probable that the measurements of K^+ utilized in calculating the K^+/O_2 ratio represent primarily increments in the influx of K^+, because it is unlikely that efflux is significantly altered during the brief initial rate period. Protein concentrations varied over a range of 4 to 10 mg of protein per milliliter but had no discernible effect on the K^+/O_2 ratio.

The rapid initial phase of K^+ uptake is mediated by a K^+-induced stimulation of the ouabain-sensitive Na^+,K^+-ATPase. This is consistent with the observation that the medium K^+ concentration prior to injection is well below reported K_m values (Michaelis constant) for the ouabain-sensitive Na^+,K^+-ATPase in the presence of 150 m*M* sodium (*3*). Figure 1B illustrates the total inhibition of K^+ uptake induced by the prior addition of 60 μM ouabain. In fact, immediately after ouabain administration to K^+-depleted tubules there is a decrease in respiration and an efflux of K^+, indicating the inhibition of extant Na^+,K^+-ATPase activity by the glycoside.

The effect of 20 m*M* succinate supplementation on the K^+/O_2 ratio was examined because flavoprotein-linked substrates are expected to produce fewer ATP molecules per O_2 consumed. The average K^+/O_2 ratio obtained with succinate was 8.4 ± 0.6 (range 6.7 to 9.5, see Table 1). This value is consistent with the observation in isolated mitochondria that flavoprotein-linked respiration proceeds with two-thirds of the ATP-producing capacity of NADH-linked respiration. The possibility of a significant contribution from NADH-linked respiration was not precluded in these experiments since rotenone was not present. However, experiments with isolated mitochondria shown in Table 2 indicate that the efficiency of oxidative phosphorylation for succinate alone or in combination with NADH-linked substrates approximates that of succinate and rotenone.

The mitochondrial phosphorylation ratios presented in Table 2 demonstrate that the rabbit renal cortex possesses an orthodox phosphorylation system and that the effect of ouabain on the renal tubules is not the result of mitochondrial toxicity. The ratios were obtained by the polarographic technique (*2*) and values were calculated by using both the total oxygen excursion (ADP/O) and the incremental oxygen excursion (ADP/ΔO) observed during an ADP-induced period of state 3 respiration. This distinction is noteworthy (*1*, *6*) because the K^+/O_2 ratio calculated for K^+ transport in the intact tubules is based on incremental oxygen consumption. The phosphorylation ratios obtained are in excellent agreement with those reported by Hinkle and Yu (*1*) for NADH-linked (glutamate and malate) and succinate-supported respiration in rat liver mitochondria. Earlier observations (*7*, *8*) indicating that ouabain has no direct effect on the rate of mitochondrial respiration were also confirmed (not shown).

The K^+/O_2 ratio of 11.8 ± 0.2 obtained in this study indicates that an extremely efficient coupling exists between Na^+,K^+-ATPase-mediated ion transport ($K^+/ATP = 2$) and NADH-linked oxidative phosphorylation ($ATP/O_2 = 6$) within the intact cell. This value is appreciably higher than the K^+/O_2 ratio of 4 obtained by Whittam and Willis (*5*) for kidney cortex slices and reflects both the rapid kinetic capability and superior oxygenation characteristics (*9*) of our experimental preparation. A review of the literature indicates that K^+/ATP ratios determined in three different red cell preparations (*10*–*12*) yielded values somewhat higher than 2 (2.2 to 2.4), whereas the results of experiments in a reconstituted renal ATPase preparation (*13*) lacked sufficient resolution to discriminate between integral and fractional values. In view of some uncertainty within the literature regarding the precise evaluation of K^+/ATP and ADP/O coupling ratios, we propose that the 11.8 ± 0.2 value be not strictly viewed as the product of 2 and 6; rather it may be indicative of numbers 10 to 20 percent greater than 2 and 10 to 20 percent less than 6, respectively. We conclude that the excellent correlation between phosphorylation ratios and K^+/O_2 ratios for both NADH-linked and succinate-supplemented respiration indicates that in vitro mitochondrial studies are indeed valid indices of energy metabolism within the intact cell.

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Intercellular Adhesion: Coconstruction of Contractile Heart Tissue by Cells of Different Species

Abstract. *Dissociated embryonic rat myocardial cells and chick myocardial cells labeled with radioactive isotope coaggregate and establish intercellular junctions. These bispecific cells reconstruct synchronously beating myocardial tissue within 24 hours of culture.*

By conducting experiments with dissociated embryonic cells and observing the subsequent aggregation of the cells and tissue reconstruction, biologists have been able to learn something about the mechanism of cell movement and organization that leads to the differentiation of tissues in embryos. The dissociated cells from two or more different tissues of the same species segregate according to tissue type (*1*, *2*). This cellular process is widely regarded as a form of histotypic self-recognition. One way to understand histotypic recognition is to examine its operation among different animal species. Moscona and his collaborators (*3*, *4*) studied aggregates containing mouse and chick embryonic cells derived from the same type of tissue. They used embryonic limb precartilage, liver, skin, neural retina, and kidney and reported that the mouse and chick cells of the same type did not sort out according to species. From these studies it was hypothesized that, for any one embryonic cell type, the properties responsible for cell sorting were indistinguishable among even very distantly related warm-blooded vertebrate species. Later, Burdick and Steinberg (*5*), working with the embryonic heart cells from mouse and chick at the light microscopic level, reported that mouse and chick myocardial cells segregated

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according to species. From this study it was hypothesized that species differences controlled the selective intercellular adhesion or sorting-out phenomenon in the animal species. Thus, the intercellular adhesion phenomena between dissociated embryonic cells derived from the same tissue but different species remain unresolved.

In the present study, we observed by electron microscopic autoradiography that embryonic rat and chicken myocardial cells coaggregate and form intercellular junctions of the type present in intact vertebrate myocardial tissue. We used 13-day embryonic rat ventricles and 3-day embryonic chick ventricles for this study. The chick cells were labeled with tritiated thymidine by the administration of 12 μCi of tritiated thymidine through an opening in the shell, at 48, 72, and 98 hours of incubation. Each group of ventricles was fragmented and incubated in 0.25 percent trypsin in calcium- and magnesium-free Tyrode's solution at 37°C for 45 minutes. After incubation, the fragmented tissues were rinsed in a culture medium containing 88 percent Eagle's basal medium, 10 percent fetal calf serum, 1 percent glutamine solution, and 1 percent penicillin-streptomycin mixture. The tissues were then transferred to the fresh medium and dispersed into a suspension of single cells by pipetting.

Two sets of experiments were performed, differing in the ratio of intermixed cells. The experiments were repeated several times. In one set, the rat and chick cells were mixed together in a 1:1 ratio (chick:rat) at total cell concentrations of 1.0×10^6 cells per milliliter of medium. In another set, heart cells from the two species were mixed in a 4:1 ratio (chick:rat) at 1.0×10^6 cells per milliliter of medium. The cells were allowed to aggregate in 25-ml Erlenmeyer flasks containing 3 ml of medium on a gyratory water bath shaker at 70 rev/min at 37°C (*8*, *9*). After the cultures were initiated, the cell aggregates were examined microscopically for evidence of contractility and were subsequently processed at selected intervals between 1 and 72 hours for scanning electron microscopy and transmission electron microscopic autoradiography.

The rat and chick hearts used in this study appeared comparable in their stages of development and cellular composition (*6*, *7*). Muscle cells were the most numerous cell type in both kinds of heart. With the exception of mature blood cells and some fibroblastic cells, all chick heart cells, including muscle cells, were labeled with radioactive isotope. As the culture continued, the aggregates increased in size by continuous addition of cells and joining of small clusters. The cells within these mixed aggregates exhibited synchronous contractility from 1 hour after initiation of the culture to the terminal time point of the culture. Most of the aggregation in both control and mixed aggregates was completed within a 24-hour period. The control aggregates consisted of cells from a single species, either chick or rat. Close examination of the control chick aggregates revealed that the labeled chick cardiac muscle cells closely adhered with one another after 1 to 72 hours of culture (Fig. 1A). Close membrane contacts and desmosomes were observed between these cells. Similar results were obtained in previous experiments with rat cell aggregates (*8*, 9).

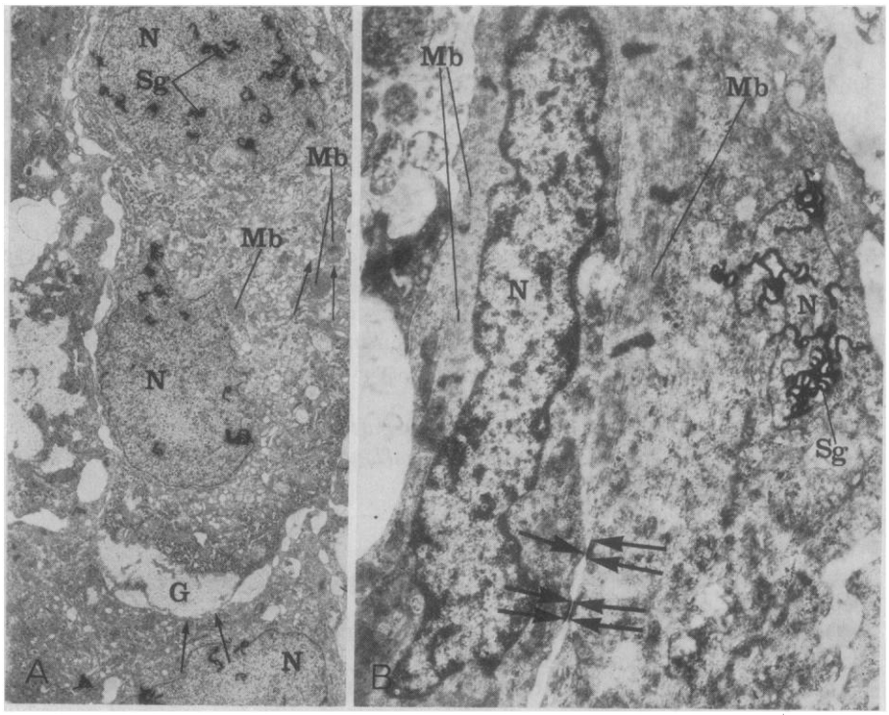

Fig. 1. (A) Autoradiograph of part of a 24-hour control aggregate of cardiac muscle cells from a chick. The labeled cells are shown closely adhered with some of them having intercellular contacts (arrows) (×5760). (B) Autoradiograph of part of a 24-hour mixed aggregate of chick and rat cardiac muscle cells (1:1, chick:rat cells). Well-developed desmosomes are visible (arrows) between the labeled chick cardiac muscle cell (right) and the nonlabeled rat cardiac muscle cell (left) (×12,000). Symbols: *G*, gylcogen particles; *Mb*, myofibril; *N*, nucleus; *Sg*, silver grains.

In the present experiments with the mixed aggregates containing 1:1 mixed cell ratios, most of the cardiac muscle cells of the two species were interspersed randomly throughout the aggregates after 1 to 72 hours of culture. In the early to late stages of aggregation approximately three-fourths of the labeled chick cardiac muscle cells came in close apposition with the nonlabeled rat cardiac muscle cells and established close intercellular contact (Fig. 1B). Distinct desmosomes were formed between the labeled chick cardiac muscle cells and the nonlabeled rat cardiac muscle cells (Fig. 1B). The dimensions of the intercellular spaces at the cell adhesion regions ranged from 20 to 100 Å. The intercellular spaces in some close membrane contact regions were not discernible. These observations did not conform with the concept of Burdick and Steinberg (*5*), who reported on the basis of their light microscopic studies that the embryonic mouse and chick myocardial cells underwent segregation according to species. Our findings demonstrate that the embryonic rat and chick cardiac muscle cells coaggregate and form a joint bispecific myocardial tissue that is capable of beating synchronously.

In aggregates containing chick and rat cells in a ratio of 4:1 we observed, as expected, numerous labeled chick cells along with scanty nonlabeled rat cells. Although the labeled chick muscle cells were in close proximity because of their large numbers, the scattered nonlabeled rat muscle cells also established intercellular contacts with the labeled chick muscle cells through membrane appositions as well as through the formation of desmosomes and hemidesmosomes. These findings demonstrate that the embryonic rat and chick cardiac muscle cells establish intercellular junctions between them, irrespective of cell num-

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.

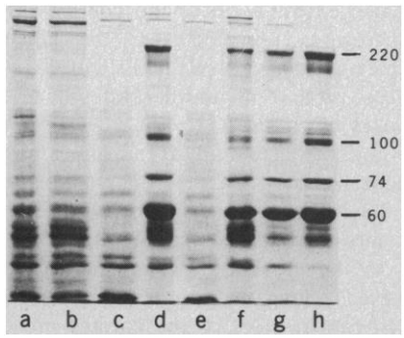

Fig. 1. Purification of squid brain IF's. (a) Whole brains homogenized in disassembly buffer. (b) First 250,000*g* pellet. (c) First 250,000*g* supernatant. (d) First 150,000*g* (first repolymerized) IF pellet. (e) First 150,000*g* supernatant. (f) Second 250,000*g* pellet. (g) Second 250,000*g* supernatant. (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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