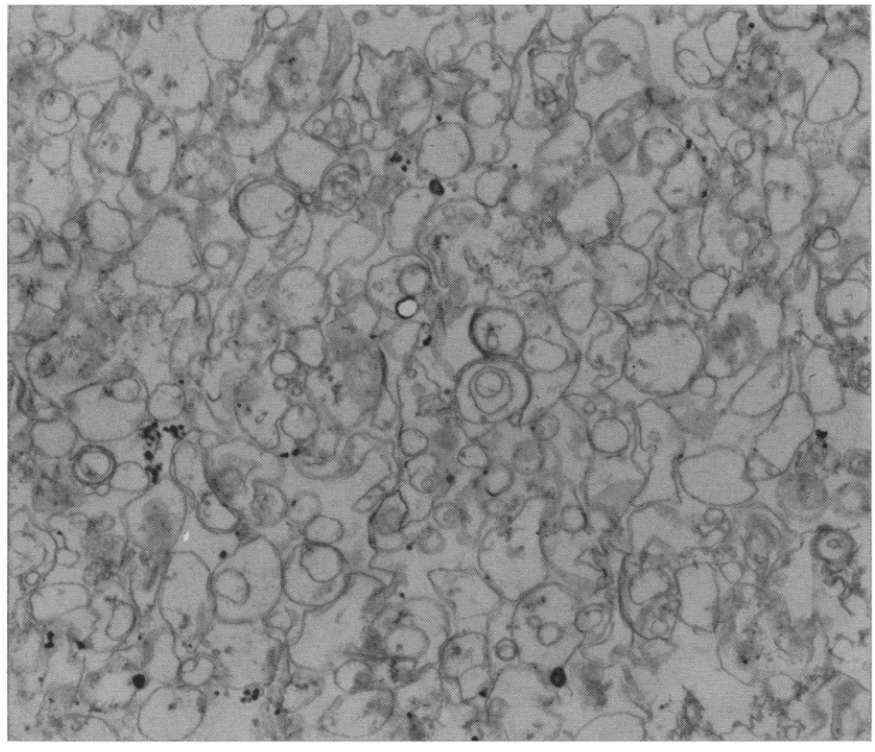

Fig. 2. Particles isolated directly from homogenate treated with 1 percent deoxycholate (× 29,400). Particles were collected by centrifugation of samples from a gradient as in Fig. 1B. They were fixed in 1.5 percent glutaraldehyde buffered in collidine at *p*H 7.4, then postfixed in 2 percent OsO_4, dehydrated, and imbedded in Maraglas.

borders of the tubular cells. The composition of the particles and their appearance in electron microscopy would identify them as derived from the membranes. Thus, the particles may be considered to be derived from the membranes of the brush borders of the tubular cells and, in view of their constant composition, consistent behavior in centrifugation, and their unique and constant enzymatic content, we propose the term "nephrosomes" as a convenient designation.

FRANCIS BINKLEY, NEVA KING
ELIZABETH MILIKIN, REBA K. WRIGHT
C. H. O'NEAL, INA JANE WUNDRAM
Departments of Biochemistry and
Anatomy, Emory University,
Atlanta, Georgia 30322

References and Notes

1. F. Binkley, *J. Biol. Chem.* **236**, 1075 (1961).
2. N. G. Anderson, *Nat. Cancer Inst. Monogr.* **21**, (1966).
3. F. Binkley, N. L. King, F. H. Leibach, *Arch. Biochem. Biophys.*, in press.
4. R. C. Nordlie and J. F. Soodsma, *J. Biol. Chem.* **241**, 1719 (1966).
5. J. C. Skou, *Physiol. Rev.* **46**, 593 (1966).
6. S. A. Rosenberg and G. Guidotti, *J. Biol. Chem.* **243**, 1985 (1968).
7. O. H. Lowry, N. W. Rosebrough, A. L. Farr, R. J. Randall, *ibid.* **193**, 265 (1951).
8. E. G. Bleigh and W. J. Dyer, *Can. J. Biochem. Physiol.* **37**, 911 (1959); N. Crawford, *Clin. Chim. Acta* **3**, 357 (1958).
9. R. J. L. Allen, *Biochem. J.* **34**, 858 (1940).
10. F. W. Fales, *J. Biol. Chem.* **193**, 113 (1951).
11. D. Aminoff, *Biochem. J.* **81**, 384 (1961).
12. E. A. Peterson and D. M. Greenberg, *J. Biol. Chem.* **194**, 359 (1942).
13. A. Korner, *Biochem. J.* **81**, 168 (1961).
14. M. B. Hoagland, E. B. Keller, P. C. Zamecnik, *J. Biol. Chem.* **221**, 45 (1956).
15. Supported by PHS research grant AM06089. Publication No. 892 of the Division of Basic Sciences.

18 July 1968

Morphine: Single-Dose Tolerance

Abstract. *Rats show a significant degree of tolerance to a second dose of morphine, with the degree of tolerance increasing the longer the delay between the two doses of morphine. To measure the morphine effect a foot-shock attenuation procedure that allowed the animal to adjust the shock intensity was used in studying delays of up to 180 days.*

A single dose of morphine sulfate (MS) in the rat results in a significant degree of tolerance to a second dose given several months later (*1*). We now report results of a study designed to determine whether this single-dose tolerance is independent of the time interval between the first and second dose.

Seven groups of experimental animals and seven groups of control animals (male, albino Holtzman; 175 to 250 g) consisted of eight animals, with the exception of one control and one experimental group that had five animals in each. On day 1, all experimental rats were given a single subcutaneous dose of 10 mg of MS (in 1 ml of saline) per kilogram of body weight and all control rats were given 1 ml of saline alone. Animals were then returned to their home cages. On day 2 (24 hours later) one group of experimental and one group of control subjects were tested on a shock attenuation procedure (*2*) after receiving 5 mg of MS per kilogram of body weight. On day 4, a second experimental and a second control group were tested after a dose of 5 mg/kg. Other groups were tested on days 8, 16, 32, and 180. (The groups tested on day 180 consisted of five experimental and five control subjects.)

To test the effects of the drug, we used a chamber (20.3 by 22.8 cm) with a paddle wheel 15.2 cm long and 7.6 cm in diameter, placed 3.8 cm above the grid floor in the shorter wall of the chamber. On a test day, animals were trained to escape from a gradually increasing shock by rotating the wheel one-quarter of a revolution. The shock intensity increased 0.02 ma every 15 seconds. When the animal turned the wheel, he terminated the shock (escaped) for 15 seconds. After 15 seconds, the shock returned, but at the next lower intensity. By this means the animal could maintain a "comfortable level" of shock intensity (Fig. 1). Animals were tested for 150 minutes after the drug was given. By means of a planimeter, the area under the time-effect curve was determined in square centimeters. The data were transformed by obtaining the square root of this area as well as the square root of the area during the 30-minute period prior to administration of the drug.

Since it was found that the score after administration of the drug was significantly correlated with the score prior to administration of the drug ($r=.53$) we obtained an adjusted difference score (*3*) that allowed further statistical computation in which the variance due to the initial level was removed. A "predicted" score for an individual animal for the period was determined by the use of the regression equation for drug scores before and

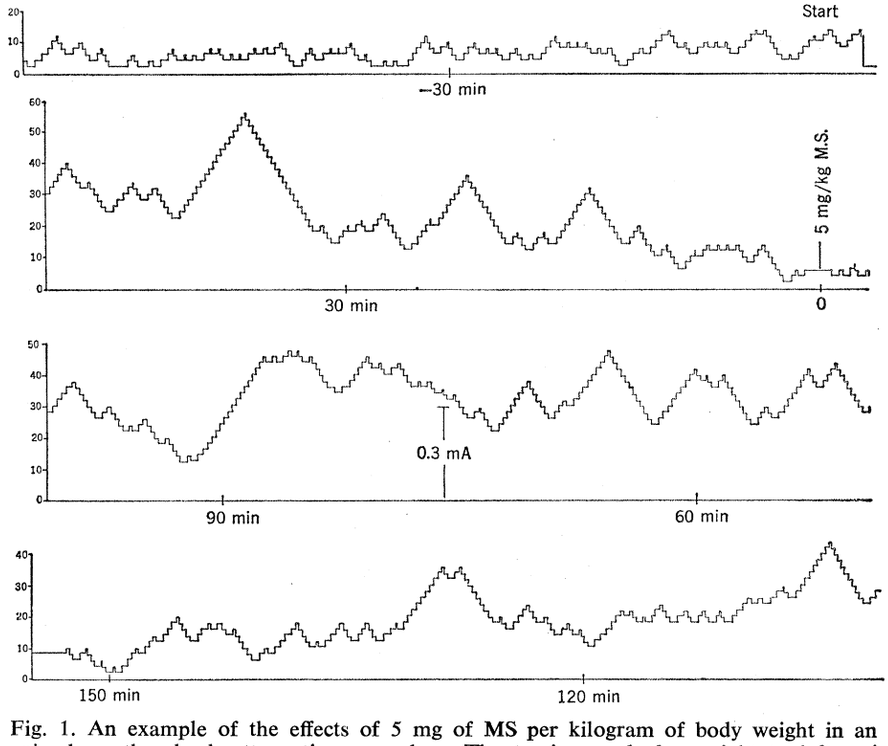

Fig. 1. An example of the effects of 5 mg of MS per kilogram of body weight in an animal on the shock attenuation procedure. The tracing reads from right to left and from top to bottom. The vertical distance is a linear representation of the shock intensity, and the horizontal distance represents time.