cause an increase in the adrenaline contents of various tissues. However, the adrenaline levels were decreased consistently by insulin in animals from which the suprarenal medullae previously had been removed. These results provide strong support for the hypothesis that the increased amounts found in intact animals represent catecholamine released from the suprarenal medullae in response to the insulin-induced hypoglycemia and subsequently accumulated from the blood stream in the tissues studied.

The mechanism of action of insulin in reducing the adrenaline content of organs of suprarenal-demedullated rats is not entirely clear. However, the observation that ganglionic blockade prevents the effects of insulin suggests that these are mediated *via* the autonomic nervous system. This does not imply that all tissue storage sites for adrenaline are directly under the control of the autonomic nervous system. It is possible that a part of the effect of insulin on the adrenaline contents of tissues of suprarenal-demedullated animals is due to a flooding of the organism with noradrenaline which might displace some of the stored adrenaline. We previously have shown that injection of noradrenaline can decrease the adrenaline content of the organs studied(4).

The results presented above demonstrate that a catecholamine released from one site can be transported to and accumulated in other organs and tissues. This observation requires that events in other areas of the body, particularly in the adrenal medullae, as well as local synthesis, storage and release, be considered in interpreting the results of tissue analyses for adrenaline and noradrenaline.

Summary. Injection of insulin into intact rats increased the adrenaline contents of hearts and salivary glands. However, it decreased the adrenaline content of these tissues in animals from which the suprarenal medullae had been removed. The noradrenaline content of the heart was decreased after 7 to 11 hours of insulin action, but that of the salivary glands was unaltered. It is concluded that the increased amounts of adrenaline found in tissues of intact animals are due to the release of this catecholamine from the suprarenal medullae and its subsequent transport to and accumulation in the organs studied.

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Immunohistochemical Identification of Tissue Culture Cells. (26935)

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A major problem in tissue culture is the identification of the cells being cultured. This is important both in connection with the various cell lines now in existence and also in analysis of new cell lines derived from fresh tissue. In the case of existing cell lines there have been accidents in which cell lines have been contaminated by other propagated cultures(1,2,3,4), and passed on to other laboratories. These contaminations have been identified by chromosome morphology, immunological technics, and viral infectivity studies.

In the case of cells growing from a primary culture, where most of the cells appear to be fibroblasts, it is important to determine whether cells unique for the tissue in question are also in culture. We have used cultured muscle cells as a model system and have

found it possible to show the presence of myosin-containing cells among outgrowth of fibroblasts from a muscle explant.

Methods. Preparation of antisera: Rabbit antisera showing reactivity for human connective tissue (C.T.)(5) and rabbit anti-human myosin antisera(6) have been described. The rabbit antiserum showing reactivity with mouse connective tissue (C.T.) was prepared by injecting rabbits with the microsome fraction of a C3H mouse sarcoma in Freund's adjuvant. Microsomes were also given 3 times a week for 3 weeks by the intravenous route. The microsome fraction was prepared by the procedure outlined by Carruthers(7). Fluorescent antibody against rabbit γ globulin was prepared by conjugating fluorescein isocyanate to globulin fraction of horse anti-rabbit globulin(8). The absorption procedures with human, rat, and mouse liver sediments(9) and staining by the indirect method(10) have all been described.

Paired label staining procedure. Human C.T. reactive antiserum (globulin fraction) was labeled with tetramethylrhodamine isothiocyanate(11,12) and absorbed with mouse liver before use. The globulin fraction of C.T. reactive antiserum was labelled with fluorescein isothiocyanate(12) and absorbed with human liver before use. Tissue cultured cells fixed as described below were stained with one conjugate for 1 hr, washed 10 min. and retreated with the second conjugate for 1 hr and again washed 10 min. before observation. Since both conjugates react independently of each other, the order of addition can be reversed with little difference in results. The conjugates were not used combined, since mixing results in an additional dilution of each antibody activity. It was preferable to stain first with the fluorescein labeled antibody and subsequently with the tetramethylrhodamine labeled antibody.

Method of growing cells in tissue culture. Normal muscle cells from fetal and adult skeletal muscle of humans were explanted on sterile coverslips and overlaid with Eagle's media, containing calf serum. After 2 weeks, cultures showing good growth and containing myo-tubes by gross morphology were taken for study. The cover slips were washed in saline to remove excess proteins, fixed in ice cold acetone for 10 min., washed in distilled water and rinsed in saline before treatment with the appropriate antiserum.

Other human cells* grown from a cancer of the gall bladder, a malignant mesothelioma, and a cancer of the ovary (2 lines) were used. Mouse tissue culture cells were also used; these were 2 L lines of fibroblast grown in continuous culture (L929 and L4CZ)* and C3H muscle primary culture† explants also containing outgrowths of fibroblasts. A Gale melanoma hamster* line and a rabbit fibroblast line was also employed as control.

Staining procedure. Frozen sections of all human and mouse tissues were processed by the indirect staining procedure(5).

The tissue cultures were fixed in cold acetone and washed in saline prior to staining as described above(10).

Chromosome morphology. Since human and mouse lines can be easily distinguished by chromosome morphology, proof of the species of cells being employed with the anti-species antiserum was checked by observing the chromosomes. Explants were grown for 3-4 days and to the culture 2 γ colchicine was added per ml of media and the mixture was allowed to stand for 3.5 hrs at 37°C. The cultures were washed with Hanks' solution and treated with 25% Hanks' in distilled water for 5 minutes at 37°C. The cultures were then fixed in 60-25 methyl alcohol-glacial acetic acid, dried and stained with aceto-orcein before observation(13). Five cultures of each mouse and human were used for every chromosome morphology study.

Results. Staining properties of human and mouse connective tissue reactive antisera. The pattern of staining of rabbit antiserum reactive with connective tissue has been previously reported. This antiserum reacts with basement membrane of the human kidney tubules as well as with the glomeruli. It reacts with the central vein and sinusoids of liver and with the endomysium of skeletal muscle (Fig. 1)(6,7). It also reacts well

* These cultures were kindly made available to us by Dr. G. Moore.

† These cultures were made available to us by Dr. J. Cairns.

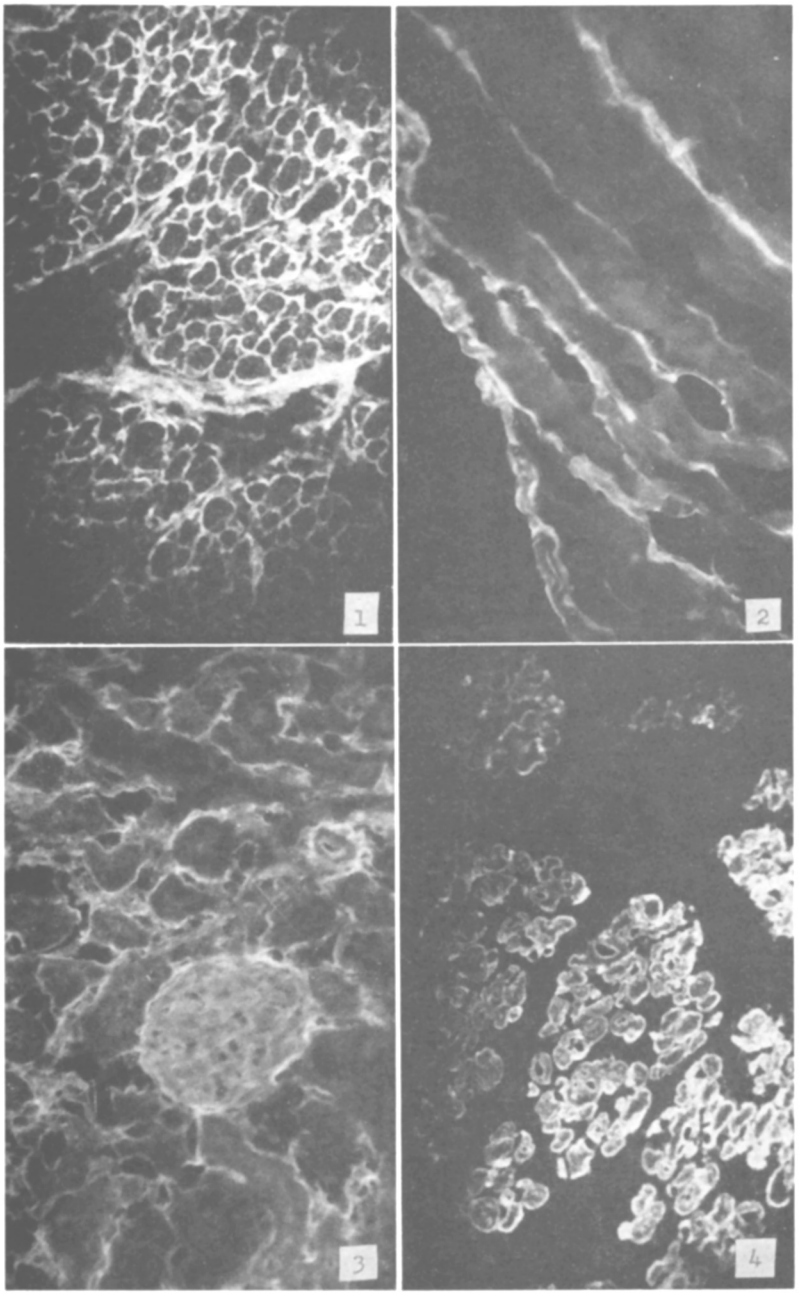

FIG. 1. Normal human skeletal muscle cross-section treated with human connective tissue reactive rabbit antiserum (absorbed with mouse liver) and subsequently stained with fluorescein labeled horse anti-rabbit globulin reagent. Staining is confined to reticular connective tissue (endomysium). No staining seen in muscle.

FIG. 2. Normal mouse skeletal muscle longitudinal section treated with rabbit anti-mouse connective tissue reactive serum (absorbed with human liver) and stained as above. Staining confined to endomysium and no staining seen in skeletal muscle.

FIG. 3. Normal mouse kidney treated as above. Staining confined to reticular connective tissue elements (basement membrane) of tubule and glomeruli.

FIG. 4. Normal human skeletal muscle cross section treated with rabbit anti-human myosin serum (adsorbed with human liver) and subsequently stained with fluorescein labeled reagent. Staining is confined to muscle elements alone. No staining seen of stroma.

TABLE I. Staining of Various Tissues (Human, Mouse, Hamster) by Several Antisera.

Antisera	Human tissue: Organ section: Normal skeletal muscle	Human tissue: Organ section: Normal liver	Human tissue: Organ section: Normal kidney	Human tissue: Tissue culture: Cancer of gall bladder	Human tissue: Tissue culture: Cancer of ovary	Human tissue: Tissue culture: Mesothelioma	Human tissue: Tissue culture: Primary normal skeletal muscle	Mouse tissue: Organ section: Normal skeletal muscle	Mouse tissue: Organ section: Normal liver	Mouse tissue: Organ section: Normal kidney	Mouse tissue: Tissue culture: 1929	Mouse tissue: Tissue culture: L40Z	Mouse tissue: Tissue culture: Primary C3H fibroblast	Mouse tissue: Tissue culture: Primary normal skeletal muscle	Hamster tissue: Tissue culture: Melanoma
Anti-human C.T.															
Absorbed with mouse liver	C.T. only	C.T. only	C.T. only	+	+	+	+	—	—	—	—	—	±	—	—
Absorbed with rat liver	C.T. only	C.T. only	C.T. only	+	+	+	+	±	—	—	—	—	—	—	—
Anti-mouse C.T.															
Absorbed with mouse liver	—	—	—	—	—	—	—	—	—	—	±	±	—	—	—
Absorbed with human liver	—	—	—	±	±	±	±	C.T. only	C.T. only	C.T. only	+	+	+	(+)	—
Anti-human myosin															
Absorbed with human liver	+*	—	—	—	—	—	+*	+*	—	—	—	—	—	+*	—
Absorbed with mouse liver†	+	C.T. only	C.T. only	+	+	+	+	+*	—	—	—	—	±	+*	—
Chromosome morphology				human	human	human	human				mouse	mouse	mouse	mouse	

* Only muscle cells staining.

† Contains also anti-human connective tissue reactive activity that was not removed by mouse liver absorption.

—, no staining; ±, slight staining, considered essentially negative; +, staining of cellular elements.

with tissue cultured cells of human origin as determined by either fluorescent antibody or cytotoxicity(5). The human connective tissue reactive serum, when absorbed either with mouse liver or rat liver, did not react with mouse, rabbit, or hamster cells in tissue culture but did so with all the human organ section or cell lines employed (Table I). When the antiserum was absorbed with human liver sediment, all reactivity with the human tissue section and tissue culture cells was removed, as shown by lack of staining of the various tissues described above.

Rabbit antiserum prepared against mouse sarcoma reacted similarly with mouse connective tissue. The antiserum stained normal mouse organs (Fig. 2, 3) in the same area as that described for the stain of human organs by the human connective tissue reactive serum. This antiserum absorbed with human liver sediments was still capable of reacting with the mouse connective tissue antigens in organ sections as well as in tissue culture (Table I). It did not react with human, hamster, or rabbit tissue culture cells. Absorption with mouse liver sediments removed the activity quite readily.

Staining properties of rabbit anti-human myosin serum. This antiserum has been shown to react with human muscle cells (Fig. 4), and also with human cells in tissue culture which contain muscle protein (Fig. 5). The antiserum after absorption with human liver sediments did not react with other human cell lines or mouse, hamster, or rabbit lines. Staining of human cells was not confined to cells that had the characteristic morphology of muscle cells in culture, but also included cells that could easily have been mistaken for fibroblasts morphologically. Cells in which longitudinal striations (myofibers) could be seen extending the length of the cell and over the nucleus were obviously derived from muscle and were strained by the antibody (Fig. 6).

None of the tissue cultured muscle cells that were stained showed the characteristic cross-striations seen in sections of adult skeletal muscle. However these cells must have contained muscle protein because they reacted with the anti-myosin antibody. Many cells in the slide contained no detectable muscle protein since they were unstained, and were presumably connective tissue cells.

It is of interest to note that not all the cells showing the appearance of the muscle cells stained to the same extent. Some were stained more weakly than others indicating different levels of myosin or muscle protein content. This difference in staining cannot be attributed to the quality of the fluorescent label, for adjacent to these weakly staining cells were found similar appearing typical muscle cells that were stained brilliantly. This may be due to variations in the amount of muscle protein being produced by individual cells in culture and it is possible that even some of the unstained cells may be derived from muscle.

A point of interest is that the anti-human myosin serum can be used to differentiate muscle cells from non-muscle cells for other species than human due to the cross-reactions between myosins of various species. Thus the antiserum after absorption with mouse liver or human liver sediment, was still capable of combining with mouse skeletal muscle and also tissue cultured mouse muscle cells (Fig. 7, 8). This observation is probably valid for other animal muscle cultures because of the cross-reactions(14). The mouse muscle cells treated with the anti-myosin serum gave essentially the same results as that for human muscle; however, the staining was weaker.

Application of the paired label technic. By employing the paired label technic in which one antibody globulin is labeled with tetramethylrhodamine isothiocyanate and the other with fluorescein isothiocyanate, the reactions of anti-mouse and anti-human antibodies can be followed on one slide culture. It can be shown that after suitable absorption each antibody reacts with the cells of the homologous species independently of the other and no cross-inhibition occurs. When human cultures, purposely contaminated with a few mouse cells (24 hr cultures), were tested by the paired label procedure, it was easy to detect the presence of even one mouse cell in the field (Fig. 9). The anti-mouse antibody labeled with fluorescein stained the

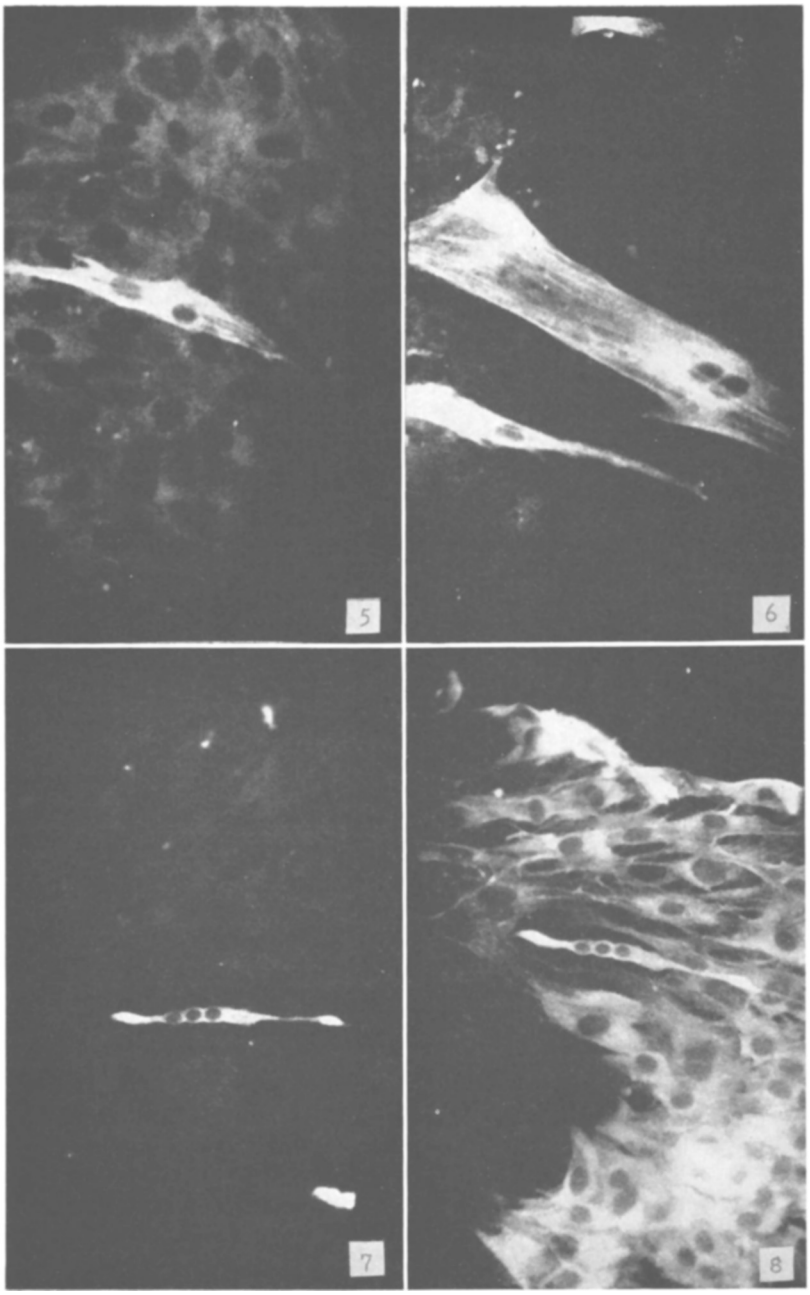

FIG. 5. Human muscle (primary explant) tissue culture treated as above. Staining confined to a single muscle cell in field. Masses of fibroblasts in background are completely negative.

FIG. 6. Human muscle (primary explant) tissue culture treated as above. Staining confined to 2 cells varying in shape and number of nuclei. Longitudinal striations can be seen fluorescing, no cross striations visible. Fibroblasts were unstained.

FIG. 7. Mouse muscle (primary explant) tissue culture treated with rabbit anti-human myosin serum (absorbed with mouse liver) and stained with fluorescein labeled reagent. A single mouse cell containing 3 nuclei is stained.

FIG. 8. Same section and field as above treated with rabbit anti-mouse connective tissue reactive serum (absorbed with human liver) and stained with fluorescein labeled antibody. Large numbers of mouse fibroblasts surrounding the single muscle cell in the field can be seen staining strongly with mouse connective tissue reactive serum.

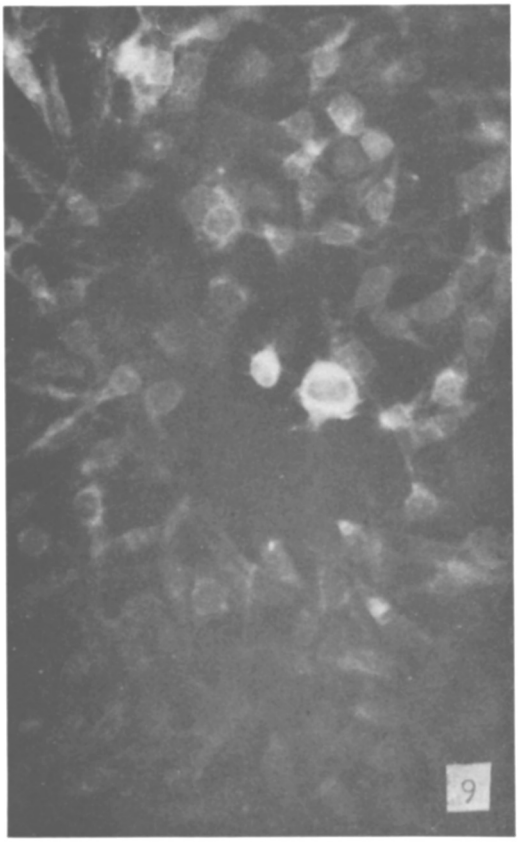

FIG. 9. Human tissue culture cells (cancer of gall bladder) purposely contaminated with a few L929 cells of mouse origin. The section was treated with fluorescein labeled anti-mouse connective tissue reactive antibody (absorbed with human liver) and subsequently with tetramethylrhodamine labeled anti-human connective tissue reactive antibody (absorbed with mouse liver). The paired labeled procedure showed 2 mouse cells which fluoresced brightly green while all background human cells fluoresced orange.

mouse cells green, whereas the anti-human antibody labeled with tetramethylrhodamine stained the human cells orange.

Summary. Cell lines of different species have been shown to be differentiated by immunohistochemical technics using properly absorbed antisera. In the work reported here the antisera were prepared against human and mouse tissues and reacted strongly with the connective tissue of the corresponding species in sections of adult human or adult mouse organs, respectively. These antisera also reacted individually with tissue culture cells of human and mouse organs. In all cases the use of the proper tissue in sufficient amounts to absorb the antiserums plays a crucial part in being able to demonstrate the specific staining of cells. Differentiation can be made even within the cell lines of a single species when unique components are present in one of them. The use of anti-myosin serum to detect muscle cells is an example. The technic of using paired labeled antiserum quickly and effectively demonstrates that very slight contamination can be recognized. Such a small contamination would be difficult to detect by other tests such as chromosome morphology technics where the cell must be in the metaphase before it can be observed and chromosomes of each cell counted.

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