precise location of individual atoms (within 1 to 2 Å) is in part due to the cone-averaging procedure utilized in the fractal surface calculation. A second factor is that surface area calculations also appear to be insensitive to the fine details of residue structure and conformation, as indicated by the successful application of several simplified and statistical approaches for the calculation of surface area (*6, 7*).

We also examined whether D may be related to such properties as residue mobility or exposed surface area to establish whether fractal surfaces provide an independent characterization of surface properties. Comparisons of D to the refined temperature factors for myoglobin (protein databank set 1MBD) and superoxide dismutase indicate that these parameters are unrelated. As measured with small probes ($R < 2$ Å), the value of D also appears to be unrelated to exposed surface area. Regions of proteins accessible to larger probes, however, tend to be associated with smooth regions of the fractal surface. Intuitively, this relation appears to be reasonable since residues on smooth surfaces are more likely to be able to contact large probes than residues on irregular surfaces. These considerations suggest that the calculation of fractal surfaces, like the calculation of residue mobility (*8, 9*), may be a useful technique for describing the antigenic determinants on a protein.

Fractal surfaces provide a means for characterizing the irregularity of protein surfaces. These surfaces are irregular when viewed on an atomic scale, with an average fractal dimension of about 2.4. A high degree of irregularity in proteins is consistent with direct experimental measurements of the fractal dimension of the polypeptide backbone (*10*). Rather than being uniformly irregular, however, the degree of irregularity varies across the protein surface. Regions involved in the formation of tight complexes (such as interfaces and possibly antibody-combining regions) appear to be more irregular than regions involved in the formation of transient complexes (such as active sites). Recognition of these geometric factors provides a new approach to describing the interaction of macromolecules with one another.

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17 May 1985; accepted 23 September 1985

Synthesis and Evaluation of a Prototypal Artificial Red Cell

Abstract. *A new process allows microencapsulation of purified human hemoglobin and 2,3-diphosphoglycerate to form neohemocytes. The microcapsule membrane is composed of phospholipids and cholesterol. Neohemocytes are substantially smaller than erythrocytes, contain 15.1 grams per deciliter of hemoglobin, and have a* P_{50} *value (the partial pressure of oxygen at which the hemoglobin is half-saturated) of 24.0 torr. All rats given 50-percent exchange transfusions survived with only limited evidence of reversible toxicity. Normal serum glutamate-pyruvate-transaminase values at 1, 7, and 30 days after transfusion were consistent with minimal hepatotoxicity. The concentration of blood urea-nitrogen was elevated by 35 percent after 1 day but returned to normal by day 7. However, histopathology revealed normal kidneys on day 1 as well as on days 7 and 30. Neohemocytes cleared from the circulation of transfused rats with an apparent half-life of 5.8 hours.*

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We now report the synthesis of artificial red cell prototypes that meet the six essential specifications for such cells: (i) the microcapsule membrane must be biodegradable and physiologically compatible; (ii) the encapsulation process must avoid significant hemoglobin (Hb) degradation; (iii) when encapsulated, the oxygen affinity of Hb must be reduced relative to that of free human Hb; (iv) the encapsulated Hb must be sufficiently concentrated, that is, more than 33 percent of that in erythrocytes; (v) there should be no evidence of overt intravascular coagulopathy; and (vi) the artificial cells must be small enough to pass unrestricted through normal capillaries. We call these prototypal artificial red cells neohemocytes (NHC).

Microcapsules with lipid bilayer membranes rather than biodegradable polymer (*1*) or polymerized protein membranes (*2*) were used to make the artificial cells, and the procedure (*3*) was based on one used for the preparation of liposomes (*4*). The preparation of each batch started with 0.293 mmol of human hemoglobin and 3.75 mmol of lipid consisting of egg yolk phosphatidylcholine, dipalmitoylphosphatidic acid, cholesterol, and α-tocopherol in a molar ratio of 4:1:5:0.1, respectively. The resulting NHC were relatively homogeneous in size but less than homogeneous in content (Fig. 1). The pellet from a 25 percent suspension of NHC contained an average (n = 10 batches) of 151 mg/ml [(standard deviation (SD), 4)] of Hb and 2.21 mg/ml (SD, 0.11) of total lipid. The mean concentration of Hb within NHC was 15.8 g/dl. Only about 4.4 percent of the total displaced volume of NHC was membrane, if a lipid density of 1 g/cm^3 is assumed. Additional characteristics of the cells are listed in Table 1.

The pharmacokinetic properties of NHC were analyzed by following coencapsulated tracer amounts of ^{14}C-labeled sucrose in five rats that had undergone a 50 percent exchange transfusion (*5*). The amount of ^{14}C found in the blood of these rats thus reflected the amount of circulating, encapsulated sucrose and was taken as a direct measure of those NHC remaining intact in circulation (*6*). After 8 hours the mean amount of ^{14}C in the blood dropped to 40 percent of its value immediately after transfusion, with an

Table 1. Properties of NHC. Oxygen disassociation curves were determined (*15*) at 37°C. In each case the data were fitted to the Hill equation: $Y = (PO_2/P_{50})^n/[1 + (PO_2/P_{50})^n]$, where Y is the fraction of Hb with O_2 bound to it, PO_2 is the O_2 pressure, P_{50} is the partial pressure of O_2 at which hemoglobin is half-saturated, and n is the Hill number, which allowed calculation of P_{50} and n. Results are means ± SD for three Hb solutions and ten batches of NHC. The lipid content was determined from two separate batches of NHC (*16*). Viscosities were determined at 37°C (n = 3). The expected ability of a 100-ml preparation to deliver oxygen to tissues, referred to as a-v O_2 delivery, was calculated assuming arterial and venous PO_2 values of 100 and 30 torr, respectively: a-v O_2 delivery = 1.4[Hb](ΔY), where the constant has units ml (O_2)/g, [Hb] is the concentration of Hb in grams per deciliter, and ΔY is the difference in Y values calculated at the two different PO_2 values. The [lipid] value is the concentration of total NHC lipid and viscosity is measured in centipoise (cp).

Transfusion material	P_{50} (torr)	Hill number, n	Methemoglobin (%)	[Hb] (g/dl)	[Lipid] (g/dl)	Viscosity (cp)	a-v O_2 delivery (ml/dl)
Stroma-free Hb	13.2 (±1.1)	2.4 (±0.1)	0.8 (±0.3)	7.2 (±0.01)	—	—	1.16
25% suspension of NHC	24.0 (±2)	2.1 (±0.1)	1.5 (±0.7)	3.78 (±0.1)	1.11 (±0.06)	1.0 (±0.2)	1.78
50% suspension of NHC	24.0 (±2)	2.1 (±0.1)	1.5 (±0.7)	7.55 (±0.4)	2.21 (±0.11)	1.9 (±0.2)	3.35
Normal stored human blood	27	2.6	<1	13.5	*	2.7	7.5

*A typical range for total lipids in blood is 0.38 to 0.74 g/dl.

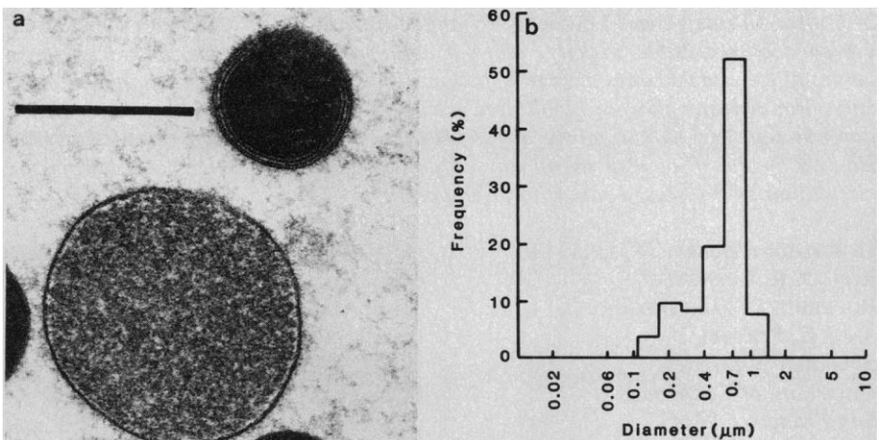

Fig. 1. (a) Thin-section electron micrographs of neohemocytes from an electron microscope (Philips model 201). The bar designates 0.5 μm. Differences in electron density reflect differences in entrapped hemoglobin concentration. (b) Size-frequency distribution of NHC. The size spectrum was determined with a photon correlation spectrometer (Coulter model N4) at a 90° scattering angle. Results are the average of three 5-minute counts of light intensity scattered by particles of the indicated size.

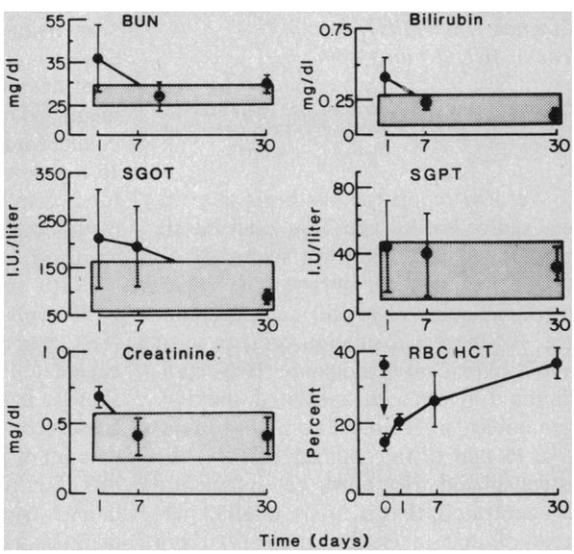

Fig. 2. Partial clinical chemistry results for blood or serum from n male rats [122 to 150 g; Crl:CD® (SD)BR] receiving 50 percent exchange transfusions of NHC. Clinical chemistries were performed using a clinical chemistry analyzer (Technicon SMA-12 model). The blood urea-nitrogen (BUN), bilirubin, creatinine, serum glutamate-oxalacetate transaminase (SGOT), and SGPT values shown are the mean ± SD at either 1 (n = 4), 7 (n = 4), or 30 (n = 5) days after transfusion, just before the rats were killed. The shaded region is the range for the mean ± SD for values from sham-transfused rats (n = 7) killed on day 1 after transfusion. Red blood cell hematocrit (RBC HCT) values are for blood taken before and immediately after transfusion and, on the indicated day, immediately before the rats were killed. Blood glucose, uric acid, alkaline phosphatase, cholesterol, protein, phosphorous, albumin, and lactate dehydrogenase values for transfused rats were equivalent to control values at all three times.

apparent half-life of 5.8 hours (SD, 0.3), whereas during that time the cumulative amount of ^{14}C recovered from urine was approximately 18 percent of the dose of ^{14}C-labeled sucrose with an apparent half-life of less than 1 hour. In contrast, free Hb is cleared from the circulation of transfused rats by an apparent zero-order process, and about 4 hours are required for the amount of circulating Hb to fall to 40 percent of its original value (*7*). Liposomes are known to be cleared from the circulatory system, in part, by uptake by the reticuloendothelial system; adsorption to vascular endothelial cells is also thought to remove them. Significant extravascularization of liposomes has not been reported and would not be expected for particles as large as NHC. Because NHC are not excreted in urine, urinary recovery of ^{14}C is a measure of the stability of NHC in vivo. Apparent clearance of NHC from the circulatory system is therefore a combination of irreversible loss of intact NHC from circulation and loss of quantified contents. The latter accounted for less than 18 percent of the total apparent clearance.

Acute toxicity was evaluated in three experiments. In the first, a 50 percent suspension of NHC was injected intravenously into rats (0.5 to 8.0 ml per kilogram of body weight). There was no significant difference in the behaviour of control and experimental rats in the 48 hours after injection. Next, 13 rats were transfused (*5*) with a 25 percent suspension of NHC until their red blood cell hematocrit level fell to 50 percent or less of its original value. Rats were killed 1, 7, and 30 days after transfusion; their blood was taken for analysis; and their lungs, brain, liver, spleen, heart, and kidney were removed, fixed, and stored for subsequent pathological examination. The blood chemistry results are presented in Fig. 2 and the pathology results in Table 2. The mean serum gluta-

mate-pyruvate transaminase (SGPT) values were normal, despite evidence of acute focal hepatic necrosis in one of four rats evaluated on day 1 (*8*), which was evidence that there was no gross toxicity from the NHC. The slightly elevated concentration blood urea-nitrogen suggested a transient reduction in kidney function, but both the creatinine values and the kidney histology on all 3 days were normal. There was no evidence of either hemaglobinemia or hemoglobiniuria. Improving the stability and the vascular retention time of NHC would be ways to further improve safety and reduce acute toxicity (*9*).

In the final toxicity study the survival times of rats were compared after 95-percent exchange transfusions with either 7.2 g/dl of Hb, 3.6 g/dl of Hb, or neohemocyte suspensions (3.6 to 3.8 g/dl of Hb). Figure 3 shows that rats transfused with Hb solutions had a mean survival time of less than 10 hours. Rats receiving 95 percent transfusions with suspensions of NHC survived more than 18 hours, with two of five rats being long-term survivors, even though the mean total concentration of Hb in their "blood" after transfusion averaged 4.4 g/dl, as compared to the mean value of 7.8 g/dl observed in rats transfused with 7.2 g/dl of stroma-free Hb. This improvement in survival presumably resulted because of the lowered oxygen affinity of Hb in NHC, the prolonged circulation time of encapsulated Hb as opposed to free Hb (*10*), and the absence of significant acute toxicity.

Several of the basic attributes of an artificial red cell (*11*) were achieved when Djordjevich and Miller (*12*) demonstrated that erythrocyte lysates could be reencapsulated in liposomes. Our NHC qualify as prototypal artificial red cells because they meet the previously listed six essential specifications (*13*). The membrane components are all nontoxic and biodegradable, and the data demonstrate reasonable biocompatibility. Unlike previous efforts (*12, 14*), the encapsulation process does not significantly degrade Hb, although the amount of methemoglobin consistently increased relative to the amount present just after encapsulation. The oxygen affinity of NHC was less than that of free human Hb and approached that of normal blood. Because of the osmotic adjustment during the microencapsulation process, the average final internal Hb concentration is higher than that of the starting Hb solution. There was no histopathological evidence of overt intravascular coagulopathy. Neohemocytes are small enough to pass freely through normal, and possibly moderately restricted, capillaries. A nontoxic resuscitation fluid that combines the functions of a plasma expander with the ability to carry and deliver oxygen to tissues could prove useful in treatment of trauma, as a temporary substitute for red cells, and for the treatment of tissue ischemia.

Table 2. Results from pathological examination of organs from *n* rats after 50 percent exchange transfusion (*5*) with 25 percent suspensions of NHC. A single-blind histopathological evaluation of organs from the 20 animals was conducted. Only the five listed categories of pathology were observed. All lungs, hearts, brains, and kidneys were judged normal. The trauma or abnormality was either absent (−), mild (+), moderate (++), or marked (+++); no severe trauma was detected. Seven control rats were sham-transfused (withdrawal and replacement of 2 ml of blood) and killed after 1 day. Mild and moderate extramedullary hematopoiesis is periodically seen in normal rats (122 to 150 g) as well as in rats affected with chronic, low-grade anemic, or hypoxic conditions.

Pathology	Severity of trauma in *n* rats			
	Days after transfusion			Control at day 1 (*n* = 7)
	1 (*n* = 4)	7 (*n* = 4)	30 (*n* = 5)	
Acute focal hepatic necrosis	1 ++, 3 −	4 −	5 −	7 −
Liver and spleen erythrocytophagy	2 ++, 2 +	4 −	5 −	7 −
Extramedullary hematopoiesis	4 −	2 +++ 2 +	2 ++ 3 +	1 +++, 1 + 1 ++, 4 −
Lymphoid hyperplasia of spleen	4 −	1 +, 3 −	5 −	2 +
Spleen sheath hyperplasia	4 −	4 −	4 +++, 1 −	7 −

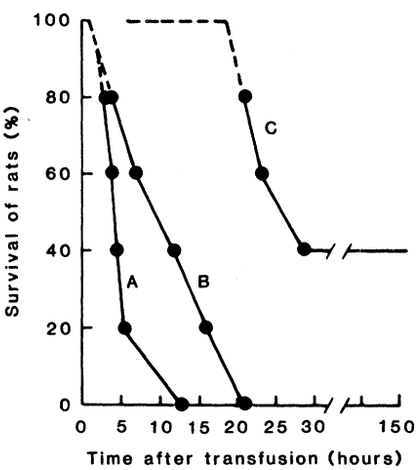

Fig. 3. The percent of rats surviving a 95 percent exchange transfusion as a function of time after transfusion (*10*). This second group of male rats (150 to 250 g) were maintained under pentobarbital anesthesia for the duration of the transfusion, which was completed when each animal's red blood cell hematocrit level was equal to, or less than, 5 percent of its original value. Curve A resulted from transfusion with a solution consisting of 3.6 g/dl of Hb in Ringer's solution (*p*H 7.4, 300 mOsm). Curve B resulted from transfusion of a similar solution with 7.2 g/dl of Hb. Curve C resulted from transfusion with 25 percent suspensions of NHC (as described in Table 1) in normal saline containing 5 g/dl of HSA (*p*H 7.4, 300 mOsm). We also found that rats transfused with a suspension of washed red blood cells with a hematocrit value of 25, where the red blood cells were suspended in the same solution used for NHC, all survived. The mean concentrations of Hb in "blood" immediately after transfusion were: curve A, 4.3 g/dl; curve B, 7.8 g/dl; and curve C, 4.4 g/dl.

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3. Aseptic techniques were used, and in all buffers pyrogen-free (USP) water was used. Purified human Hb [F. DeVenuto, T. F. Zuck, A. I. Zenga, W. Y. Moores, *J. Lab. Clin. Med.* **89**, 509 (1977)] was concentrated to between 16 and 18 g/dl and extensively dialyzed against phosphate buffer (*p*H 7.4, 30 mOsm). Disodium 2,3-diphosphoglycerate (DPG) was added to give a DPG to Hb molar ratio of 1.5 and a Hb concentration of 15 g/dl. The final Hb solution had a *p*H value of 7.4 and an osmolality (Osm) value of 40 mOsm or less. Each preparation of NHC for subsequent studies in vivo began with 125 ml of this Hb solution and 3.75 mmol of lipid (2.03 g) that was free of oxidation products. The lipids were dissolved in a 125-ml mixture of peroxide-free diethyl ether and 1,1,2-trichloro-1,2,2-trifluoroethane (TCTFE), and an emulsion of the aqueous Hb solution in the volatile nonaqueous mixture was prepared (*17*). The lipids served as surfactants. As the volatile mixture was removed under vacuum at 37°C in a rotary evaporator, the dispersed aqueous microdroplets were concentrated until they formed a matrix. Agitation under vacuum was continued to break and collapse the matrix such that part of the Hb solution coalesced to form a new continuous phase with complex, multichambered particles suspended within. Extrusion of this suspension through 1-μm polycarbonate membranes [M. E. Bosworth, C. A. Hunt, D. Pratt, *J. Pharm. Sci.* **71**, 806 (1982)] fixed an upper limit on diameter and resulted in particles suspended in a Hb solution. Addition of a 2.7*M* NaCl solution (approximately 7.5 ml) at a constant rate over 12 hours at 4°C increased the osmolality to 300 mOsm. After the osmotic adjustment, 20 to 35 percent of the original Hb was encapsulated by 70 to 80 percent of the original lipid. The NHC were separated from nonencapsulated Hb on a 4 by 40 cm column (Biogel A5M) eluted with normal saline (0.9 percent USP). The NHC collected in the void volume, which accounted for approximately 15 percent of the original Hb and 55 percent of the original lipid, were centrifuged at 4°C and 6000*g* for 20 minutes. The resulting supernatant, which contained less than 12 percent of the Hb and about 20 percent of the lipid added to the centrifuge tube, was discarded. The Hb-rich pellet was suspended in 0.15*M* NaCl containing 5 g/dl human serum albumin (HSA) to give the final 25-percent neohemocyte suspension (*p*H 7.4, 300 mOsm). The suspension value was determined at 8900*g* for 5 minutes in hematocrit tubes using a hematocrit centrifuge

(International Equipment Company). Purified bovine albumin was used in all process optimization studies. Substitution of HSA for bovine albumin has no impact on currently measured neohemocyte properties.

F. Szoka and D. Papahadjopoulos, *Proc. Nat. Acad. Sci. U.S.A.* **75** 4194 (1978).

Five male rats [200 to 250 g; Cr1:CD® (SD)BR] were maintained under pentobarbital anesthesia for the duration of the study. Each rat received a 50 percent transfusion of a 25 percent neohemocyte suspension. Transfusions consisted of 2-ml withdrawals and replacements from a jugular vein catheter until each animal's red blood cell hematocrit level was equal to, or less than 50 percent of, its original value. The 25 percent suspension of NHC had the properties listed in Table 1. Immediately after the last 2-ml blood exchange, each rat was injected through the same jugular vein catheter with 0.2 ml of a second batch of NHC, which was identical to the first in all respects except that it was prepared from a Hb solution to which a tracer amount of ^{14}C-labeled sucrose had been added; all sucrose was encapsulated at the time of injection. Urine was collected quantitatively from urinary catheters implanted in each subject for the duration of the study. Blood samples were taken at 0.25, 0.5, 1, 2, 4, 6, and 8 hours after transfusion. There was neither a loss of encapsulated sucrose nor a significant increase in methemoglobin formation for NHC stored for 5 days at 4°C. An apparent half-life was calculated from a nonlinear least-squares fit to the log of the blood concentration values with equal weights given to each value.

Intravenously administered sucrose, like inulin, is rapidly cleared from circulation, $t_{1/2} < 10$ minutes [T. M. Allen and J. M. Everest, *J. Pharmacol. Exp. Therap.* **226**, 539 (1983)], by glomerular filtration and is excreted quantitatively in urine. Because free sucrose is cleared from blood much faster than encapsulated sucrose, the amount of ^{14}C in the blood reflects the amount of circulating, encapsulated sucrose.

F. DeVenuto, W. Y. Moores, T. F. Zuck, *Transfusion (Philadelphia)* **17**, 55 (1977).

The one rat that showed signs of moderate acute focal hepatic necrosis on day 1 after transfusion (Table 2) had the highest clinical chemistry values in each category listed. The SGOT and SGPT values were 3.1 and 2.9 times the mean control values, respectively.

The vascular retention times of liposomes having the same composition as these NHC increase with decreasing diameter for a dose of constant surface area [R. M. Abra and C. A. Hunt, *Biochim. Biophys. Acta* **666**, 493 (1981)]. However, the maximum encapsulated volume per mole of lipid decreases with decreasing diameter. Increased intravascular retention times are therefore possible. Neither the membrane composition nor the size of the NHC has been optimized for a specific set of desired functional properties in vivo, because there may not be a single set of preferred properties. For example, the degree to which increases in intravascular retention of NHC would contribute to improved efficacy may depend on the intended function of the NHC [A. G. Greenburg, G. W. Peskin, D. B. Hoyt, W. Y. Moores, *Crit. Care Med.* **10**, 266 (1982)].

Rats transfused with normal saline died before the hematocrit value was reduced to 5 percent of the initial values. The amount of Hb in the red blood cells of each group in Fig. 3 after transfusion was less than 1 g/dl, too low to sustain life. Death would be expected if the total effective amount of Hb (based on a-v O_2 delivery) dropped below a critical value higher than 1 g/dl. Rats in groups A and B died, presumably, because the effective amount of Hb in their blood dropped below this critical value. A similar explanation may account for the deaths of three of five rats in the group transfused with NHC. Death would be expected if the total amount of effective Hb fell below the critical level as a result of neohemocyte clearance or oxidation of Hb to methemoglobin in vivo. Survival would only occur if the combination of the rising amount of Hb in the red blood cells and declining amounts of Hb in the NHC maintained the total effective amount of Hb above the critical value.

T. M. S. Chang, *Science* **146**, 524 (1964).

L. Djordjevich and I. F. Miller, *Exp. Hematol.* **8**, 584 (1980).

Now that these specifications have been met, it is desirable to create a more demanding list of specifications. For example, one may set a minimum on the rate of methemoglobin formation in vivo. We had no control over this process, and its rate may be a major factor governing the functional lifetime of NHC as they are currently formulated. Meeting new specifications may require coencapsulating agents such as methemoglobin reductase along with its required cofactors. Another more rigorous specification may be the mean concentration of Hb within NHC. In preliminary studies we have increased the mean concentration of encapsulated Hb from the current value of 15.8 g/dl to as much as 28.6 g/dl. Significantly more demanding specifications will require reoptimization of the procedure.

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15. J. R. Naville, *J. Appl. Physiol.* **74**, 198 (1974).
16. The two preparations of NHC had identical lipid compositions. One batch included tracer amounts of ^{3}H-dipalmitoylphosphatidylcholine, and the other included tracer amounts of ^{14}C-cholesterol. We assumed that the combined average recovery of these two markers was identical to the average total lipid recovery.
17. Vigorous mixing of either peroxide-free diethyl ether or TCTFE with purified Hb for up to 1 hour did not result in degradation of Hb as measured by precipitate, methemoglobin, or hemichrome formation. The primary independent variables governing emulsion stability are particle size of the dispersed phase, surfactant concentration and properties, differences in surface tension, viscosity and density of the two phases, and osmolality of the aqueous phase. Of these, only the last two could be varied in this procedure. The use of TCTFE made it possible to match the densities of the volatile and aqueous phases, a step that is essential for subsequent success. If detectable coalescence occurred within the 5 minutes after 5 minutes of vigorous shaking, additional ether or TCTFE was added. The mixture was placed in screw-cap bottles such that the air space was minimized, and then the bottles were shaken for 30 minutes on an industrial paint shaker (Red Devil). If excess methemoglobin or hemichromes were present, lipid-protein aggregates with intended membrane lipids could form. If the combined amount of these two proteins exceeded 5 percent of the total Hb content, the yield of NHC was reduced.

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Biosynthesis and Secretion of Proatrial Natriuretic Factor by Cultured Rat Cardiocytes

Abstract. Rat atrial natriuretic factor (ANF) is translated as a 152–amino acid precursor preproANF. PreproANF is converted to the 126–amino acid proANF, the storage form of ANF in the atria. ANF isolated from the blood is approximately 25 amino acids long. It is demonstrated here that rat cardiocytes in culture store and secrete proANF. Incubation of proANF with serum produced a smaller ANF peptide. PreproANF seems to be processed to proANF in the atria, and proANF appears to be released into the blood, where it is converted by a protease to a smaller peptide.

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Atrial natriuretic factor (ANF) is a peptide hormone with potent diuretic and natriuretic properties that probably plays an important role in controlling blood pressure (*1*, *2*). ANF is released from the atria in response to an increase in blood volume (*3*). Understanding the mechanisms that control release of this peptide from the atria is critical to understanding the mechanisms that control blood pressure. One approach to identifying these mechanisms is to study the peptide's biosynthesis. Analysis of the ANF gene and messenger RNA (mRNA) suggests that rodent ANF is translated as a 152–amino acid precursor, preproANF (Fig. 1) (*4*–*7*). PreproANF has many structural features common to other peptide hormone precursors (*8*) and thus may be processed similarly. PreproANF is converted to a 126–amino acid form, proANF (Fig. 1), by removal of the amino terminal hydrophobic leader segment and two residues from the carboxyl terminus. ProANF is the storage form of ANF in the atria (*9*) and has less diuretic activity than smaller ANF-related peptides (*2*). The subsequent steps in the