structured ways what respondents have "on their minds."

There is one practical solution to the problems pointed to in this report. The solution requires giving up the hope that a question, or even a set of questions, can be used to assess preferences in an absolute sense or even the absolute ranking of preferences and relies instead on describing changes in responses over time and differences across social categories (*3*). The same applies to all survey questions, including those that seem on their face to provide a picture of public opinion (*8*).

REFERENCES AND NOTES

1. See S. Sudman and N. M. Bradburn [*Asking Questions* (Jossey-Bass, San Francisco, 1982)] for a discussion of the open-closed distinction. H. Schuman and S. Presser [*Questions and Answers in Attitude Surveys* (Academic Press, New York, 1981)] and a few earlier but marginally relevant reports cited therein provide partial exceptions to the statement about lack of research for questions about public opinion.
2. Although relatively simple questions were used in this investigation in order to provide precision in results, there is little reason to think that the basic conclusions will differ when questions are more complex.
3. We cannot compare correlates of the four focal issues on the two question forms, since there are too few cases in those categories on the open form to allow comparison. Considering the closed form alone, there is no significant relation between education and choosing one of the listed alternatives rather than giving an unlisted problem. However, among the four closed alternatives taken as a set, there are statistically significant associations with education, for example, choice of quality of public schools increases with respondent educational level.
4. The closed question was itself divided into five randomly administered forms, each with a different ordering of the five alternatives. No significant order effect was discovered in this sub-experiment, though such effects occur for some questions; see H. Schuman and S. Presser in (*1*).
5. H. Schuman, J. Ludwig, J. Krosnick, *Public Opin. Q.* **50**, 519 (1986).
6. Although we cannot compare correlates of the computer response on the two question forms—there are only five such cases on the open form—the closed form does yield a highly significant correlate for the computer response versus all other closed choices. "The invention of the computer" was chosen especially by the youngest (18 to 29) of four age categories. Closer study of the two forms suggests that young people tended to give space-related responses to the open question, but shifted to the computer response on the closed form.
7. There is evidence that such a sequence can produce close correspondence between open and closed question distributions. See Schuman and Presser in (*1*) and Schuman, Ludwig, and Krosnick (*5*).
8. Exactly this point was made explicitly in one of the first major uses of survey data [S. A. Stouffer *et al.*, *The American Soldier: Adjustment During Army Life* (Princeton Univ. Press, Princeton, NJ, 1949)]. Of course, all such comparisons assume that the form of the question has been held constant.

9. This report draws on research supported by NSF grants SES-8411371 and SES-8410078. Helpful suggestions on the manuscript were made by P. E. Converse, R. M. Groves, and J. Ludwig.

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Lipoprotein Uptake by Neuronal Growth Cones in Vitro

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Macrophages that rapidly enter injured peripheral nerve synthesize and secrete large quantities of apolipoprotein E. This protein may be involved in the redistribution of lipid, including cholesterol released during degeneration, to the regenerating axons. To test this postulate, apolipoprotein E–associated lipid particles released from segments of injured rat sciatic nerve and apolipoprotein E–containing lipoproteins from plasma were used to determine whether sprouting neurites, specifically their growth cones, possessed lipoprotein receptors. Pheochromocytoma (PC12) cells, which can be stimulated to produce neurites in vitro, were used as a model system. Apolipoprotein E–containing lipid particles and lipoproteins, which had been labeled with fluorescent dye, were internalized by the neurites and their growth cones; the unmetabolized dye appeared to be localized to the lysosomes. The rapid rate of accumulation in the growth cones precludes the possibility of orthograde transport of the fluorescent particles from the PC12 cell bodies. Thus, receptor-mediated lipoprotein uptake is performed by the apolipoprotein B,E(LDL) (low density lipoprotein) receptors, and in the regenerating peripheral nerve apolipoprotein E may deliver lipids to the neurites and their growth cones for membrane biosynthesis.

INJURED MAMMALIAN PERIPHERAL nerves can regenerate for long distances through a distal sheath populated by Schwann cells, macrophages, and other nonneuronal "sheath" cells (*1*). When transplanted into injured central nervous system (CNS) pathways, these peripheral nerve sheaths can support growth of normally nonregenerating CNS axons (*2*). Attention has therefore been directed at identifying factors present in the injured peripheral nerve that might initiate or facilitate the growth of the damaged fibers (*3*). One candidate is a soluble protein of M_r 37,000. The rate of synthesis of this protein increases dramatically after injury to an adult rat sciatic nerve; this protein can account for nearly 5% of the total protein secreted by the nerve 3 weeks after injury (*4*). This protein, identified as apolipoprotein E (apo-E) (*5*), is produced by the macrophages that enter the damaged nerve within 3 days of injury (*6*).

Apolipoprotein E is associated with various plasma lipoproteins, including high density lipoproteins (HDL), and participates in the transport of cholesterol into various cells. It serves as a ligand for the apo-B,E(LDL) (low density lipoprotein) receptor, which mediates the uptake of the apo-E–containing lipoproteins and provides cells with lipids for various metabolic pathways, including membrane biosynthesis (*7*). Thus, apo-E may participate in the redistribution of lipids to various cells in neural tissue through similar mechanisms.

After nerve injury, the cholesterol released from myelin membranes is reused in the reassembly of both myelin and axonal membranes in the regenerating nerve (*8*). It has been suggested (*5, 9*) that apo-E secreted by macrophages in the injured nerve provides the mechanism for lipid reutilization by facilitating the production of apo-E–containing lipoproteins that could be bound and internalized via lipoprotein receptors on both Schwann cells and regenerating axons. We have asked whether the secreted apo-E in injured nerve is complexed with lipid, whether these apo-E complexes and apo-E–containing plasma lipoproteins can be taken up by neuronal growth cones, and whether the uptake is mediated by apo-B,E(LDL) receptors.

Conditioned medium containing apo-E was obtained from cultures of injured segments of rat sciatic nerves 2 weeks after crush injury, as described (*4*). Newly synthesized and secreted apo-E was obtained by incubating the injured segments with [^{35}S]methionine. To determine whether both the accumulated and newly synthesized apo-E were associated with lipid, the conditioned medium was subjected to density-gradient ultracentrifugation (Fig. 1). SDS–

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polyacrylamide gel analysis (*10*) of density fractions demonstrated that both unlabeled apo-E and apo-E labeled with [^{35}S]methionine were present at densities of 1.06 to 1.12 g/ml. Identification of the apo-E was confirmed by immunoblotting (*11*) of a duplicate gel. Material obtained 1 and 3 weeks after the injury gave identical results. Although insufficient quantities of this material were available for the determination of specific lipid classes, the apo-E in the injured nerve segments floated at densities expected for apo-E complexed with lipid. Both apo-E–phospholipid complexes and apo-E–containing HDL, which contain both phospholipid and cholesterol, float at a density of 1.06 to 1.12 g/ml (*7, 12*).

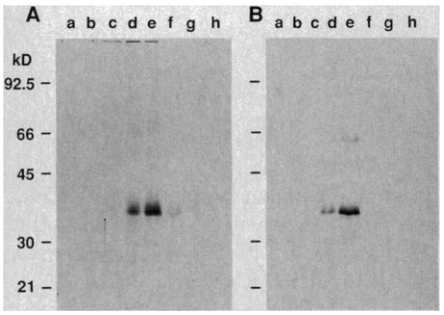

Fig. 1. Isolation by density-gradient ultracentrifugation of apo-E–associated lipid complexes in medium obtained from segments of injured rat sciatic nerves. Injured segments of sciatic nerves were removed from rats 2 weeks after crush injury and incubated for 4 hours in methionine-free Dulbecco's modified Eagle's medium to which 10 μCi of [^{35}S]methionine was added; one nerve segment was incubated per milliliter of medium. The conditioned medium was dialyzed against a solution of 50 m*M* sodium phosphate and 140 m*M* sodium chloride at *p*H 7.6 overnight and then was adjusted to a density of 1.21 g/ml with potassium bromide (the assumed starting density was 1.006 g/ml). A 4-ml volume of this material (equivalent to the conditioned medium from four nerve segments) was added to the bottom of the centrifuge tube and overlaid with 7.5 ml of a 1.21 g/ml solution of potassium bromide containing 2 m*M* EDTA. After ultracentrifugation for 48 hours at 250,000*g* in a Beckman SW41.TI rotor, the top 1 ml of the fluid was collected from the tube and placed at the bottom of a step gradient of potassium bromide composed of 1.21, 1.063, 1.02, and 1.006 g/ml (2 m*M* EDTA was present in all solutions). These samples were centrifuged as before, and then 1-ml fractions were collected and their densities determined by weighing a measured volume of each fraction. Each fraction was analyzed on 10% SDS-polyacrylamide gels (*10*). The gel was stained with Coomassie blue and autoradiography performed as described (*4*). (**A**) Coomassie blue–stained gel showing the density distribution of the unlabeled protein. (**B**) The corresponding autoradiograph of the newly synthesized, labeled material. Densities of the fractions in grams per milliliter were as follows: lane a, 1.20; lane b, 1.18; lane c, 1.15; lane d, 1.12; lane e, 1.09; lane f, 1.08; lane g, 1.06; and lane h, 1.03. Both the labeled and unlabeled apo-E were present in fractions with densities of 1.06 to 1.12 g/ml.

To determine whether apo-E–containing lipoproteins could be bound and internalized by nerve cells and whether growing neurites possessed apo-B,E(LDL) receptors, pheochromocytoma (PC12) cells were used as a model system (Fig. 2). PC12 cells were grown in the presence of nerve growth factor for 8 days to stimulate neurite formation (*13*). The cells were then incubated with apo-E–containing HDL (referred to as apo-E HDL$_c$, a lipoprotein from cholesterol-fed dogs) (*7*) labeled with the fluorescent probe 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI) (*14*).

An intense granular fluorescence pattern was observed within the neuronal cell body, neurites, and growth cones after 1 to 2 hours of incubation of the cells with DiI-labeled apo-E HDL$_c$ (Fig. 2, A, C, and D). In other cell types, this pattern of fluorescence reflects receptor-mediated internalization of the DiI-labeled apo-E HDL$_c$ and the subsequent accumulation of the unmetabolizable DiI in the lysosomes (*14*). Fluorescent labeling could be blocked by incubating the cells with a 100-fold excess of unlabeled apo-E HDL$_c$ along with the DiI-labeled apo-E HDL$_c$ (Fig. 2E). Thus, the binding and internalization by the PC12 cells represented a specific interaction. Furthermore, acetoacetylation of the apo-E moiety of the HDL$_c$, a procedure known to prevent receptor-mediated binding of these lipoproteins (*14*), prevented most of the binding

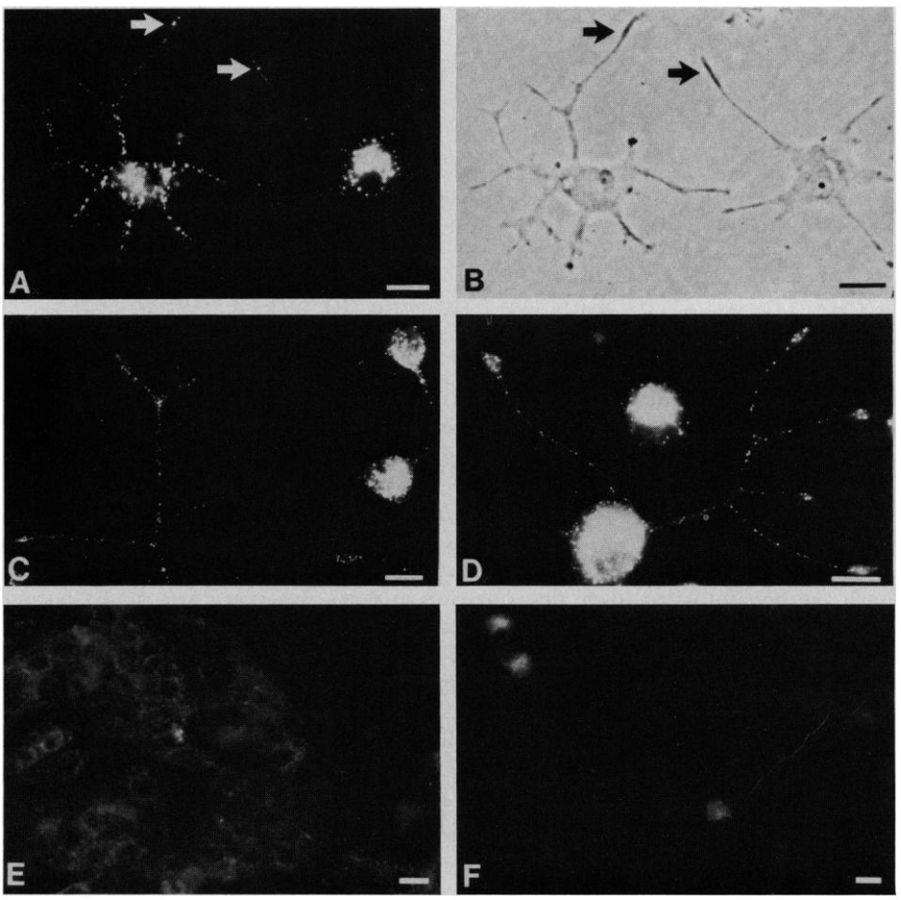

Fig. 2. Labeling of PC12 growth cones, neurites, and cell bodies with DiI-labeled apo-E HDL$_c$. The PC12 cells were grown for 8 days in the presence of 50 ng of nerve growth factor (NGF) per milliliter in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. On day 8, when the cells had produced extensive neurites, the medium was replaced with serum-free medium containing NGF, and experiments were begun on day 9. After a 1-hour or 2-hour incubation with DiI-labeled apo-E HDL$_c$ (5 μg of protein per milliliter) at 37°C, the cells were washed five times for 1 minute each with phosphate-buffered saline (PBS) containing 1% bovine serum albumin and once with PBS. They were then fixed with 4% paraformaldehyde for 1 hour. All procedures were performed at room temperature. Cells were photographed at ×50 to ×200 magnification with a Zeiss microscope with epifluorescent illumination, with a rhodamine filter. Incubation with DiI-labeled lipoproteins for 1 hour (**A**) or 2 hours (**C**, **D**) resulted in extensive labeling of the cells and their processes. (**B**) The phase micrograph corresponding to (A), demonstrating that the labeling in (A) is evenly distributed over the entire cell body, including the neurites and growth cones (arrows). The labeling detected under these conditions represented aggregates of DiI sequestered in lysosomes. (**E**) A 100-fold excess of unlabeled apo-E HDL$_c$ was added along with the DiI-labeled apo-E HDL$_c$ (1 hour, 37°C). In a second control (**F**), DiI-labeled apo-E HDL$_c$ containing acetoacetylated apo-E at 10 μg of protein per milliliter was added. Little specific fluorescence is seen in (E) and (F). Scale bars, 10 μm.

and internalization of the DiI-labeled apo-E HDL_c (Fig. 2F). In addition, unlabeled low density lipoproteins from the dog—lipoproteins resembling HDL_c in lipid composition but possessing apo-B instead of apo-E—effectively inhibited the interaction of these lipoproteins with the PC12 cells. Thus, the PC12 cells possess lipoprotein receptors capable of interacting with both apo-E– and apo-B–containing lipoproteins, a characteristic of the apo-B,E(LDL) receptor (*7*).

The granular staining seen in the growth cones and neurites in these experiments after relatively long incubation times might have been caused by orthograde axonal transport of DiI-labeled apo-E HDL_c internalized in the cell body. To rule out this possibility, cells were incubated with DiI-labeled apo-E HDL_c for 5 to 10 minutes, and only those growth cones over 250 μm from the cell body were analyzed. DiI-labeled lipoproteins transported by orthograde movement, even via fast axonal transport, could not have covered this distance in the time allotted (*15*). Fluorescent labeling, although faint, was definitely present at 5 minutes and was associated with 90% of the growth cones greater than 250 μm from the cell body. Growth cones more than 300 μm from the cell body were labeled within 10 minutes after incubation with DiI-labeled apo-E HDL_c (Fig. 3, C and D, arrows). Therefore, there are apo-B,E(LDL) receptors in the growth cone, as well as in the cell body.

Additional studies were performed with DiI-labeled apo-E complexes isolated from injured nerve segments by ultracentrifugation. The granular fluorescent pattern obtained with the PC12 cells following a 2-hour incubation with the DiI-labeled apo-E complexes closely resembles that seen with DiI-labeled apo-E HDL_c (Fig. 4A). Most of the fluorescent labeling could be prevented by simultaneous incubation of the cells with a 100-fold excess of unlabeled apo-E HDL_c (Fig. 4B).

These experiments demonstrate that PC12 growth cones are able to take up apo-E–containing lipoproteins obtained from either serum or the injured nerve. Since growth cones contain coated pits, lysosomes, and endoplasmic reticulum (*16*), which are necessary to metabolize lipids, it is reasonable to postulate that the internalized lipid is used in the assembly of new axonal membranes. Indeed, it has been shown that the generation of new membrane in a growing axon most likely occurs at the base of the growth cone (*17*). However, it has been suggested that all the lipid required for axonal elongation during development [a growing axon is estimated to require 0.5 to 1.0 μm^2 of plasma membrane per minute (*18*)] is supplied from the cell body via axonal transport (*19*). On the other hand, following nerve injury, when there is a marked accumulation of lipids and apo-E, the apo-E complexes may interact with the apo-B,E(LDL) receptors on growth cones to provide an additional, local source of lipid for axon regeneration. Because regenerating axons of injured nerves may traverse greater distances than occur during development, such a mechanism could reduce the demand for axonal transport of cholesterol and phospholipid from the cell body and hasten the regeneration.

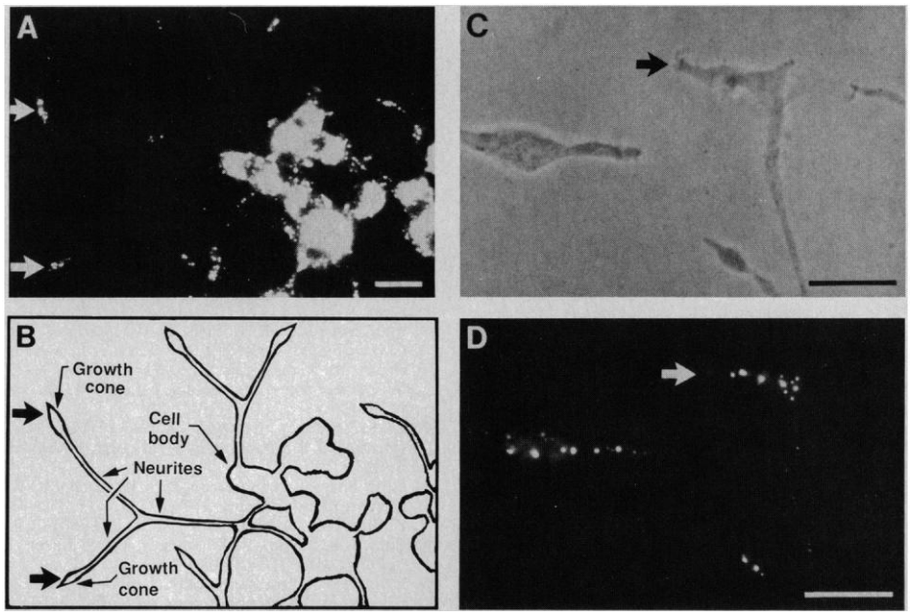

Fig. 3. Pulse labeling of neurite-bearing PC12 cells with DiI-labeled apo-E HDL_c showing uptake by growth cones. Cell culture, labeling, fixation, and microscopy were performed as in Fig. 2, except that the incubation time was 10 minutes. (**A**) A low-power fluorescence micrograph showing the labeled apo-E HDL_c in growth cones (arrows), with little fluorescence in the neurite separating the growth cone from its labeled cell body. The neurites are difficult to visualize because of this low level of fluorescence between the cell body and growth cones. (**B**) A sketch of (A) showing the position of the neurites. (**C**) A high-power phase micrograph of several growth cones, shown in the corresponding fluorescence micrograph (**D**) to be heavily labeled. The large arrow in (C) and (D) points to a growth cone that was estimated to be more than 300 μm from its cell body. Scale bars, 10 μm.

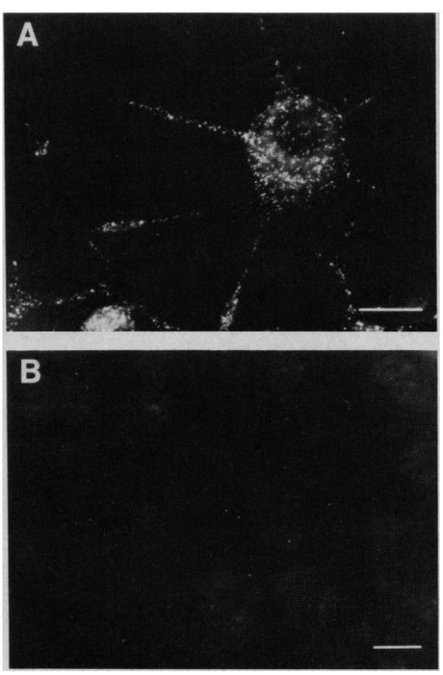

Fig. 4. PC12 cells bind DiI-labeled apo-E–containing lipoproteins isolated directly from injured nerve. Conditioned medium from injured nerves was incubated with DiI, the density was raised to 1.21 g/ml with potassium bromide, and the apo-E–containing lipoproteins were isolated by ultracentrifugation (48 hours, 100,000*g*). The sample was dialyzed against Dulbecco's modified Eagle's medium and then incubated with PC12 cells for 2 hours with lipoprotein protein (10 μg/ml). Fixation and photography were as in Fig. 2. (**A**) The uptake of the DiI-labeled lipoproteins was extensive throughout the cell and its processes. (**B**) A 100-fold excess of unlabeled apo-E HDL_c prevented most of the binding and uptake of the DiI-labeled apo-E complex. Scale bars, 10 μm.

Further consideration of the overall importance of apo-E in lipid transport or in other, as yet unidentified roles may provide insights into the factors controlling and regulating nerve regeneration. For example, there is a marked difference in the amount of apo-E accumulated in injured peripheral nerves, which are capable of regeneration, and CNS fiber tracts, which lack the ability to regenerate. Within the CNS tracts (rat optic nerve) there is little apo-E accumulation within the first 18 weeks after injury (*20*). It is possible that the lack of apo-E and

its associated lipid may be one of the factors that retards the ability of these fiber tracts to sustain effective sprouting and axon elongation.

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15. Two steps and their rates have to be considered: (i) receptor-mediated uptake [via the apo-B,E(LDL) receptor] and (ii) fast axonal transport. Ferritin-LDL uptake by normal human fibroblasts into coated pits takes 3 minutes, and within 6 to 8 minutes LDL can be seen in lysosomes [M. S. Brown and J. L. Goldstein, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3330 (1979)]. An in vivo rate of 2.7 μm per second for fast axonal transport in the rabbit visual system has been determined [T. Lorenz and M. Willard, *ibid.* **75**, 505 (1978)]. At this rate, particles could travel 810 μm in 5 minutes or ~160 μm per minute. Factoring in the delay of 3 to 6 minutes for uptake and compartmentalization, a particle could travel from 0 to 320 μm in 5 minutes, with 80% of the material able to travel less than 160 μm.
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Sequence-Specific Packaging of DNA in Human Sperm Chromatin

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The DNA in human sperm chromatin is packaged into nucleoprotamine (~85%) and nucleohistone (~15%). Whether these two chromatin fractions are sequence-specific subsets of the spermatozoon genome is the question addressed in this report. Sequence-specific packaging would suggest distinct structural and functional roles for the nucleohistone and nucleoprotamine in late spermatogenesis or early development or both. After removal of histones with 0.65*M* NaCl, exposed DNA was cleaved with Bam HI restriction endonuclease and separated by centrifugation from insoluble nucleoprotamine. The DNA sequence distribution of nucleohistone DNA in the supernatant and nucleoprotamine DNA in the pellet was compared by cloning size-selected single-copy sequences and by using the derived clones as probes of nucleohistone DNA and nucleoprotamine DNA. Two clones derived from nucleohistone DNA preferentially hybridized to nucleohistone DNA, and two clones derived from nucleoprotamine DNA preferentially hybridized to nucleoprotamine DNA, which demonstrated the existence of sequence-specific nucleohistone and nucleoprotamine components within the human spermatozoon.

DURING THE FINAL STAGES OF spermatogenesis in placental mammals, the histones associated with DNA in spermatid chromatin are replaced by smaller, extremely arginine-rich protamine molecules. As a result, the nucleosomal-based packaging of DNA characteristic of somatic chromatin is transformed into a highly condensed form of chromatin referred to as nucleoprotamine. In most mammals, this displacement is generally thought to be complete. In humans, however, approximately 15% of the histones remain associated with sperm DNA (*1*). Because the semen of normal human males contains large numbers of abnormal sperm types, it has been difficult to positively link histone with the packaging of chromatin in normal, mature sperm.

The nucleoprotamine complex is structurally distinct from nucleohistone (*2*), insoluble in buffers of physiological ionic strength, transcriptionally inactive (*3*), and resistant to enzymatic digestion (*4*). Histones can be quantitatively extracted from human sperm chromatin with a mixture of 8*M* urea and 0.2*M* NaCl without an accompanying release of DNA, which suggests that nucleohistone is present in each sperm nucleus and is bounded by regions of nucleoprotamine (*5*). Using blot hybridization techniques, we demonstrated that DNA is packaged in the two chromatin fractions in a sequence-specific manner, confirming the presence of sequence-specific nucleohistone and nucleoprotamine components in the spermatozoon.

Sperm previously stored at −20°C (*6*) were separated from semen by washing with 100 m*M* NaCl, 10 m*M* tris (*p*H 8.0), and 1 m*M* EDTA containing 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF). Chromatin was isolated without sonication by solubilizing the tail, acrosome, and nuclear membrane with mixed alkyltrimethylammonium bromide (Sigma M7635) (*7*). To determine the NaCl concentration necessary for histone extraction without disrupting nucleoprotamine, we extracted the chromatin with increasing NaCl concentrations and analyzed the dissociated proteins in Triton-acid-urea (TAU) gels. As shown in Fig. 1A, histones began dissociating from sperm chromatin at 0.6*M* NaCl. Protamines were not released at NaCl concentrations up to at least 0.9*M*. The ease with which histones were removed from sperm chromatin in this study differs from an earlier report that

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