bers from each species in the culture.

The cardiac muscle cells in the present study did not show the active segregation or sorting-out phenomenon according to species that was reported by Burdick and Steinberg (*5*) with their light microscopic studies. The active segregation involves a sorting out of cells of one species from those of another without the establishment of intercellular junctions. Whether or not intercellular contact or adhesion through intercellular junctions has taken place cannot be determined with certainty with the light microscope. Since we observed intercellular contacts through close membrane appositions and adhesion through intercellular junctions between embryonic rat and chick cardiac muscle cells, it is evident that true segregation did not occur in these experiments.

Our findings partially support the hypothesis of Moscona and his collaborators (*3*, *4*) who maintained that bispecific combinations of homologous cells did not sort out sharply according to species. However, in our bispecific homologous cell aggregates containing chick and rat cells in the ratio of 1:1, a number of cardiac muscle cells of the same species were in close association with one another and exhibited intercellular junctions. In association with these monospecific cell groups were scattered cells of the other species (rat or chick) that did not exhibit intercellular contact. These observations raised the question of whether some of the cells were sorted out. The small groups of monospecific cells did not impair or interfere with the coaggregation of most of the groups of bispecific cells. Although the random collision of cells during rotation culture probably gives rise to the monospecific association of a small number of cells that cannot be expected to adhere to cells of other species, the possibility that some species specific cell recognition occurred in our experiments cannot be ruled out completely. Nevertheless, the small monospecific cardiac muscle cell groups in our study did not show the pattern of sorting out reported by Burdick and Steinberg (*5*), who mentioned that the mouse cells were sorted out at the periphery and the chick cells were located internally.

We believe that the resolution used in the past studies (*5*) was not sufficient to detect intercellular contact and adhesion, and that the light microscopic criteria, that is, the morphology and size of the nucleus, used (*5*) to differentiate chick cells from those of rat were not wholly reliable. We observed that the morphology and size of the nuclei varied within the cells of the same species.

Our findings provide evidence that embryonic rat and chick cardiac muscle cells can coaggregate and form a bispecific tissue that consists of mosaics of cells and cell groups from two species. Such bispecific myocardial tissue is capable of beating synchronously. These data demonstrate that homologous cells from different species can recognize their histotypic similarity and form joint tissues across the species differentials.

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In vitro Reassembly of Squid Brain Intermediate Filaments (Neurofilaments): Purification by Assembly-Disassembly

Abstract. *Intermediate filaments from squid brain tissue were reassembled in vitro and purified by two cycles of assembly and disassembly. Purified squid brain filaments contained one major polypeptide (60,000 daltons), which constituted about 70 percent of the total protein, and three minor polypeptides (74,000, 100,000, and 220,000 daltons). Squid brain intermediate filaments were reconstituted from rod-shaped protofilamentous subunits. In addition to the intermediate filaments, dense bodies which may function in intermediate filament nucleation or organization were retained through two purification cycles.*

Intermediate filaments (IF's) (neurofilaments) constitute a major cytoplasmic fiber system in nerve cells. These tubular structures are approximately 10 nm in cross-sectional diameter. They are thought to be a major cytoskeletal component in the formation and maintenance of the asymmetric shape of neurons (*1-3*). In spite of their structural similarities (*4-6*), the identity of the polypeptides constituting IF's in general, and neural IF's in particular, represents an area of considerable confusion. Polypeptides associated with neural IF's obtained from different species do not exhibit generalized immunological cross-reactivity (*2*, *3*), and the reported molecular weights of IF polypeptides from different sources vary widely. We have been attempting to elucidate the composition, structure, and biological functions of IF's in cultured fibroblasts of baby hamster kidney (BHK-21) (*7-10*). In one approach, we have reconstituted IF's in vitro (*10*). As with microtubules (*11*), advantage can be taken of the different sedimentation rates of intact IF's and their constituent subunits. This permits their rapid purification by cycles of assembly and disassembly (*10*) and ensures the isolation of polypeptides that retain their ability to reassemble into IF's. These preparations can then be used to obtain quantitative information about the assembly reaction.

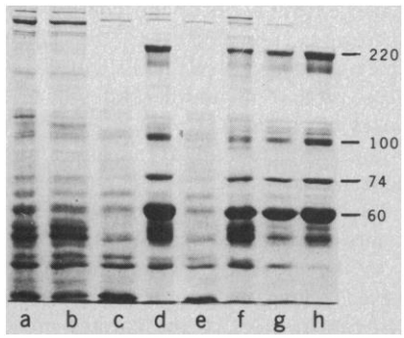

Fig. 1. Purification of squid brain IF's. (a) Whole brains homogenized in disassembly buffer. (b) First 250,000*g* pellet. (c) First 250,000*g* supernatant. (d) First 150,000*g* (first repolymerized) IF pellet. (e) First 150,000*g* supernatant. (f) Second 250,000*g* pellet. (g) Second 250,000*g* supernatant. (h) Second 150,000*g* (second repolymerized) IF pellet. Lanes were loaded with 25 μg of protein. The SDS polyacrylamide (7.5 percent) gel electrophoresis was performed according to Laemmli (*26*).

Preliminary attempts at in vitro reconstitution of IF's from neural tissue have met with limited success (*12-17*). As a result, details of the assembly process have not been described. We now report the in vitro reassembly of squid brain IF's and their purification by cycles of assembly and disassembly. We have also analyzed the ultrastructural events accompanying the assembly pro-

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cess as well as the protein subunit composition of the reassembled IF's.

Our initial attempts to solubilize preparations from extruded axoplasm of squid (*Loligo paelii*) giant axons confirmed previous reports that IF's were disrupted with 0.8 to 1.0*M* KCl (*12, 13, 18*). These observations suggested that restoration of ionic strength to concentrations at which isolated IF's remained stable might also promote their reassembly. Although the axoplasm preparations provided a highly enriched source of IF's for these pilot solubility studies, there was insufficient protein to permit attempts at reassembly. Therefore, all reconstitution experiments were carried out with squid brain tissue.

Brains from 20 to 30 freshly killed squid were homogenized in 0.3 ml of disassembly buffer [0.25*M* 4-morpholineethanesulfonic acid (MES), 1*M* KCl, 5 m*M* ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 m*M* phenylmethylsulfonyl fluoride (PMSF) at *p*H 6.6] at 0°C with several strokes of a glass-glass homogenizer. The volume of disassembly buffer used was 0.3 ml per brain. An elastic, semisolid material formed during homogenization was dialyzed for 5 to 6 hours at 0° to 4°C against 200 volumes of disassembly buffer. This material was then centrifuged at 4°C for 90 minutes at 55,000 rev/min (Beckman type 65 rotor; average, 250,000*g*). The supernatant, which was devoid of detectable IF's as determined by negative staining, was diluted with 9 volumes of assembly buffer (0.25*M* MES, 5 m*M* EGTA, 5 m*M* EDTA, 1 m*M* PMSF, at *p*H 6.6), and IF's were reassembled by incubation for 1 hour at 20°C. Reassembled IF's were harvested by centrifugation at 20°C for 30 minutes at 45,000 rev/min (150,000*g*). The pelleted IF's comprised 5 to 10 percent of the original protein in the extract. These IF pellets were resuspended by being homogenized in a small volume of disassembly buffer (protein concentration, approximately 4 to 8 mg/ml) and incubated at 0° to 4°C for 6 to 10 hours. This solution was clarified by centrifugation at 4°C for 60 minutes at 55,000 rev/min.

For a second assembly cycle, the clarified supernatant was diluted with 9 volumes of EDTA-free assembly buffer, and IF's were reassembled and harvested. Approximately 50 percent of the protein present in the first IF pellets was recovered in the supernatant after subsequent depolymerization and clarification. Ap-

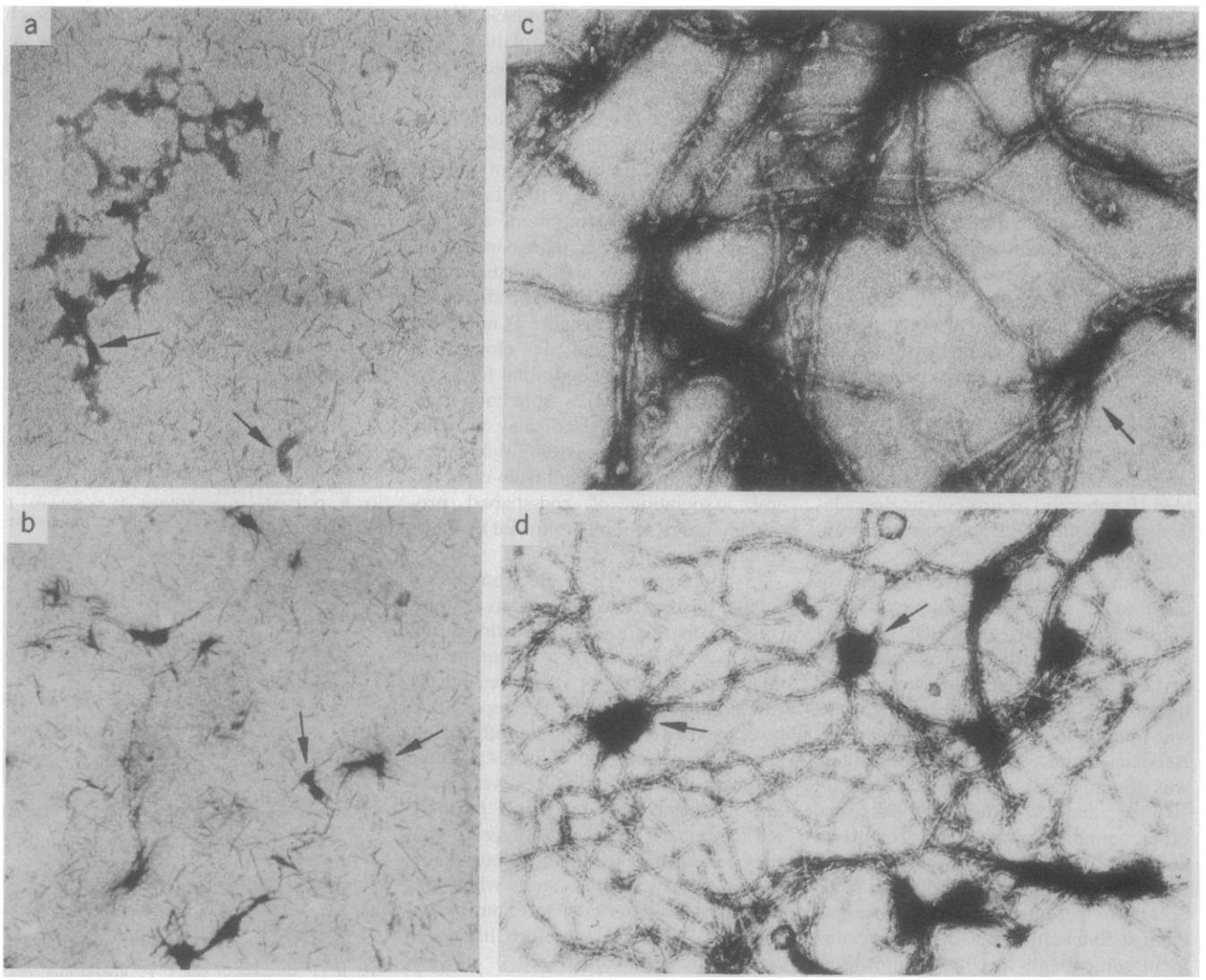

Fig. 2. Ultrastructural analysis of squid brain IF assembly. (a) Protofilamentous IF subunits obtained after a cycle of assembly-disassembly, and clarification (×145,000). Note also the presence of dense bodies (arrows). (b) The sample shown in (a) 1 minute after addition of assembly buffer (×22,000). Short filaments about 10 nm in diameter are present, many of which are associated with the dense bodies (arrows). (c) and (d) Forty minutes after addition of assembly buffer. The IF's have undergone elongation, and many remain associated with the dense bodies (arrows). Negative staining with 3 percent uranyl acetate was performed on carbon- and Formvar-coated grids. (c) ×145,000. (d) ×67,400.

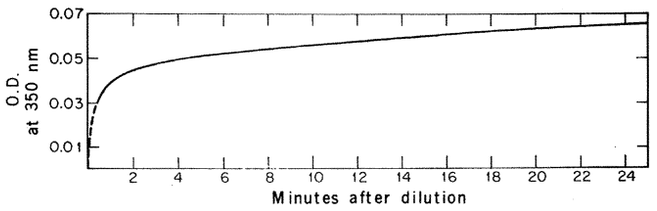

Fig. 3. Turbidimetric analysis of squid brain IF assembly. At time 0, a sample of clarified extract protein was diluted with assembly buffer, and the optical density (*O.D.*) at 350 nm was monitored through the use of a blank with protein in disassembly buffer at the same concentration. The dashed initial portion of the curve is an extrapolation to the origin, and represents the optical density change during mixing, which was too rapid to monitor.

proximately 60 percent of this clarified protein was harvested in the pellets of IF's reassembled for the second time. From 30 brains, about 0.4 mg of protein was obtained in the two-cycle purified IF pellets.

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis of fractions at successive stages in the purification (Fig. 1) revealed that four polypeptides were already greatly enriched in the neurofilaments reassembled from the extract. These included one major band with a mobility corresponding to 60,000 daltons and three minor bands at 74,000, 100,000, and 220,000 daltons. Some material at a lower molecular weight (< 60,000 daltons) was also present in the gels from this fraction. After a second cycle of clarification and reassembly, the four polypeptides were retained in the IF pellets. However, the lighter material was found predominantly in the pellet of insoluble material obtained during the second clarification. These pellets contained very few IF's, as determined by negative staining. Therefore, this material might contain rapidly sedimenting contaminants, aggregated but not assembled IF subunits, or proteolytic degradation products of IF-associated polypeptides.

Estimates of the relative amounts of the four IF-associated polypeptides by densitometric analyses of SDS gels (not shown) indicated that approximately 70 percent of the total protein recovered after two assembly-disassembly cycles was found under the 60,000-dalton peak. Between 3 and 6 percent of the protein was found under each of the 74,000-, 100,000-, and 220,000-dalton peaks, and other minor components comprised about 10 to 20 percent of these preparations. The IF's purified from squid axoplasm by gel filtration and sucrose gradient sedimentation also displayed prominent components at 60,000 daltons and at high molecular weights (~ 200,000 daltons) (*19*). However, we cannot yet rule out the possibility that some of the purified IF may originate from glial or other cells, rather than from neurons.

Analysis of squid brain IF reassembly by electron microscopy and by turbidity suggests that the reaction proceeds by way of rapid lateral association of protofilaments approximately 3 to 5 nm in diameter and 10 to 50 nm long (Fig. 2a). Upon addition of assembly buffer, the turbidity of the solution increased immediately (Fig. 3), and short IF's were observed (Fig. 2b). As the IF's grew longer (Fig. 2, c and d), a slower phase of turbidity developed until a plateau was reached (Fig. 3). The IF's reassembled under these conditions exhibited the light-scattering properties of long rods (*20*). The turbidity should therefore be a direct measure of the mass of polymerized protein (*21*). These results suggest a rapid lateral association of the protofilamentous subunits, increasing the diameter of the rods and, consequently, scattering more light (*22*). The short IF's thus formed appear to serve as nuclei for a slower phase of polymer elongation. Although the ionic conditions for polymer stability differ, IF's from baby hamster kidney fibroblasts display virtually identical biphasic reassembly kinetics; these also appear to result from rapid lateral association of rod-shaped protofilamentous subunits followed by slower elongation (*10*).

In addition to IF's, electron microscopic observations of negative stains (Fig. 2) as well as thin sections (not shown) of the in vitro preparations indicate the presence of structures closely resembling the dense bodies reported to be associated with IF's in smooth muscle (*23*) and in epithelial cells (*24*). Large numbers of these structures were present at all stages of the squid brain IF purification. This result suggests that they remain largely in the supernatants during the clarification steps when IF's are in a depolymerized state, but are associated with and therefore harvested with the reassembled IF's.

Analysis of negatively stained preparations indicates that the dense structures are present, along with the ~ 3-nm protofilaments, in the purified and solubilized IF preparations of the first cycle (Fig. 2a). At early stages of the assembly process, short IF's appear to radiate from the dense structures (Fig. 2b). However, newly formed IF's are not exclusively associated with these bodies, and apparently "free" IF's can be found (Fig. 2b). Squid IF's associated with structures similar to the dense bodies have also been seen in preparations of freshly extruded axoplasm (*20, 25*). These structures may play a role in nucleation or organization of IF's in axons. We have not yet determined which of the major or minor polypeptides in our preparations may be localized in the dense bodies.

The in vitro reassembly and purification conditions described here should now make it possible to study the basic mechanism of brain IF assembly and to investigate the effects of various biological variables important to the regulation of IF assembly in nerve axons. Such studies should ultimately permit new insight into the molecular basis of the role of IF's in maintaining the structural integrity of axons; it should also become possible to determine their interactions with other axonal fibrous proteins, such as those related to microtubules.

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Cholecystokinin Receptors in the Brain: Characterization and Distribution

Abstract. *Specific cholecystokinin binding sites in particulate fractions of rat brain were measured with iodine 125-labeled Bolton-Hunter cholecystokinin, a cholecystokinin analog that has full biological activity. Binding was detected in brain regions known to contain immunoreactive cholecystokinin. Binding was saturable, reversible, of high affinity (dissociation constant, 1.7 × 10^{-9} M), and was inhibited by cholecystokinin analogs but not by unrelated hormones.*

Certain polypeptide hormones are present both in endocrine cells of the gastrointestinal tract and in cells of the nervous system (*1*). Cholecystokinin (CCK), originally isolated as a 33 amino acid polypeptide (CCK_{33}) from the small intestine, stimulates both pancreatic exocrine secretion and gallbladder contraction (*2*). The biological activity of this hormone is contained in the carboxyl terminal octapeptide (CCK_8) portion of the molecule. This smaller molecule is also present in the mucosa of the small intestine along with a larger variant, CCK_{39} (*3*, *4*).

Vanderhaeghen *et al.* (*5*) described a polypeptide in the brain that reacts with antibodies to gastrin, a 17 amino acid gastrointestinal hormone that stimulates acid secretion in the stomach. The terminal carboxyl pentapeptide sequences are identical in CCK and gastrin. Although CCK occurs naturally only in the sulfated form, gastrins occur in both sulfated and unsulfated forms. In subsequent studies the gastrinlike immunoreactivity in brains was found to be CCK_8 (*6*). Using antibodies that react to various segments of CCK, Larsson and Rehfeld (*7*) found that the central and peripheral nervous systems of the guinea pig contain molecular components of CCK having gel chromatographic elution coefficients corresponding to those of CCK_{33}, CCK_{12}, CCK_8, and CCK_4. Antiserums with carboxyl terminal specificity react with all these forms of CCK, and through the use of such antiserums immunoreactive material has been found in nerves, fibers, and cell bodies of the brain (*7*, *8*).

If CCK regulates brain functions, then brain cells should possess CCK receptors analogous to those for other polypeptides (*9*). However, due to difficulties in radioactively labeling CCK to high specific activity, brain receptors for the hormone have not been previously described. We have reported that the action of CCK on the exocrine pancreas is initiated by occupancy of a high-affinity CCK receptor (*10*). That study was made possible by a new preparation of radioactive iodine-labeled CCK of high specific activity obtained by the conjugation of ^{125}I-labeled Bolton-Hunter (BH) reagent to CCK_{33} (*11*). This ligand has the full biological activity of CCK as determined by assays of amylase release in isolated pancreatic acini of rats and mice (*11*). In the present study, we used this new ligand to characterize high-affinity CCK binding sites in particulate fractions prepared from rat brain.

When the particulate fraction of rat cerebral cortex (*12*) was incubated with 100 p*M* ^{125}I-labeled BH-CCK at 24°C, binding was half-maximal after 10 minutes and maximal after 60 minutes. A binding plateau was maintained for up to 180 minutes (Fig. 1A). When an excess of CCK_8 (10^{-6}*M*) was added with the label, binding of the tracer was reduced to 70 percent or less (nonspecific binding). Specific binding was reversible, since there was rapid displacement of labeled hormone when 10^{-6}*M* unlabeled CCK_8 was added after 60 minutes of incubation (Fig. 1A).

Unlabeled CCK_{33} competed with ^{125}I-labeled BH-CCK for these binding sites (Fig. 1B). Detectable inhibition of specific binding was seen at 0.3 n*M* CCK_{33}, half-maximal inhibition at 1.0 n*M* CCK_{33}, and maximal inhibition at > 100 n*M* CCK_{33}. Scatchard plots (*13*) of the CCK_{33} binding data were linear, a finding compatible with the presence of a single class of binding sites (Fig. 1C). In four brain-membrane preparations, CCK binding had a dissociation constant of 1.7 ± 0.7 n*M* and a binding capacity of 27.3 ± 5.4 fmole per milligram of protein. Other CCK analogs also inhibited the binding of ^{125}I-labeled BH-CCK. As estimated from the midpoint of parallel displacement curves, CCK_8 was three times more potent than CCK_{33}, desulfated human gastrin was half as potent as CCK_{33}, and desulfated CCK_8 and CCK_4 were one-fourth as potent as CCK_{33} (Fig. 1B). Unrelated peptides including insulin, secretin, pancreatic polypeptide, substance P, and β-endorphin all failed to inhibit binding of ^{125}I-labeled BH-CCK at a concentration of 10^{-7}*M*.

Like the CCK receptors in the brain, those in pancreatic acini have the highest affinity for CCK_8. However, the affinity of the pancreatic receptors is much reduced for analogs without a sulfated tyrosine in position 7 from the carboxyl terminus. Thus the affinity for CCK_8 in rat pancreatic acini is about 5000 times greater than that for either desulfated CCK_8 or gastrin, while the affinity for CCK_4 is at least ten times weaker than that for desulfated CCK (*10*). Accordingly, neither gastrin nor CCK_4 is a potent agonist in rat pancreatic acini. In contrast, the mammalian stomach responds to both gastrin and CCK. Receptors in rat stomach have the highest affinity for gastrin; CCK_{33} is one-half as potent (*14*). Also, in the stomach the

Table 1. Distribution of CCK receptors in rat brain and the regional content of CCK. Rat brains were dissected on ice and the specified regions separately homogenized. Specific binding was determined as described in the legend to Fig. 1, except only 50 p*M* ^{125}I-labeled BH-CCK was used. All values for binding are the mean ± standard error for three experiments. N.A., not assayed.

Brain region	Specific CCK binding (fmole/mg protein × 10^2)	CCK content* (pg/mg, wet weight)
Cerebral cortex	106 ± 13	550
Olfactory bulb	112 ± 3	185
Caudate nucleus	90 ± 13	N.A.
Hippocampus	52 ± 9	103
Hypothalamus	51 ± 15	179
Hindbrain	14 ± 6	45
Midbrain	9 ± 6	N.A.
Cerebellum	0	0

*Data are taken from Schneider *et al.* (*16*).

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