TRANSFER OF POTASSIUM

A New Measure of Cell-Cell Coupling

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ABSTRACT

Mammalian cells of different species differ in sensitivity to ouabain. This sensitivity is expressed as reduced intracellular K^+ content, reduced rates of protein synthesis, and cessation of cell multiplication. Using $^{86}Rb^+$ as a measure of intracellular K^+, we found higher levels of radioactivity in mixtures of ouabain-sensitive and -resistant cells cultured in the presence of ouabain than predicted from pure cultures of the two component cell types. The simplest explanation is that K^+ and $^{86}Rb^+$ are being transferred from ouabain-resistant to ouabain-sensitive cells, enhancing the total intracellular $^{86}Rb^+$ in the culture. A function, "index of cooperation," expresses this enhancement as a number ranging from 0 to 1, and permits comparisons to be made under various culture conditions and using various cell types. An index of cooperation >0 requires cell contact, since no enhancement occurs when contact between two cell types in the same culture is prevented. The index of cooperation for a number of different cell combinations agrees with other measures of cell-cell interaction associated with gap junctions, such as electrical coupling and metabolic cooperation.

Coculture of ouabain-sensitive and ouabain-resistant cells in the presence of ouabain also leads to restoration of the capacity for protein synthesis. Autoradiography shows that this restoration occurs in the sensitive cell type and is dependent upon contact with ouabain-resistant cells. Furthermore, sensitive cells are able to multiply in the presence of ouabain when cocultured with resistant cells. Thus K^+, presumably transferred to sensitive cells through gap junctions, is able to counteract the toxic effects of ouabain on intracellular K^+ levels and protein synthesis, and to restore growth.

KEY WORDS cell-cell communication · gap junctions · potassium · protein synthesis · cultured cells

It is well-established that K^+ is an essential requirement for protein synthesis and growth of cells, both procaryotic and eucaryotic (13, 26, 30, 31). We have reported that, in cultured human fibroblasts, reduction of intracellular K^+ content limits the rate of protein synthesis and cell growth (26). The possibility that this mechanism is involved in growth control led us to investigate the extent to which cells could influence one another's K^+ content.

Most cultured fibroblastic cells and many epithelioid cells can establish specialized junctional contact with other cells of diverse derivation. The junctional membranes permit the transmission of

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inorganic ions and other low molecular weight molecules from cell to cell at a rate similar to that of diffusion through the cytoplasm itself, and much faster than that through nonjunctional membranes (15, 27, 28). Such junctions between cells permit low-resistance coupling that may be detected with intracellular microelectrodes. These junctions also appear to be the basis of the phenomenon known as metabolic cooperation (4, 43) or contact feeding (7), in which a metabolic deficiency in cells of one type may be overcome by mixing them with normal cells under conditions where they may establish contact.

The structure responsible for cell-cell communication is probably the gap junction, a specific array of membrane components of the two apposed cell surfaces. Gap junctions are found in most cases where electrical coupling or metabolic cooperation has been described, and have not been demonstrated in cases where no such functional contact is seen (16, 22, 25, 32, 33, 37).

We present here the results of experiments on mixtures of cells that differ in the relative resistance of their Na^+-K^+ ATPase to inhibition by ouabain, and that thus can be made to differ markedly in K^+ content in the presence of a selected concentration of ouabain. For example, rodent cells require 10^4 times as much ouabain for inhibition as cells of human origin (3). We wished to see whether the cells containing high K^+ could transmit sufficient K^+ to restore protein synthesis and cell division in depleted recipient cells. In carrying out these experiments, we developed a method to express the degree of functional coupling between two cell types, which we call "index of cooperation." Our method has certain advantages for the quantitative determination of cell-cell coupling.

MATERIALS AND METHODS

Cell Cultures

Cells were cultured in Dulbecco's modified Eagle medium (Grand Island Biological Co. [GIBCO], Grand Island, N. Y.; H-16) supplemented with 10% fetal calf serum (Reheis Chemical Co., Kankakee, Ill.) and antibiotics (GIBCO). Cultures were checked for mycoplasma contamination by the method of Schneider et al. (38), which compares the incorporation of uracil and uridine. The designation and sources of cells used are as follows:

(*a*) *Human cells:* HF strains were derived from newborn human foreskin by O. R. McIntyre (HF-1) (26) and by C. E. Brinckerhoff (HF-2). L-N, derived from patients with the Lesch-Nyhan syndrome, are fibroblasts that lack the enzyme HPRT; they were supplied by R. Erbe. SV/HF are fibroblasts transformed by SV40 virus: the SV/HF-3 line was obtained from R. Hoffman and SV/HF-4 from J. Epstein. All of these human strains showed half-maximal depression of K^+ content at ~0.03 μM ouabain.

HeLa strain S3 (ATCC no. CCL 2.2) was given to us in suspension form by G. Michaels. It was subsequently grown under conditions permitting substrate attachment. The ouabain resistance of HeLa cells is similar to that of normal human fibroblasts.

(*b*) *Hamster cells:* CHO cells were originally obtained from ATCC (no. CCL 61) and were subsequently mutagenized and selected for resistance to 2.0 mM ouabain in our laboratory. BHK cells were given to us by J. Littlefield. 1 μM ouabain had no effect on their intracellular K^+ content, and we found about a 50% inhibition of intracellular K^+ at 0.1 mM ouabain,

(*c*) *Mouse cells:* 3T3 are Balb/3T3 cells of the line originally developed by Aaronson and Todaro (1). We obtained the cells from S. Aaronson (3T3-2) and from ATCC (no. CCL 163) (3T3-1). Half-maximum K^+ content was seen at ~0.3 mM ouabain. THO2 is a ouabain-resistant mutant isolated by Jha and Ozer (21) from NTG2, a thioguanine-resistant mutant of Balb-3T3 clone A31, after mutagenesis and selection in 3 mM ouabain. That level of ouabain had no effect on intracellular K^+. L cells are of the 1R line obtained from W. Bodmer and recloned; they are resistant to 8-azaguanine.

(*d*) *Terato Cl-25* is a clonal derivative which we isolated from the transplantable teratocarcinoma line OTT-6050 (41) supplied as an ascites tumor by L. C. Stevens. In culture the cells are large, flat, and grow to a uniform monolayer. Injection of 5×10^5 cultured cells into 129/SV male mice failed to produce tumors; this dose is at least two orders of magnitude larger than the tumorigenic dose of the malignant embryonal carcinoma stem cell of this tumor. The K^+ content of terato Cl-25 is unaffected by 1 μM ouabain, and we assume that it has the typical high ouabain resistance characteristic of other rodent cells.

Determination of $^{86}Rb^+$ Content

$^{86}Rb^+$ appears to be a valid tracer for cell K^+ in animal cells (26), and we have referred to the two ions interchangeably in this paper. Replicate cultures in 35-mm plastic tissue culture dishes were incubated 20–24 h in medium containing $^{86}Rb^+$ (0.01–0.04 μCi/ml; 0.8–3.5 mCi/mol K^+). At the end of the incubation, the cultures were rinsed rapidly three times with 2 ml of ice-cold phosphate-buffered saline (PBS) and extracted with 0.5 to 1.0 ml of cold 5% TCA. A 100- to 400-μl portion of this extract, containing the intracellular $^{86}Rb^+$ and K^+, was dissolved in PCS scintillation fluid (Amersham Corp., Arlington Heights, Ill.) and counted in a Beckman LS-335 liquid scintillation counter (Beckman In-

struments, Inc., Fullerton, Calif.). Values for a typical experiment are given in Table I.

The TCA-insoluble material remaining was rinsed with water and dissolved in 0.5 ml of 0.2 N NaOH with 0.3% sarkosyl NL30 (ICN K & K Laboratories Inc., Plainview, N. Y.). Protein determinations were performed on 100-μl portions of this solution with bovine serum albumin as a standard (29), and in experiments

TABLE I

Calculation of Index of Cooperation

A. Calculation of $^{86}Rb^+$ content per μg protein

	$^{86}Rb^+$ (*cpm/dish*)	Protein (μg/*dish*)	(*cpm*/μg)
HF (human)			
+Ouabain	1,596	55	29.0
	1,214	52	23.4
	1,152	47	24.5
−Ouabain	23,008	61	377
	21,684	57	380
	19,774	52	380
3T3 (mouse)			
+Ouabain	18,365	64	287
	17,657	64	276
	16,371	55	298
−Ouabain	19,432	65	299
	18,030	70	258
	17,676	69	256
Mixture (75% human + 25% mouse)			
+Ouabain	12,712	67	190
	12,600	63	200
	10,650	57	187
−Ouabain	22,045	65	339
	22,144	62	357
	20,943	59	355

B. Statistical parameters for each group of replicates (mean cpm $^{86}Rb^+/\mu$g protein ± SEM at 12 degrees of freedom)

	Ouabain	No ouabain
HF	25.4 ± 2.2 (*A*)	379 ± 8.4
3T3	287 ± 7.3 (*B*)	270 ± 7.1
Mixture	192 ± 6.0 (*C*)	350 ± 8.1 (*D*)

C. Calculation of index of cooperation

Expected if no cell-cell-interaction

	Ouabain	No ouabain
r(HF) + (1 − r) (3T3) (r = 0.75)	91	352

$$\text{Index of cooperation} = \frac{\text{observed} - \text{expected}}{\text{maximum} - \text{expected}} = \frac{C - (rA + (1-r)B)}{D - (rA + (1-r)B)} = \frac{192 - 91}{350 - 91}$$

$$= \frac{C - B - r(A - B)}{D - B - r(A - B)}$$

$$= 0.39 \pm 0.028$$

TABLE I—*continued*

D. Estimate of reproducibility of index of cooperation	
Replicate measurements of index of cooperation	0.40, 0.39, 0.54, 0.42, 0.69, 0.32, 0.48, 0.45, 0.49, 0.55, 0.42, 0.39
Mean: 0.46	
SD: 0.10	
Coefficient of variation: 22%	

Human fibroblasts and mouse 3T3 cells were mixed in the proportion 3 to 1 ($r = 0.75$) and inoculated into 35-mm culture dishes, 10^5 cells per dish. Parallel cultures containing only HF cells or only 3T3 cells were also set up at the same cell density. All dishes received $^{86}Rb^+$. The next day ouabain was added to half the dishes in each set, and 6 h later the experiment was terminated as described in Materials and Methods. Since ouabain was present for only this short period, and since ouabain does not have its full effect on $^{86}Rb^+$ content for several hours, no significant differential effect of ouabain on the proportion of cells was expected (see first time point of Fig. 4). In part A the observed values for $^{86}Rb^+$ cpm and μg protein determined on each dish are given as well as the resulting cpm/μg. In part B the results of the analysis of variance are given in the form of the mean for each group of observations ±SE (12 degrees of freedom). In part C the equation for index of cooperation is derived, using four of the elements of the table. The two remaining elements, the values for the pure cultures without ouabain, are not used except to provide an internal control on the effect of the ouabain treatment. Standard error of index of cooperation was calculated using error propagation from the standard errors of the component measurements (6). In part D indices of cooperation measured twelve times over a one year period under the conditions described above are given, together with the mean and SD.

where [^{3}H]leucine was included in the incubation, other portions were neutralized with acetic acid and dissolved in PCS for determination of [^{3}H]leucine incorporation by scintillation counting. Data used for calculations are expressed as $^{86}Rb^+$ content/μg protein or [^{3}H]leucine incorporation/μg protein.

Autoradiography

The general procedures described by Appleton (2) were used. Cultures were grown on sterile 10.5 × 22 mm cover slips in 35-mm tissue culture dishes. After incubation in [^{3}H]leucine or [^{3}H]proline (10 μCi/ml), cultures were rinsed three times with ice-cold PBS, and the cover slips were transferred to absolute methanol for fixation. They were transferred successively to fresh portions of methanol (5 min), methanol (2 h to overnight), 5% TCA (15 min to 1 h), and quickly rinsed in distilled water before drying in air. Cover slips were mounted on microscope slides two at a time (treated and corresponding control); duplicate cover slips were kept on separate slides. After an overnight rinse in running tap water, the slides were covered with Kodak AR10 stripping film and exposed at room temperature 4–8 days. The film was developed with Microdol X and the cells were stained through the emulsion with Giemsa.

Cell Marking and Growth Curves

Cells were marked by the methods of Stoker (42) and Krondahl (23): incubation overnight in a 2% suspension of carmine (Alum Lake, National Aniline Corp.) or polystyrene latex beads, 0.982 μm diameter (Polysciences, Inc., Warrington, Pa.) in Dulbecco's medium. Excess dye or beads was then removed by repeated rinses with Puck's saline A, and cells were trypsinized, centrifuged, and resuspended several times in fresh medium, and finally replated in tissue culture dishes. Cells thus treated were indistinguishable from untreated controls in ability to attach to the substrate, gross morphology, growth rate, $^{86}Rb^+$ content, and rate of incorporation of [^{3}H]leucine (data not shown). Carmine or latex particles in the cytoplasm were readily visible in >90% of the cells, particularly in the case of carmine, even after the marked cell population had undergone several cycles of mitosis. (For photomicrographs of cells marked by this method, see references 23 and 42.) Gradually, however, the proportion of marked cells declined, owing to dilution of the particles in progeny cells. No release of particles to the medium was seen, and cells could be maintained fully marked and quiescent as long as a week after by reducing the serum concentration in the medium to 0.5%.

For growth curves of the components of cocultures, labeled and unlabeled cells were plated either separately or as mixtures in replicate dishes. 1 day later, ouabain (Sigma Chemical Co., St. Louis, Mo.) was added to the medium in half the dishes, and sample dishes were trypsinized then and at intervals thereafter. Total cells per dish and the proportion of marked cells were determined with a hemocytometer.

RESULTS

Index of Cooperation

Human cells are known to be much more sensitive to ouabain than are rodent cells (3). We found that when certain combinations of ouabain-sensitive and ouabain-resistant cells were cocultivated in the presence of 1 μM ouabain, the steady-state $^{86}Rb^+$ content of the culture was substantially greater than expected from the $^{86}Rb^+$ content of the two cell types treated separately with ouabain. Even when ouabain-sensitive cells constituted 75% of the ouabain-treated mixture, the $^{86}Rb^+$ content exceeded the expected value. Apparently, the resistant cells in the mixture could transfer $^{86}Rb^+$ and K^+ to the sensitive cells. Thus, the resistant cells were behaving as K^+ donors and the sensitive cells as recipients, and cell types are so designated in the tables that follow.

To compare results obtained in different experiments with different types of cultured cells, we expressed our data by a function, index of cooperation. The $^{86}Rb^+$ content of a noninteracting culture was assumed to be given by the sum of the proportional $^{86}Rb^+$ contributions of the separate ouabain-treated cell cultures. A fully interacting culture would have been expected to have an $^{86}Rb^+$ content as high with ouabain as without. The actual $^{86}Rb^+$ content of the mixed culture usually was somewhere between these two extremes. Thus, index of cooperation is a ratio of the observed enhancement in $^{86}Rb^+$ content to the maximum possible enhancement. A small enhancement of $^{86}Rb^+$ content in cocultures would result in a low index of cooperation while nearly complete enhancement would give an index approaching 1. Illustrative data and a sample calculation are given in Table I.

The calculation of index of cooperation was carried out in three parts:

(*a*) The experimental design included six different kinds of cultures: either cell type alone and a defined mixture of these two types, with or without ouabain. At least two, usually three, replicates of each kind of culture were prepared. At the end of the experiment, for each sample, the $^{86}Rb^+$ content of the cells was divided by their protein content, to control for minor variability in the number of cells inoculated into the dishes (Table I, part A).

(*b*) We computed statistical parameters corresponding to the means of each of the six groups of replicates for each experiment by one-way analysis of variance, yielding 12 degrees of freedom for the residual variance (Table I, part B). Plots of the residual errors showed that the variance appeared to be proportional to the square of the group mean. Therefore, each observation was assigned a weight proportional to the inverse of the square of the observation. Examinations of histograms showed that the resulting errors appeared to be normally distributed.

(*c*) The index of cooperation was calculated according to the equation:

$$\text{index of cooperation} = \frac{C - B - r(A - B)}{D - B - r(A - B)},$$

where A is the mean cpm $^{86}Rb^+/\mu g$ protein for the group of ouabain-sensitive cultures with ouabain; B is the mean cpm $^{86}Rb^+/\mu g$ protein for the group of ouabain-resistant cultures with ouabain; C is the mean cpm $^{86}Rb^+/\mu g$ protein for the group of mixed cultures with ouabain; D is the mean cpm $^{86}Rb^+/\mu g$ protein for the group of mixed cultures without ouabain; and r is the proportion of ouabain-sensitive cells in the initial mixture (Table I, part C).

The data for the ouabain-sensitive and -resistant cultures in the absence of ouabain did not enter the calculation of the index of cooperation but were included as controls to show the effect of ouabain treatment on cell $^{86}Rb^+$ content. The error of the index of cooperation was determined by error propagation from the statistical parameters calculated by analysis of variance (6).

In the calculation of error, the parameter r was assumed to be exactly equal to the proportion of sensitive cells seeded. For this assumption to be strictly true, (*a*) there must have been a minimum error in preparation of the cell mixtures used to set up the cocultures, and (*b*) there must have been minimum divergence from the initial proportion of cells between the time of inoculation and the time, 24 h later, when the cultures were analyzed. By using the same pooled cell mixture to inoculate all mixed cultures, we avoided random error within each experiment arising from variability in constructing the mixture. There was, however, a possible systematic error in the true proportion of sensitive cells at the end of the experiment due to unequal plating efficiencies of the two cell types or unequal growth rates over the course of the experiment. Such an error would affect the value of the index itself. This error is probably not substantial, as may be seen by

examining the data for cultures without ouabain: Both the $^{86}Rb^+/\mu g$ protein and the protein contents themselves determined on cocultures agree closely with those calculated from the values for the separate cell types using the initial input ratio. Measurements on pure cultures confirmed that, for most cell types studied, differences in extent of growth over the first 24 h were small, perhaps because there is a lag in resumption of growth after cells are trypsinized.

Over the course of a year, the index of cooperation was measured between 3T3 and HF cells a number of times. All measurements made under identical conditions are given in Table I, part D. Some of these data are also given in subsequent tables. The assay, at least for this combination of cell types, appears to be quite reproducible, and we therefore did not undertake a systematic control of day-to-day variability in most of our experiments; a similar reproducibility was assumed to apply to other measurements as well.

Optimum Conditions for Determining Index of Cooperation

Two parameters expected to influence the degree of enhancement of $^{86}Rb^+$ in the cocultures and hence the index of cooperation were (*a*) the total density of cells in the culture and (*b*) the proportion of recipient cells in the mixture, *r*. Furthermore, these two variables were also expected to influence the precision with which index of cooperation could be measured. We therefore carried out the experiments illustrated in Tables II and III to determine values for these parameters that would yield a maximum index of cooperation with a minimum error.

Table II illustrates the effect of cell density. With 10^4 cells/35-mm dish, index of cooperation was low and its standard error was high, but with 10^5 cells/dish the index was greater and the error substantially reduced. From similar experiments we determined that 10^5 cells/dish was a convenient density; denser cultures could be used, but the index of cooperation did not increase significantly as the cultures approached confluence. Fewer than 10^4 cells/dish gave indices approaching 0 (see Table IV).

In the experiments of Table III we varied the proportion of recipient cells while keeping the total cell density constant at 10^5 cells/dish. As anticipated, a vast excess of donor cells ($r = 0.1$) gave an index of cooperation with a high standard error, because the calculation involved a relatively small difference between two large numbers, i.e., the observed and expected quantities of $^{86}Rb^+$ in the mixed, ouabain-treated culture. On the other hand, when recipient cells were in vast excess ($r = 0.9$), the index of cooperation was low, although determined with much greater precision. This probably reflected the inability of the few donor cells to pump K^+ for so many recipients. We found the optimum balance between maximum index of cooperation and minimum standard error when mixtures contained between 50 and 75% recipient cells, and we used the latter value ($r = 0.75$) in subsequent experiments.

TABLE II

Effect of Cell Density on Ability to Share K^+

Donor	Recipient	*r*	Inoculum	Index of cooperation
BHK	HF-1	0.5	10^4	0.19 ± 0.11 n.s.
			10^5	0.51 ± 0.14
3T3-1	HF-1	0.5	10^4	0.20 ± 0.13 n.s.
			10^5	0.69 ± 0.23
3T3-2	HF-1	0.75	10^4	0.49 ± 0.21
			10^5	0.69 ± 0.15

Cultures were seeded so that a 35-mm dish contained either 10^4 or 10^5 cells, composed either entirely of the donor cell type, entirely of the recipient cell type, or a mixture of recipient and donor cells in the proportion *r*. $^{86}Rb^+$ was added and the cultures were incubated overnight. Then ouabain was added to three of the dishes of each set to a final concentration of 1 μM, and sterile PBS to the other three. After another 6 h of incubation, all dishes were assayed for the $^{86}Rb^+$ content of the TCA extract and the protein content of the TCA precipitate. From the resulting values of cpm $^{86}Rb^+/\mu g$ protein for each dish, the index of cooperation was calculated as described in Table I.

n.s., Not significantly different from 0. The remaining values are not significantly different from each other ($P > 0.05$).

Other factors that may influence the index of cooperation are cell size, area of cell surface, relative rate of cell growth, and number of cell extensions. We made no attempt to account for these factors, although clearly they could influence the conditions selected as optimum.

Cooperation for K^+ Requires Cell Contact

Although the results presented so far are consistent with exchange of K^+ between cells in contact, an exchange which certainly can occur

TABLE III

Effect of Proportion of Recipient Cells on Ability to Share K^+

Donor	Recipient	r	Index of Cooperation
BHK	HF-1	0.1	1.34 ± 1.87 n.s.
		0.5	0.51 ± 0.14
		0.9	0.07 ± 0.018
3T3-1	HF-1	0.1	0.51 ± 0.72 n.s.
		0.5	0.69 ± 0.23
		0.9	0.19 ± 0.059
3T3-2	L-N	0.5	0.77 ± 0.19
		0.75	0.40 ± 0.08
		0.9	0.09 ± 0.022

Donor and recipient cells were suspended in growth medium at 10^6 cells/ml. Mixtures of the two suspensions were prepared which consisted of various proportions of recipient cells (r in the Table). These mixtures and the unmixed donor and recipient cell suspensions were used to inoculate replicate 35-mm dishes and $^{86}Rb^+$ was then added. After overnight incubation, 1 μM ouabain was added to half of each set of cultures and PBS to the other half. Incubation was continued for 6 h, and then all dishes were assayed for $^{86}Rb^+$ and protein content. The data for $r = 0.5$ involving BHK with HF and 3T3 with HF also appear in Table II.

n.s., Not significantly different from 0 ($P > 0.05$).

between two cells that are electrically coupled, it was necessary to rule out the possibility that ouabain resistance was transferred from resistant to sensitive cells through the culture medium. Experiments to show that cell contact was required for K^+ transfer are summarized in Table IV. HF and 3T3 cells were cultivated separately on glass cover slips in medium containing $^{86}Rb^+$. After 2 days, two cover slips, each containing one of the cell types, were combined in a single dish in medium containing $^{86}Rb^+$ at the same specific activity. The next day ouabain was added.

The first two lines of Table IV show that when the cell types were grown on separate cover slips in the same dish, the index of cooperation was indistinguishable from 0. The relatively high variance can be accounted for by variability in the number of cells per cover slip, adjusted only at the time the initial separate cultures were inoculated, which could lead to differences between the estimate of r and its true value. Cells cultivated at a density of 5×10^3/dish, where contacts between cells were seen to be rare, also showed a negligible index of cooperation. Hence we conclude that a positive index of cooperation requires opportunity for contact formation between the two cell types.

TABLE IV

Conditions which Interfere with the Sharing of K^+

	Index of cooperation
A. Cultivation of cells on separate cover slips	−0.10 ± 0.09 n.s.
	−0.097 ± 0.19 n.s.
B. Cultivation of cells at low density (5×10^3 per 35-mm dish)	−0.041 ± 0.084 n.s.

A. HF cells and 3T3 cells were seeded into 60-mm dishes, each of which contained four 10.5 × 22 mm glass cover slips at densities of 9×10^4 cells/dish (HF), 6×10^4 cells/dish (both HF and 3T3), or 3×10^4 cells/dish (3T3). After 2 days of incubation, cover slips were transferred to 35-mm dishes. Each dish received either one concentrated HF and one dilute 3T3 cover slip, two intermediate cover slips of HF, or two intermediate cover slips of 3T3. Six dishes of each sort were prepared and growth medium and $^{86}Rb^+$ were added. The next day ouabain (1 μM) was added to half the dishes of each set and PBS to the other half. 6 h later, the cultures were assayed for $^{86}Rb^+$ and protein content. Index of cooperation was calculated assuming that after 2 days in culture, r remained 0.75.

B. HF and 3T3 cell suspensions were each diluted in growth medium to 5×10^4 cells/ml and a mixture containing 75% HF cells was prepared. Twenty-four 35-mm dishes were inoculated with 5×10^3 cells from this mixture or from one or the other of the original pure cultures, and all dishes received $^{86}Rb^+$. After overnight incubation, 1 μM ouabain was added to half the dishes in each set, and the other half received PBS. All dishes were terminated 6 h later. To obtain sufficiently concentrated extracts from such sparse cultures, the following procedure was used: Each group of 12 dishes was divided into three groups of four dishes each, and each of these was extracted sequentially with 0.5 ml 5% TCA to obtain a pooled extract containing the $^{86}Rb^+$ content of four dishes; similarly 0.5 ml of 0.2 N NaOH + 0.3% sarkosyl was used to dissolve sequentially the TCA precipitate in each of the four dishes in turn.

n.s., Not significantly different from 0 ($P > 0.05$).

Index of Cooperation for Various Cell Types

Using standard conditions of 10^5 cells/dish and 75% ouabain-sensitive cells, we examined various combinations of cultured cells (Table V). The table is arranged in order of decreasing index of cooperation and is divided into three classes: combinations showing high index of cooperation, combinations showing reduced but measurable index of cooperation, and a combination that shows no cooperation. The elements within each of the first two classes are statistically indistinguishable (chi-square; $P > 0.05$). In the first class, 3T3 cells from two sources and human fibroblasts from three sources are included. No significant

difference was found among these combinations; thus the ability to cooperate may be a characteristic of cells of similar derivation.

Certain modifications of the cells do not affect their ability to express a high index of cooperation. Thus, allowing HF cells to take up colloidal carmine particles as a visible cytoplasmic label has no effect on the index of cooperation. SV40-transformed human fibroblast lines from two different sources show as high an index of cooperation as untransformed fibroblasts. Fibroblasts from Lesch-Nyhan patients are also capable of cooperation, and 3T3 cells selected for increased ouabain-resistance (THO2) are as able to cooperate with HF as are the wild-type 3T3 lines. Also in the first group is a cell line that we derived from cultured mouse teratocarcinoma cells and that shows a very high index of cooperation with HF cells.

In an intermediate class are combinations of 3T3 or BHK cells with HeLa and combinations of CHO cells with HF. Since 3T3, BHK, and HF are all capable of cooperating with one another, the failure to cooperate as donor or as recipient with CHO or with HeLa must be a property of the latter strains. Others have noted that these two lines also show reduced electrical coupling (20) and metabolic cooperation (7, 10, 11, 34, 36).

The third class in Table V includes the combination of L cells with HF cells showing no enhancement of the $^{86}Rb^+$ content of mixtures over that expected from the sum of the component cell types. L cells are often used as negative controls in studies on contact-mediated cooperation and gap junction formation since they appear to have an inherent deficiency in these functions. Some experiments with duck embryonic fibroblasts, pig kidney epithelial cells, and mouse peritoneal macrophages also showed no cooperation in our assay (data not shown).

Donor Cells can Restore Protein Synthesis in Recipient Cells by Feeding K^+

We had previously shown that inhibition of protein synthesis is a direct and primary consequence of the K^+ depletion resulting from ouabain treatment (26). To see if protein synthesis in cells

TABLE V

Ability of Various Cell Strains to Share K^+

Donor	Recipient	Index of Cooperation	Remarks
3T3-2	HF-1	0.75 ± 0.079	3 days of co-cultivation
Terato Cl-25	HF-1	0.70 ± 0.081	
3T3-1	SV/HF-3	0.66 ± 0.093	
3T3-1	HF-carmine	0.59 ± 0.04	
3T3-1	SV/HF-4	0.56 ± 0.083	
3T3-1	HF-1	0.54 ± 0.10	0.3 μM ouabain
BHK	HF-1	0.51 ± 0.14	$r = 0.5$
3T3-1	HF-2	0.49 ± 0.058	
THO2	HF-1	0.47 ± 0.065	
BHK	HF-1	0.41 ± 0.10	$r = 0.5$
3T3-1	HeLa	0.16 ± 0.054	
BHK	HeLa	0.10 ± 0.049	
CHO	HF-2	0.10 ± 0.021	
L	HF-1	−0.012 ± 0.024 n.s.	

Replicate cultures of the indicated recipient and donor cell types were set up, as well as cultures of mixtures containing 75% recipient cells. The cell types are defined in Materials and Methods. HF-carmine refers to HF cells allowed to take up colloidal carmine, then washed and trypsinized to remove residual extracellular carmine. After addition of $^{86}Rb^+$, the cultures were incubated overnight. Ouabain was added to half the cultures in each set (1 μM) and PBS to the other half. Exceptions to these standard conditions are noted under Remarks. Incubation was continued for 6 h and all cultures were terminated. In most of the experiments [^{3}H]leucine was added to all the cultures during the final hour of incubation and leucine incorporation was determined on TCA precipitates. Those data are presented in Table VI. In this table, the data are arranged in order of decreasing index of cooperation. The cell types are divided into three groups according to whether they show high, low, or zero index of cooperation.
n.s., Not significantly different from 0 ($P > 0.05$).

depleted of K^+ could be restored by cell-cell coupling, we measured the incorporation of [^{3}H]leucine during cocultivation of ouabain-sensitive and -resistant cell types in the presence of ouabain. Precursor specific activity in cultured HF cells was unaffected by ouabain treatment (26). In these experiments, the procedure for assay of index of cooperation was modified by the addition of 2 μCi/ml [^{3}H]leucine to the incubation medium during the last hour of the 6-h ouabain treatment. The results of a series of such experiments are shown in Table VI in which incorporation of [^{3}H]leucine per μg protein is used in the equation to measure index of cooperation. (The corresponding $^{86}Rb^+$ data are given in Tables II–V.) In general, the precision of the assay in this form was lower than for measurements made with $^{86}Rb^+$. Part A shows that cell combinations whose index of cooperation for K^+ is high also have a high index as measured by incorporation of [^{3}H]leucine. In part C it can be seen that L cells, which show no cooperation for K^+, also show no stimulation of leucine incorporation upon cocultivation. In part D conditions that prevent physical contact among cells block the enhancement of leucine incorporation.

In part B, the two cell lines with a reduced index of cooperation for K^+ nevertheless show a high level of cooperation as measured by [^{3}H]leucine. In one case the error is too great to permit distinction of the measured value from 0, but in the other the difference between the measured value and 0 is highly significant. This result may reflect the nonlinear relationship between decreased cell K^+ and protein synthesis, so that the incorporation of leucine is not suppressed unless K^+ drops below 50–70% of control levels (5, 26).

The increase in leucine incorporation as a result of feeding of K^+ from donor to recipient cells

TABLE VI

Ability of K^+ from Donor Cells to Restore Incorporation of Leucine in Recipient Cells

Donor	Recipient	r	Index of cooperation (calculated from [^{3}H]leucine incorporation)
A. Cells capable of cooperating for K^+:			
3T3-2	L-N	0.5	0.69 ± 0.32
		0.75	0.90 ± 0.15
		0.9	0.25 ± 0.18 n.s.
3T3-2	HF-1	0.75	0.87 ± 0.099
Terato Cl-25	HF-1	0.75	0.94 ± 0.14
B. Cells of intermediate ability to cooperate for K^+:			
3T3-1	HeLa	0.75	0.61 ± 0.18
CHO	HF-2	0.75	1.06 ± 0.90 n.s.
C. Cells incapable of cooperating for K^+:			
L	HF-1	0.75	0.026 ± 0.062 n.s.
D. Cells grown under conditions preventing cooperation for K^+:			
3T3-1	HF-1	0.75 (5×10^3 cells/dish)	0.12 ± 0.22 n.s.
3T3-1	HF-1	0.75 (10^5 cells on separate cover slips)	−0.07 ± 0.051 n.s. 0.18 ± 0.23 n.s.

These data were obtained in some of the experiments whose index of cooperation for $^{86}Rb^+$ is reported in Tables II, III, IV, and V. [^{3}H]leucine (2 μCi/ml) was added for the last hour of the 6-h ouabain treatment and incorporation of tritium into each TCA precipitate was determined. Interference of $^{86}Rb^+$ with this determination was avoided by extraction of the bulk of the $^{86}Rb^+$ with TCA, and the use of a double-label scintillation counting procedure (22). Index of cooperation was calculated using the equation given in Table I but with values for [^{3}H]leucine incorporation/μg protein used as the elements.

n.s., Not significantly different from 0 ($P > 0.05$).

could be strikingly illustrated by autoradiography. 3T3 cells were prelabeled by incubation for 2 days in [^{3}H]thymidine (2.0 μCi/ml). They were then washed and mixed with unlabeled HF cells, and the mixture was cultivated on cover slips. After a 4-h incubation to allow cell attachment, ouabain (1 μM) was added to half the cultures, and 6 h later all cultures received 10 μCi/ml [^{3}H]leucine. After another 11 h of incubation, the cover slips were fixed, extracted with TCA, and prepared for autoradiography. Fig. 1 shows as controls the results obtained with pure cultures of HF (*a* and *c*), or 3T3 (*b* and *d*), in the absence (*a* and *b*) or presence (*c* and *d*) of ouabain. 3T3 cells are readily distinguishable from HF by their dense nuclear label. In cultures without ouabain, both cell types show clear cytoplasmic labeling, but this labeling is abolished in the HF cells treated with ouabain. 3T3 cells show no difference in labeling with or without ouabain, since 1 μM was far below the concentration needed for inhibition. Fig. 2 illustrates results with mixtures of 3T3 and HF. In the absence of ouabain (*a* and *b*) both cell types are well-labeled whether or not they were in contact. In the presence of 1 μM ouabain, however, 3T3 cells show strong cytoplasmic labeling, as expected, but HF cells are labeled only when in contact with 3T3 cells (*c* and *d*). Thus, restoration of K^+ levels as a result of cell interaction permitted restoration of normal leucine incorporation.

In Fig. 3, a similar experiment was performed between HF cells and mouse L cells. No prelabeling of the L cells was necessary because of their differential affinity for the stain used (Giemsa), and their morphological distinctness from HF cells. It is clear that even when an HF cell is in close proximity to a number of L cells, no cytoplasmic labeling in the HF cell is seen. The image in the upper left corner of this photomicrograph is particularly clear. It shows a cell process of the HF cell lying completely over a process from an L cell, and preventing the tritium disintegrations from reaching the emulsion.

Restoration of Growth by Cell-Cell Contact

Since reduction of intracellular K^+ blocks cell growth, we tested whether growth of ouabain-sensitive cells could be restored by contact with ouabain-resistant cells. HF cells were marked with latex beads as described in Materials and Methods. Three days later they were trypsinized and mixed with an equal number of 3T3 cells. Mixtures and pure cultures of unmarked 3T3 and marked HF cells were distributed to replicate cultures in 35-mm dishes. (The intended inoculum was 10^5 cells per dish, but the mixed cultures appear to have received twice that.) The next day ouabain was added to half the dishes in each set, and 6 h later the first samples were trypsinized and counted. In the controls (pure HF cultures) 96% of the cells were marked; by the end of the experiment the number of detectably marked cells had fallen to 54% because of progressive dilution of beads among progeny cells (Fig. 4, upper panel). By counting the number of marked and unmarked cells in dishes sampled over a period of time, it was possible to estimate the growth rate of each of the components of the mixture. The lower panel of Fig. 4 shows that HF cells grew well in drug-free medium, but were drastically inhibited in 1 μM ouabain. As expected, the growth rate of unmarked 3T3 cells was the same whether or not ouabain was present and whether or not HF cells were mixed with them (data not shown). In the presence of ouabain the admixture of 3T3 cells to HF cells restored normal growth rate to the latter.

In other versions of this experiment, we marked the 3T3 cells, or we used carmine dye particles as the labeling agent. In these cases as well, HF cells were stimulated to grow in ouabain-containing medium only when there was the possibility of forming contacts with the ouabain-resistant 3T3 cells (data not shown).

DISCUSSION

We have shown that among cells capable of forming functional contacts, differences in levels of intracellular K^+ induced by treatment with ouabain may be corrected. This correction is sufficient to restore the capacity for protein synthesis and replication to cells otherwise inhibited by ouabain. We presume that K^+ levels are similarly buffered in all communicating groups of cells, both in culture and in tissues of intact animals.

We have reported our results in terms of K^+ levels, as measured by the intracellular tracer $^{86}Rb^+$ under steady-state conditions. K^+, however, is only one of several ions whose concentration in the intracellular environment may be changed by blocking the plasma membrane Na^+-K^+ ATPase with ouabain. In the presence of ouabain, sensitive cells are known to have higher than normal Na^+ content and intracellular Ca^{++} probably in-

creases (24), at least in some types of cells. Na^+ is likely to be transmitted through functional cell-cell contacts, and Ca^{++} might be as well; we presume that exchange of these ions in cocultures with a high index of cooperation for K^+ occurs also.

We have ruled out the possibility that ouabain resistance is transmitted through the medium by showing that restoration of K^+ to ouabain-sensitive cells depends on physical contact with resistant cells. Thus at low culture density, or when the cell types are grown on separate cover slips in the same medium, no K^+ transfer occurs. Similarly, autoradiography shows that protein synthesis is restored only in those ouabain-sensitive cells in contact with ouabain-resistant cells.

It is probably the direct transmission of ions through gap junctions from donor to recipient that mediates the correction. A very unlikely alternative possibility is that the donor cells are supplying ouabain resistance in some other form, perhaps as an inhibitor of ouabain binding to the sensitive ATPase or as a resistant ATPase itself. This latter mechanism would involve molecules too large to be transmitted through cell-cell junctions (39).

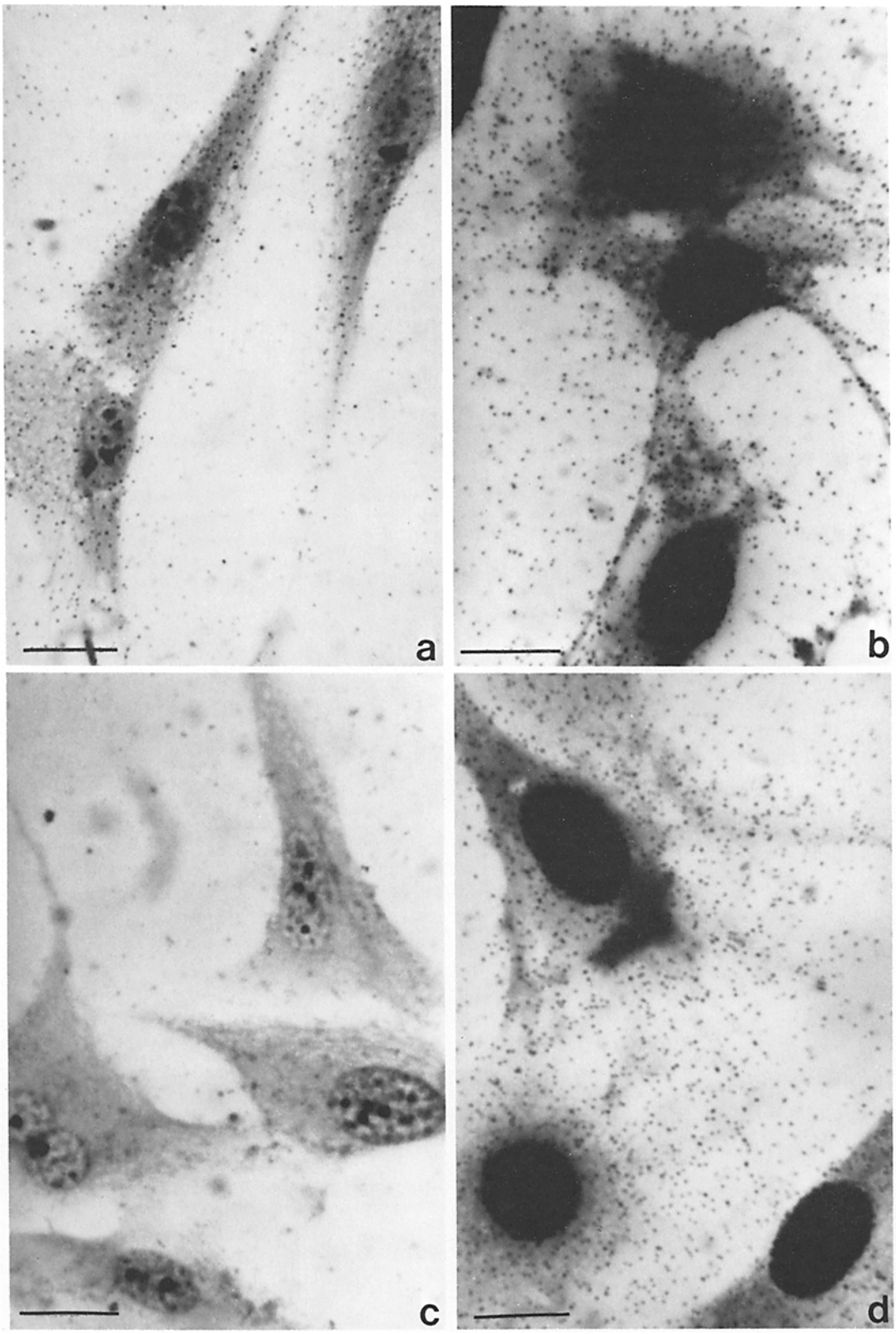
We do not believe that our results could be explained by changes in the ability of HF cells to transport K^+ actively. Such an assumption would require that contact with 3T3 cells had induced a new transport system which was no longer inhibitable by ouabain, and we know of no precedent or evidence for this.

In analyzing our data, we used a function, index of cooperation, to quantitate the effectiveness of correction of ouabain sensitivity. The measure of index of cooperation depends on the steady-state levels of K^+ in the cocultures. The cell types that we have classified by index of cooperation (Table V) fall into the same groups that were found by others using electrical coupling (20), metabolic cooperation (12, 34), and contact feeding (7) to measure the contact-dependent transmission of small molecules from cell to cell through gap junctions. These other methods all require involved or time-consuming techniques which are avoided in our assay of index of cooperation. The assay is simple to perform, requiring small numbers of cells of each of the two types being tested and a minimum of culture medium and dishes. It is rapid: a complete assay requires only overnight cocultivation with the $^{86}Rb^+$ label and a 6-h ouabain treatment the next day. Several such assays may readily be performed at once, avoiding the need to consider variability arising from successive determinations. It is noninvasive: the tracer levels of $^{86}Rb^+$ used do not appear to interfere with cell growth, and the ouabain treatment has no apparent effect on the resistant cell types. Sensitive cells are not permanently damaged by the brief ouabain treatment, since K^+ levels and growth rate return to normal after removal of ouabain, even after treatment as long as 24 h (26). The method is quantitative and the effect of various treatments is thus readily assessed. The only restriction on the choice of cell types is that they must differ in resistance to ouabain so that a drug concentration can be chosen that will deplete K^+ in one cell type but not the other. Since cells of different species display naturally occurring differences in resistance to ouabain, there is no need to select resistant mutants unless one wishes to measure cooperation between cells of the same species. Although we have limited this investigation to cells growing in monolayer, there is no reason why the method could not be extended to cells normally growing as single cells or aggregates in suspension, which have been shown to form communicating junctions (19, 22, 32).

The experiments of Fig. 2, Fig. 4, and Table VI

FIGURES 1 and 2 Autoradiographs: Inhibition of protein synthesis in HF cells by ouabain and restoration by contact with 3T3 cells. 3T3 cells were incubated for 2 days in medium containing 2 μCi/ml [^{3}H]thymidine, a procedure which gave heavy nuclear labeling of virtually all cells. The medium was then removed and the cells were trypsinized, mixed with a threefold excess of unlabeled human fibroblasts, and plated in 35-mm tissue culture dishes containing two 10.5 × 22 mm cover slips at a total cell inoculum of 10^5 per dish. Control dishes contained either pure HF cells or pure 3T3 cells. By 4 h all the cells had attached, and ouabain (1 μM) was added to half the dishes of each type. 7 h later, all the dishes received 10 μCi/ml [^{3}H]leucine, and incubation was continued for 11 h more. Cover slips were processed for autoradiography as described in Materials and Methods.

FIGURE 1 Pure cultures of: (*a*) HF cells without ouabain; (*b*) 3T3 cells without ouabain; (*c*) HF cells with ouabain; and (*d*) 3T3 cells with ouabain. Bars, 20 μm. (*a* and *d*) ×750; (*b* and *c*) × 825.


a
b
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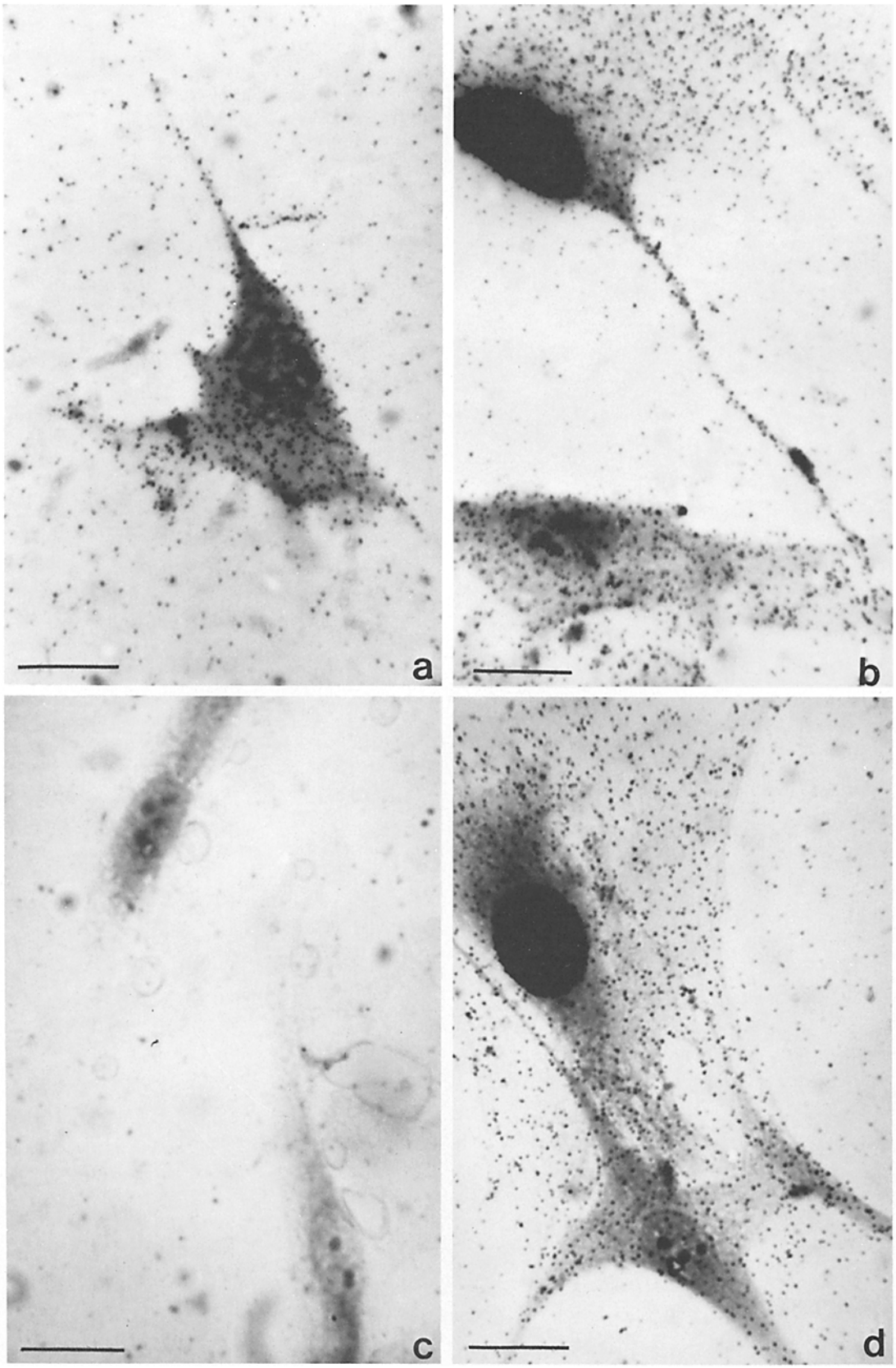

FIGURE 2 Mixed cultures containing 75% HF and 25% 3T3 cells. (*a*) Isolated HF cells without ouabain; (*b*) HF cells contacting 3T3 cells without ouabain; (*c*) Isolated HF cells with ouabain; and (*d*) HF cells contacting 3T3 cells with ouabain. Bars, 20 μm. (*a* and *d*) × 840; (*b*) × 810; (*c*) × 850.

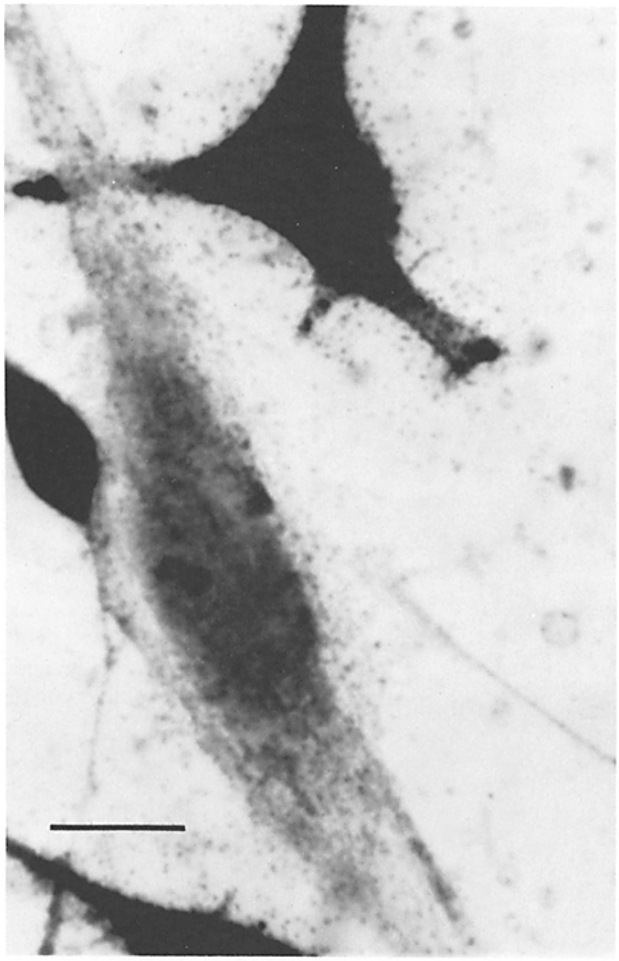

FIGURE 3 Autoradiograph: Failure of L cells to restore protein synthesis in HF cells inhibited by ouabain. L cells were mixed with human fibroblasts ($r = 0.75$) and inoculated into 35-mm tissue culture dishes with two 10.5 × 22 mm cover slips. The next day, half the dishes received 1 μM ouabain, and 5 h later all dishes received 12.5 μCi/ml [^{3}H]proline. After another 18 h all dishes were terminated and the cover slips processed as described in Materials and Methods. Bar, 20 μm. × 915.

all show that contact between donor and recipient cells increases the K^+ content of the latter and restores protein synthesis and growth. Our explanation is that feeding of K^+ from donor to recipient cells relieves the block to translation caused by low levels of K^+. One possible alternative interpretation of our data is that recipient cells, depleted of K^+, have pools of amino acids that are at too low a level to support protein synthesis, and that contact with donor cells restores these pools. This possibility is ruled out by our previous results (26) showing that in K^+-deficient HF cells, transport of amino acids is not a factor limiting the rate of protein synthesis.

While our studies were in progress, Corsaro and Migeon reported the contact-mediated communication of ouabain resistance from 3T3 cells, but not from L cells, to ouabain-sensitive human fibroblasts in the presence of ouabain (9). By observing metaphase spreads containing human marker chromosomes among the metaphases of these cocultures, they concluded that the capacity for cell division had been restored. Our results agree with theirs and extend them to measurements of K^+ levels, protein synthesis, and long-term cell division, all of which are restored in

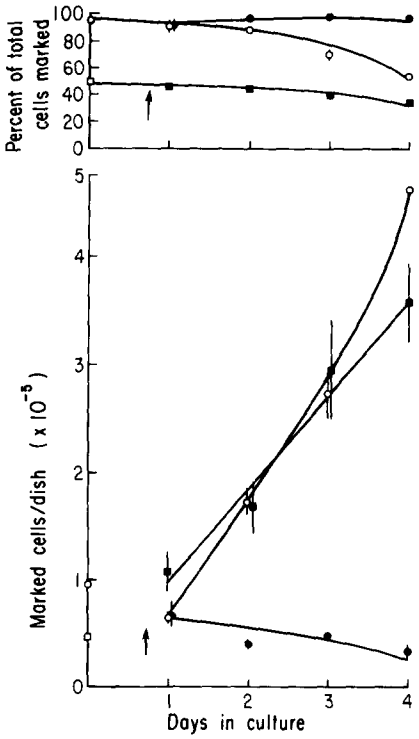

FIGURE 4 Restoration of growth of HF cells cocultured with 3T3 cells in the presence of ouabain. A confluent culture of human fibroblasts was marked with latex beads (0.98 μm diameter) overnight, and after washing and trypsinizing to remove excess beads, the cells were returned to the original flask at the original density. 3 days later the cells were again trypsinized and plated either alone or in mixtures (r = 0.5) with unmarked 3T3 cells in replicate 35-mm culture dishes (day 0). The next day (arrow), half of the pure cultures and all of the cocultures received 1 μM ouabain. Three dishes from each of the three groups were trypsinized each day beginning 5 h after ouabain addition, and the number of marked and unmarked cells in each was determined.

In the upper panel, the marked cells are given as the percent of the total cells (marked and unmarked) to indicate the degree to which marking was maintained. In the lower panel, the number of marked cells per dish is given. The zero-time values are assumed, based on the number of cells plated. ●, Marked cells/dish in pure cultures with ouabain. ○, Marked cells/dish in pure cultures without ouabain. ■, Marked cells/dish in mixed cultures with ouabain. The mean and SEM of three determinations are given for most points. (Because of loss of some cultures, the 4-day control value represents a single determination.)

sensitive cells by contact with resistant cells.

Our results with various cell types are qualitatively consistent with results obtained in other laboratories using other methods. L cells have an index of cooperation of 0 in our assay. They also fail to exhibit electrical coupling (17), fail to transmit or receive nucleotides in metabolic cooperation (17), fail to engage in contact feeding (7), and fail to form gap junctions (17). CHO cells and HeLa cells, which have been described as showing a reduced ability to engage in metabolic cooperation determined autoradiographically (12, 34) or by contact feeding (7, 10), fall into a class with low index of cooperation, statistically different from both the well-coupled and uncoupled cell combinations ($P < 0.01$). Their failure to show a correspondingly low value for leucine incorporation (Table VI) probably reflects the insensitivity of protein synthesis in some cell lines to relatively modest reductions of intracellular K^+ (5, 26).

3T3, BHK, and human fibroblasts are known to form effective intercellular junctions (34), and they show a high index of cooperation in our assay too. We see no difference between human fibroblasts and their SV40-transformed derivatives in index of cooperation, although Corsaro and Migeon (10) have reported that SV40-transformed cells are less efficient than normal adult cells in the contact feeding of thioguanine. Since thioguanine is a larger molecule than K^+, it might be transmitted less efficiently than K^+. Small changes in transmission could well lead to measurable changes in the proportion of surviving cells over the long periods required for assay of contact feeding, while having relatively little effect on index of cooperation. It has been demonstrated that coupling coefficients, measured electrically, are insensitive to changes in junctional conductance in strongly coupled cells (40); this may also apply to the assay for index of cooperation. Another possible explanation for the discrepancy is that different lines of SV/HF cells might have different abilities to engage in functional coupling.

Terato-C125, a clonal line of cells which we derived from cultured mouse teratocarcinoma, shows a high index of cooperation. An independent teratocarcinoma line, PC13, is capable of metabolic cooperation (18). PC13 is a typical undifferentiated embryonal carcinoma whose tumors contain predominantly neural derivatives, while terato-C125 has lost both its tumorigenicity and its potential to differentiate further in culture (M. L. Ledbetter, unpublished observations).

Our assay for measuring cell-cell coupling is relatively easy to perform, once suitable cell types differing in sensitivity to ouabain are chosen. The method should be useful for screening cell types,

such as epithelial cells (14, 35), that show selectivity in coupling with other cells, for characterizing the coupling ability of mutant cell lines (8), for measuring the effects of chemical or physical agents on coupling, and for determining the kinetics of coupling and uncoupling.

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