Choline Permeability in Cardiac Muscle Cells of the Cat

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ABSTRACT Permeability of the cardiac cell membrane to choline ions was estimated by measuring radioactive choline influx and efflux in cat ventricular muscle. Maximum values for choline influx in 3.5 and 137 mM choline were respectively 0.56 and 9 pmoles/cm^2·sec. In 3.5 mM choline the intracellular choline concentration was raised more than five times above the extracellular concentration after 2 hr of incubation. In 137 mM choline, choline influx corresponded to the combined loss of intracellular Na and K ions. Paper chromatography of muscle extracts indicated that choline was not metabolized to any important degree. The accumulation of intracellular choline rules out the existence of an efficient active pumping mechanism. By measuring simultaneously choline and sucrose exchange, choline efflux was analyzed in an extracellular phase, followed by two intracellular phases: a rapid and a slow one. Efflux corresponding to the rapid phase was estimated at 16–45 pmoles/cm^2·sec in 137 mM choline and at 1.3–3.5 pmoles/cm^2·sec in 3.5 mM choline; efflux in 3.5 mM choline was proportional to the intracellular choline concentration. The absolute figures for unidirectional efflux were much larger than the net influx values. The data are compared to Na and Li exchange in heart cells. Possible mechanisms for explaining the choline behavior in heart muscle are discussed.

INTRODUCTION

In frog skeletal muscle, choline ions were shown to penetrate into the cells about as fast as sodium ions (Renkin, 1961). They escape, however, more slowly from the cells, and their behavior can be compared to that of lithium ions.

A similar situation might exist in cardiac muscle. Boulpaep (1963) found a rise in K outward movement when Na ions were replaced by choline ions and interpreted this result as due to a penetration of choline into the cells. In a paper on extracellular space in heart muscle, Page (1962 *b*) noted in the discussion that choline over long periods equilibrated with a volume of tissue water in excess of 100%.

The frequent use of choline as a substitute for Na in electrophysiological work on cardiac preparations prompted us to investigate in more detail the

exchange of choline in the heart and to correlate our findings with previous work on lithium permeability in the same preparation (Carmeliet, 1964).

METHODS

Preparation Cats weighing 3–5 kg were anesthetized with ether. The thorax was quickly opened, the heart removed, and rinsed with Tyrode solution at 20°C. Papillary and trabecular muscles were dissected from the right ventricle. Papillary muscles had a diameter less than 0.5 mm. Trabecular muscles had a transverse section of about 0.1 by 2 mm. The preparations were incubated in beakers containing 10 ml Tyrode, with the solution continuously aerated by a flow of 95% O_2, 5% CO_2 gas mixture, or 100% O_2 in choline Tyrode.

Solutions The composition of normal Tyrode was in millimoles per liter: Na 149.8, K 5.4, Ca 1.8, Mg 0.5, Cl 148, HCO_3 11.8, and glucose 5. Choline Tyrode was made by substituting choline chloride for NaCl on a mole for mole basis and replacing the bicarbonate buffer by Tris-acetylglycinate buffer (5 mM); atropine sulfate was added in a concentration of 10 mg/liter. Two different choline concentrations were used: 3.5 mmoles/liter and 137 mmoles/liter. Radioactive choline-^{14}C, choline-^{3}H, sucrose-^{14}C, and sulfate-^{35}S were obtained from New England Nuclear Corporation or the Radiochemical Center of Amersham and stored at −15°C. Radiochemical purity was verified by paper chromatography. Radioactive choline was added to inactive carrier choline to obtain a final radioactivity of 5 μCi/ml. The concentration of sucrose-^{14}C or sulfate-^{35}S was 1 mM.

Experimental Procedure Choline influx was measured by putting the preparation during fixed periods of time in the radioactive choline solution. At the end of the influx period the preparation was blotted on Whatman filter paper and weighed on a Cahn electrobalance with an accuracy of 0.05 mg. Correction was made for the loss of weight due to water evaporation during the weighing procedure. The radioactivity was extracted by putting the preparation in distilled water for two periods of 2 hr. The preparation was finally put into a counting vial to determine the radioactivity left in the preparation. Radioactive choline-^{14}C was measured in a Packard Tri-Carb liquid scintillation counter. Quenching was tested by the external standard technique. Choline influx was expressed as the muscle space that equilibrated with the perfusion solution.

Choline, sucrose, and sulfate efflux was estimated by passing the preparation during timed periods through successive vials filled with 2 ml test solution. Radioactivity was measured by directly adding scintillation fluid. By adding the amounts of radioactivity which left the preparation during the individual periods to the activity present at the end of the experiment, it was possible to know the activity in the preparation at any time from the beginning of the efflux period. When choline-^{3}H and sucrose-^{14}C were used together (double tracer technique), the energy levels of the spectrometer for the ^{14}C channel were such that the radioactivity due to ^{3}H only represented a very small fraction. The sensitivity of the channels for both isotopes was determined by using standards containing only one of the isotopes. When the ratio of the counting activities in both energy levels was known, it was possible to correct the counts of a mixture for one isotope (Danielson, Delahayes, and Sjöstrand, 1966).

Paper Chromatography Tissue samples were analyzed by paper chromatography in order to identify the radioactive material recovered from tissues used for influx studies. Tissue samples weighing 1–6 mg were homogenized in a 3% trichloroacetic acid solution and centrifuged. Excess trichloroacetic acid was removed by five successive ether extracts. After evaporation the residue was redissolved in a volume of 0.1 ml and subjected to a 20 hr descending paper chromatography in a *n*-butanol:water:ethanol:acetic acid (8:2:3:1) solvent system at room temperature. After drying, the paper, 40 cm in length, was divided into 1 cm cuts, and the radioactivity determined by liquid scintillation counting. Radioactive material with the same R_f values as a sample of choline to which a muscle preparation had been added and then treated in the same way as the experimental tissue samples, was considered to be choline. In control experiments it was found that choline samples without muscle showed a larger R_f value. Thus extraneous material contained in the concentrated heart extracts reduced the R_f value. The radioactivity present in the ether extracts was less than 0.1% of the total radioactivity.

Flame Photometry After determining the wet weight, the muscle preparations were put into Teflon tubes. 1 ml of a 0.7 mmolar $AgNO_3$, 30% hydrogen peroxide solution was added and evaporated by heating to 95°C. All chloride in the tissue was precipitated as AgCl during the ashing procedure. The dry ash was dissolved with 2 ml of a solution containing 1 N HNO_3 and 0.02 M H_3PO_4. The tubes were left for at least 36 hr in the dark. This procedure dissolved all salts apart from AgCl. The Na, K, and excess Ag concentrations of the supernatant were determined by flame photometry at wavelengths of 589, 769, and 328 mμ. The flame photometer was a Zeiss spectrophotometer PMQ II with flame attachment.

Presentation of Results All data are presented as the mean followed by the standard error of the mean and the number of observations.

RESULTS

1. *Choline Influx*

Choline influx was determined at two different external choline concentrations, 3.5 and 137 mM. The results are summarized in Table I and Fig. 1. In both choline solutions the tissue radioactivity rose quickly during the first 5 min and increased thereafter at a slower rate.

After 15 min in 3.5 mM choline Tyrode, the space filled with choline approached the total water content of the preparation. This value increased to two and three and a half times the water content after respectively 1 and 2 hr of influx. In order to translate equilibration space into intracellular concentration and absolute fluxes, the extracellular space has to be estimated. The inulin space in cat ventricular muscle was found to be about 250 ml/kg wet wt (Page, 1962 *b*; Carmeliet and Janse, 1965), while the mannitol space amounted to 300 ml/kg wet wt (Page, 1962 *b*). In the present experiments the sucrose space, after correction for the slow component in the efflux curve (see the section on choline efflux), was equal to 282 ± 16 (7) ml/kg wet wt.

TABLE I

INFLUX OF CHOLINE IONS IN CAT VENTRICULAR MUSCLE

3.5 mM	5 min	15 min	30 min	60 min	120 min
Ml/kg wet wt	426±38 (8)	679±30 (9)	960±45 (8)	1,518±57 (13)	2,656±124 (10)
Mmoles/kg wet wt	1.49±0.13	2.37±0.10	3.36±0.16	5.31±0.20	9.30±0.43
Mmoles/liter intracellular water	0.98	2.93	5.13	9.47	18.33
Influx, *pmoles/cm²·sec*	0.56	0.56	0.49	0.45	0.43
137mM					
Ml/kg wet wt	352±24 (6)	418±67 (7)	527±62 (6)	602±20 (31)	668±20 (9)
Mmoles/kg wet wt	48.2±3.3	57.2±9.1	72.2±8.5	82.5±2.7	91.5±2.7
Mmoles/liter intracellular water	15.8	35.8	69.1	92.0	112.0
Influx, *pmoles/cm²·sec*	9.07	6.85	6.62	4.41	2.68

A value of 300 ml/kg wet wt was taken to calculate intracellular concentration and fluxes across the cell membrane. As can be seen from Table I choline ions accumulate against a concentration gradient after 30 min and the intracellular choline concentration, assuming choline as not being metabolized, rises more than five times above the extracellular concentration at the end of the 2 hr influx period.

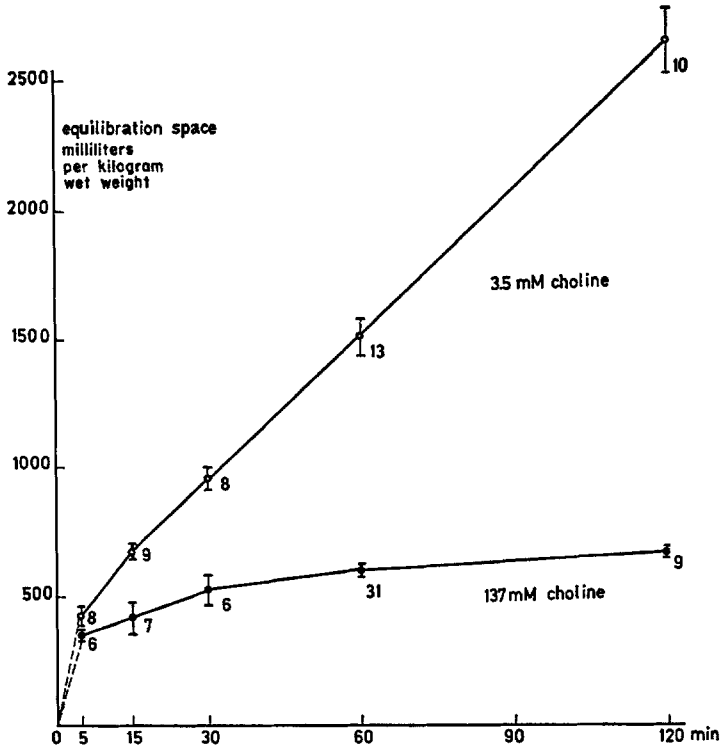

FIGURE 1. Choline-^{14}C influx in cat ventricular muscle expressed as milliliters per kilogram wet weight, as a function of time. Open circles, 3.5 mM choline, solid circles, 137 mM choline. Each point represents the mean of the indicated number of preparations; vertical bars represent standard error.

When the muscle density is taken as 1.05 g/ml, and the mean diameter as 10 μ (Draper and Mya-Tu, 1959), 1 kg of cat ventricular muscle contains 652 ml of fibers with a total surface area of 2.61×10^6 cm^2. From the change in choline content one can calculate a maximal influx of 0.56 pmole/cm^2·sec, which slowly declines to 0.43 pmole/cm^2·sec when calculated as a mean for the total experimental period.

In 137 mM choline the influx in terms of equilibration space was much smaller and slower, but was important in terms of intracellular accumulation and absolute flux. After 1 hr, for instance, a space of 602 $\pm$ 20 (n = 31)

ml/kg wet wt was filled, which corresponds to an intracellular concentration of 92 mmoles/liter intracellular water. Absolute flux was larger than 6 pmoles/$cm^2 \cdot sec$ up to 30 min, and declined to 2.7 pmoles/$cm^2 \cdot sec$ after 2 hr of influx. These values are smaller than the figure predicted from choline influx data in 3.5 mM choline, assuming choline influx to be directly proportional to the external concentration.

2. *Estimation of Na, K, and Cl Content*

An influx of 82.5 mmoles/kg after 1 hr incubation as found in the influx experiments in 137 mM choline, must result in either a swelling of the cells or in a loss of K from the cells, if choline is not metabolized or adsorbed. As shown in Table II, the results on Na, K, Cl, and water content in preparations that were bathed in 137 mM choline for 1 hr are compared to control values. The values in Na Tyrode are close to the figures obtained in a previous study on

TABLE II
ION CONTENT OF CAT VENTRICULAR MUSCLE IN SODIUM AND CHOLINE TYRODE

	No. of observations	K	Na	Cl	Dry wt:wet wt*
		mmoles/kg wet wt			
Na Tyrode	9	69.2±2.1	54.1±3.0	55.9±2.5	25.2±0.4 (27)
Choline Tyrode after 1 hr incubation	9	41.1±3.9	2.1±0.3	62.4±2.2	24.2±0.4 (29)

* The ratio dry wt:wet wt was determined in other preparations.

the same preparation (Carmeliet and Janse, 1965). The constancy of the ratio dry weight:wet weight indicates that the cells did not swell in choline Tyrode. The extracellular space may also be assumed to remain constant in choline Tyrode: for two different series, each of eight preparations, the sucrose space, corrected for the slow component in the efflux curve, was 279 ± 22 ml/kg wet wt and 329 ± 18 ml/kg wet wt as compared with 282 ± 16 (7) ml/kg wet wt in Na Tyrode.

Sodium content declined to barely detectable values; potassium decreased to less than two-thirds of its original value, while chloride content slightly increased. As most K is intracellular, and extracellular K remains constant, the results indicate a loss of intracellular K amounting to 28.1 meq/kg wet wt. The combined loss of Na and K was 80.1 meq/kg wet wt, a figure which is close to the measured choline influx of 82.5 meq/kg hr. This finding provides strong support for the thesis that choline ions penetrate the cell membrane and exchange for intracellular K and Na ions.

3. *Paper Chromatography*

Further evidence that the radioactive material is choline was obtained by paper chromatography of extracts of tissues that had been incubated in 137 and 3.5 mM choline Tyrode (see Methods). Of the four preparations that were in-

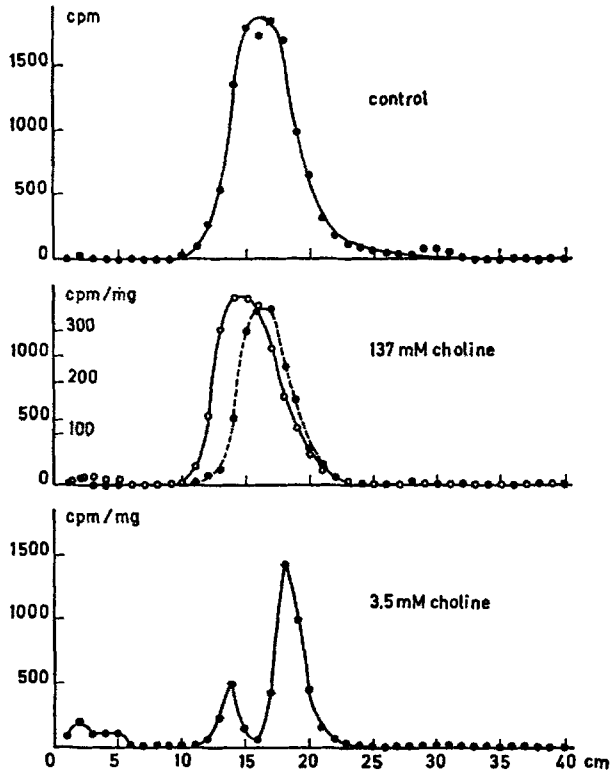

FIGURE 2. Radioactivity in strips of paper chromatograms as a function of distance from origin. The control (counts per minute) consisted of a muscle to which choline-^{14}C was added immediately before the extraction procedure. The three other chromatograms were obtained from muscles incubated in choline Tyrode for 60 min (137 mM) and 120 min (3.5 mM) and are expressed as counts per minute per milligram wet weight. The solid lines were obtained from muscles analyzed at the end of the influx period. The broken line, corresponding to the smaller ordinate scale, was obtained from a muscle at the end of a supplementary efflux period of 20 min.

cubated in 137 mM choline for 1 hr, two were analyzed at the end of the influx period, and two at the end of a supplementary efflux period of 20 min in choline Tyrode. The radioactive material of the former two consisted of an extra- and intracellular phase, the extracellular one certainly consisting of unmodified choline ions. The latter two preparations were washed for 20 min in inactive solution and contained only intracellularly located radioactive

material. Two other preparations were incubated for 2 hr in 3.5 mM choline and analyzed at the end of the influx period.

Three of the six chromatograms are shown in Fig. 2 and compared with the test substance to which a muscle was added before the extraction procedure (control). For the 137 mM choline series the peak of radioactivity coincided with the control curve; no difference was observed among the test chromatogram, the preparation cleared of extracellular choline, and the preparation containing both intra- and extracellular choline. The chromatogram obtained for the two muscles incubated in 3.5 mM choline showed a supplementary peak with a smaller R_f value and may indicate that part of the total radioactive material was not present as choline. This peak represented 19.9% of the larger peak or 14% of the total radioactive material.

4. *Choline Efflux*

In order to compare the behavior of choline with that of Na and Li, and to further substantiate the thesis of intracellular penetration of choline ions, choline efflux was studied under the same experimental conditions that were used for the influx experiments.

4. (a) 137 mM CHOLINE Choline efflux in 137 mM choline Tyrode was studied at 4° and 37°C. All preparations had been loaded for 60 min in the same solution at 37°C. In all, four series of eight experiments each were carried out, two series at 4°C, the other two at 37°C. The different series were analyzed separately, because in half the experiments choline efflux was measured simultaneously with sucrose efflux (see double tracer experiments).

Curves A and A' in Fig. 3 represent the decline of total choline in milliliters per kilogram wet weight (mean of eight experiments, double tracer series) as a function of time. The curves representing the two other series were not statistically different and for simplicity are omitted from the figure; the quantitative data on these experiments can be found in Table III (series 3 and 4). Choline efflux clearly is not exponential. By successive extrapolation and subtraction it was possible to distinguish three different phases.

About 455–488 ml/kg wet wt (sum of phases 1 and 2) exchanged during the first 10–15 min, while the rest (122–164 ml/kg wet wt) left the preparation very slowly. The sum of phases 1 and 2 was larger than can be accounted for by the inulin, mannitol, sucrose, or even the Cl and Na space; the Cl space was 378 ml/kg wet wt, and the Na space 361 ml/kg wet wt as can be deduced from the figures in Table II. The sum of phases 1 and 2 therefore cannot be due to extracellular choline alone, and part of it must be due to intracellular choline exchange. If, on the other hand, one assumes that phases 2 and 3 represent intracellular choline, then phase 1 should correspond to the extracellular choline fraction. The mean value for the four series ranged from

228 to 335 ml/kg wet wt and can be compared to the inulin, sucrose, or mannitol space in the same preparation.

It thus seems reasonable to assume that half of the intracellular choline (phase 2) exchanges very rapidly with a rate constant of 1.14–1.67 $\times$ 10^{-3} sec^{-1}, while the other half (phase 3) leaves the cells at a very slow rate.

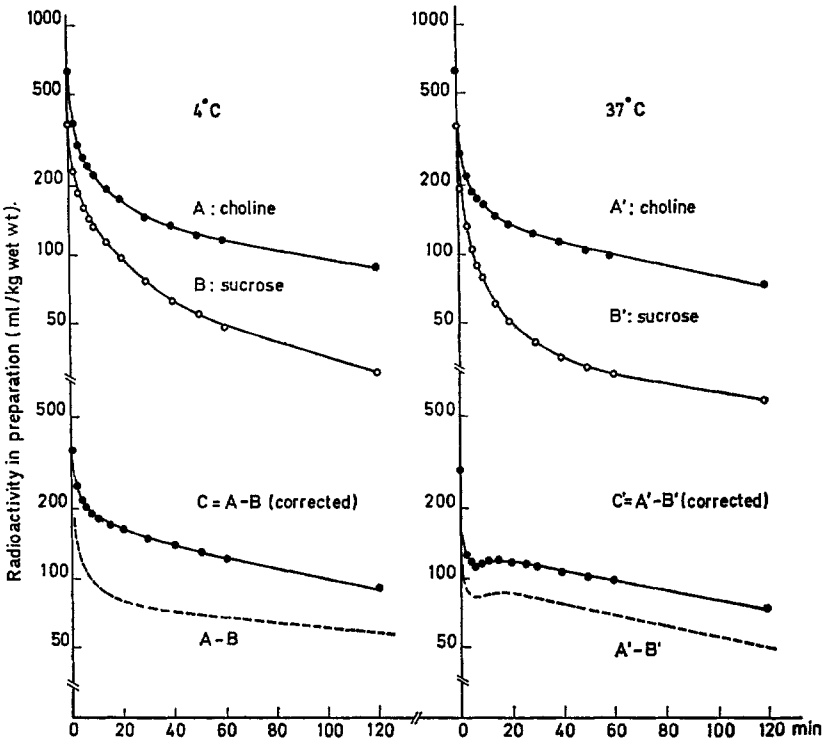

FIGURE 3. Simultaneous choline-^{3}H and sucrose-^{14}C efflux from cat ventricular muscles at 4° and 37°C. The curves show the composite efflux of two series of experiments, each of eight preparations. Influx and efflux solutions were identical (137 mM choline, 1 mM sucrose). An influx period of 1 hr at 37°C preceded the efflux. *A* and *A'*, choline-^{3}H efflux; *B* and *B'*, sucrose-^{14}C efflux; the broken line is the difference between *A* and *B*. Curves *C* and *C'* were obtained by subtraction of the sucrose efflux, after correction for the slow component (estimated by extrapolation between 60 and 120 min).

Absolute fluxes were calculated using the formula, $M = k \cdot V/A \cdot (C)_i$, where k is the rate constant in sec^{-1}, V/A the volume surface area (2.5 x 10^{-4} cm for an average diameter of 10 μ), and $(C)_i$ the intracellular choline concentration. $(C)_i$ was calculated from the intracellular choline volume (phases 2 and 3 in Table III), the extracellular space (phase 1, Table III), and the dry weight to wet weight ratio (24.17 $\pm$ 0.36, n = 29). Maximum efflux values were around 20 pmoles/cm^2·sec. It will be noted that the maximum

TABLE III

CHOLINE EFFLUX OF CAT VENTRICULAR MUSCLE IN 137 mM CHOLINE

	4°C			37°C		
	Phase 1	Phase 2	Phase 3	Phase 1	Phase 2	Phase 3
Choline efflux						
Series 1 and 2 (double tracer)						
Ml/kg wet wt	288±37 (8)	183±15	164±17	335±28 (8)	146±16	140±11
Rate constant, $sec^{-1} \times 10^{-3}$		1.20±0.09	0.084±0.009		1.67±0.29	0.088±0.009
Flux, $pmoles/cm^2 \cdot sec$		16.43	1.03		20.32	1.03
Series 3 and 4						
Ml/kg wet wt	228±22 (8)	260±24	155±30	294±27 (8)	161±28	122±13
Rate constant, $sec^{-1} \times 10^{-3}$		1.14±0.07	0.074±0.009		1.59±0.25	0.063±0.009
Flux, $pmoles/cm^2 \cdot sec$		19.45	0.75		19.23	0.58
Choline efflux minus corrected sucrose efflux						
Series 1 and 2						
Ml/kg wet wt	279±22 (8)*	189±41.2	168±23.3	329±18 (8)*	135±30	158±11
Rate constant, $sec^{-1} \times 10^{-3}$		3.27±0.38	0.088±0.008		—	0.079±0.008
Flux, $pmoles/cm^2 \cdot sec$		45.25	1.08			1.02

* Corrected sucrose space.

influx value was only 9 pmoles/cm²·sec and declined for longer equilibration times.

No great difference in choline efflux was observed between the series at 4° and 37°C (Fig. 3 and Table III). The influence of temperature was investigated more clearly by raising the temperature from 4° to 37°C during the course of choline efflux. Fig. 4 *A* represents the composite efflux of 10 preparations; efflux was started at 4°C and continued at 37°C after 30 min. The rate constant was $0.33 \pm 0.04 \times 10^{-3}$ sec^{-1} (10) between 20 and 30 min and rose to $0.70 \pm 0.12 \times 10^{-3}$ sec^{-1} (10) between 30 and 40 min, yielding a Q_{10} of 1.26 ± 0.02 (10). These experiments confirm the slight temper-

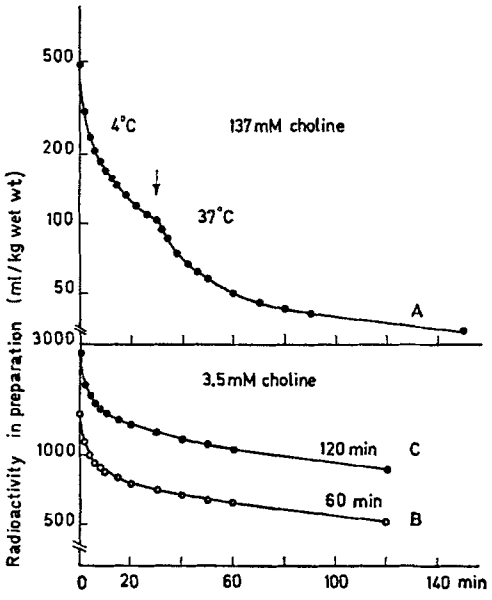

FIGURE 4. A, composite choline efflux of 10 preparations in 137 mM choline, after 1 hr influx in the same solution at 37°C. The temperature at the start of the efflux was 4°C and was raised to 37°C after 30 min. *B*, composite choline efflux of seven preparations in 3.5 mM choline after a preceding influx period of 60 min in the same solution. *C*, composite choline efflux of seven preparations in 3.5 mM choline after a preceding influx period of 120 min in the same solution.

ature dependence of choline efflux and seem to exclude an active transport mechanism for choline ions.

Double Tracer Experiments In order to better estimate the amounts of intracellular choline and its exchange kinetics, choline-^{3}H efflux was measured along with the efflux of sucrose-^{14}C, a substance that is used for estimating the extracellular space. All preparations remained for 60 min in a 1 mM sucrose-^{14}C, 137 mM choline-^{3}H Tyrode. By subtracting sucrose-^{14}C efflux expressed in milliliters per kilogram wet weight from the decline of choline content expressed in the same units, it was hoped to obtain a choline efflux curve, which would represent the release of choline from the cells.

The results of two series of experiments at 4° and 37°C, respectively, are shown in Fig. 3. Curves *A* and *A'* represent the decline of the total choline

content (mean of eight preparations), curves B and B' the decline of the sucrose content, and the broken lines the difference between both curves (total choline space minus choline space equivalent to the sucrose space).

Before analyzing these results it seems worthwhile to evaluate the double tracer technique.

The usefulness of the double tracer technique is based on the suppositions (*a*) that the rates of choline and sucrose diffusion through the extracellular space are approximately the same; (*b*) that sucrose is excluded from the intracellular compartment but fills the total extracellular space and is not adsorbed in any way. Neither of these suppositions is probably completely true:

1. The diffusion coefficient for sucrose in diluted solutions was estimated to be about 0.52×10^{-5} $cm^2 \cdot sec^{-1}$ (see Weast and Selby, 1966). No such information was found for choline ions. One of the factors determining diffusion velocity is the molecular weight. For substances with a molecular weight similar to that of choline, the diffusion coefficient lies around $0.7–1.0 \times 10^{-5}$ $cm^2 \cdot sec^{-1}$. del Castillo and Katz (1955) for instance have used 0.8×10^{-5} $cm^2 \cdot sec^{-1}$ as the diffusion coefficient for acetylcholine. If the diffusion rate of choline is higher than that of sucrose, the values corresponding to the sucrose space that are subtracted from the choline space will be too large and result in an overestimation of the choline efflux rate. This might explain (*a*) the dip in the choline efflux curve (Fig. 3) between 2 and 10 min and (*b*) the higher rate constants of the choline efflux (double tracer technique) as compared to the uncorrected choline efflux (phase 2, Table III). It was hoped to solve this difficulty by using sulfate as an extracellular marker (Keenan and Niedergerke, 1967; Page and Page, 1968). The diffusion coefficient for sulfate ions is 0.86×10^{-5} $cm^2 \cdot sec^{-1}$ (Nielsen, Adamson, and Cobble, 1952). Sulfate efflux was measured in four preparations. When subtracted from the mean choline efflux, however, the result was no better than that obtained from the corrected choline efflux, using sucrose, and a marked "dip" persisted.

2. Penetration of sucrose to some extent into the intracellular compartment or binding in the extracellular space might further induce some error and underestimate the intracellular choline content. The following facts favor the assumption that the sucrose space, after 60 min equilibration, overestimates the extracellular space. (*a*) The total sucrose space was 365 ± 29 (8) ml/kg wet wt in choline Tyrode and 356 ± 20 (8) ml/kg wet wt in Na Tyrode. This value is much larger than the inulin space (Carmeliet and Janse, 1965) and the mannitol space (Page, 1962 *b*); (*b*) in rabbit heart the sucrose space was found to be larger than the extracellular space estimated from histological sections (Johnson and Simonds, 1962); (*c*) sucrose efflux in the present experiments showed a very slow tail. This slow component might represent intracellular penetration or extracellular

adsorption. The possibility of intracellular penetration was checked in the following way. If sucrose penetrates into the intracellular medium at a slow rate, the slow tail should be largely dependent on the loading time. Sucrose efflux was therefore studied after a loading time of only 10 min instead of 60 min. In two series, each made up of eight preparations, the slow tail, however, persisted. The difference between these results and curves *B* and *B'* (Fig. 3), was due to a phase that exchanged with a half-time of 4–6 min and might represent extracellular penetration of sucrose into the deep layers of the preparation (see also Fig. 4 in Page, 1962 *b*, for mannitol and inulin). The slow tail then is best explained by adsorption to binding sites that hold the sucrose rather firmly.

A correction was therefore applied to the sucrose efflux curve by subtracting the slow tail, estimated by extrapolation between 60 and 120 min back to zero time. The extrapolated values (Fig. 3) were 70 ml/kg wet wt (4°C) and 37 ml/kg wet wt (37°C) and they result in a "true" extracellular space of 279 ± 22 (8) ml/kg wet wt and 329 ± 18 (8) ml/kg wet wt, respectively, which may be compared to phase 1 of the choline efflux curve (Table III).

Curves *C* and *C'* (Fig. 3) then represent the difference between the total choline space and the choline space equivalent to the corrected sucrose space. It is clear that the intracellular choline efflux consists of a fast and a slow phase. In Table III the amplitude of both phases, their rate constants, and the calculated absolute fluxes are summarized and can be compared to the values derived from the direct analysis of curves *A* and *A'*. All values correspond rather well, except for the rate constants and the absolute flux of the fast phase. Estimation by the double tracer technique yielded a much higher value; at 37°C an exact evaluation was even impossible because of the presence of a dip in the constructed efflux curve. These differences and the presence of a dip are thought to derive from the fact that sucrose diffuses more slowly through the extracellular phase than do choline ions.

4. (b) 3.5 mM CHOLINE Fig. 4 *B* and *C* shows the composite efflux of two series of preparations, loaded respectively for 60 and 120 min in 3.5 mM choline. As for the experiments in 137 mM choline, the curves were analyzed in three phases (Table IV). Half the total choline content (sum of phases 1 and 2) exchanged during the first 10–15 min. This amount represents a volume equal to the total water content of the preparation in the 60 min series and was double this value in the 120 min series. The largest part of it must therefore be due to intracellular choline exchange. In contrast to the findings in the 137 mM choline series, the first phase was larger than the extracellular space, as measured by sucrose or mannitol. The difference between phase 1 and the sucrose space was related to the total amount of choline taken up by the preparation: phase 1 increased from 400 to 600 ml/kg wet wt when the preceding influx was extended from 60 to 120 min. The significance of this

finding remains unclear. However, the following remarks can be made. (*a*) Our working hypothesis, i.e. the existence of an extracellular and two intracellular compartments, might be wrong. The analysis of a curve consisting of more than three exponentials, however, becomes quite arbitrary. (*b*) In the chromatograms of the two preparations loaded in 3.5 mM choline for 2 hr, the radioactive material had no uniform distribution. 14% of the total radioactivity or 375 ml/kg wet wt showed a smaller R_f value. This fraction might be due to choline that was actually metabolized. Such a metabolite may show a greater permeability than choline itself and may be partially responsible for the greater value of phase 1. The relative increase of phase 1 as a function of the loading time would be in accord with such an explanation.

From an analysis of phases 2 and 3 (intracellular choline), the following

TABLE IV
CHOLINE EFFLUX OF CAT VENTRICULAR MUSCLE IN 3.5 mM CHOLINE

	Phase 1	Phase 2	Phase 3
60 min series			
Ml/kg wet wt	400±47 (7)	347±64	762±44
Rate constant, $sec^{-1} \times 10^{-3}$		1.97±0.23	0.059±0.001
Flux, $pmoles/cm^2 \cdot sec$		1.329	0.087
120 min series			
Ml/kg wet wt	603±72 (7)	820±86	1,331±112
Rate constant, $sec^{-1} \times 10^{-3}$		1.95±0.18	0.063±0.009
Flux, $pmoles/cm^2 \cdot sec$		3.109	0.163

conclusions can be drawn: (*a*) choline efflux from the intracellular medium is characterized by a fast and slow fraction; (*b*) both fractions are functions of the loading time and practically double when the preceding influx is extended from 60 to 120 min. The rate constants, however, did not change and indicate, therefore, an increase in absolute flux. The efflux data in 3.5 mM choline are comparable to the results obtained in 137 mM choline. Supplementary information was obtained by extending the preefflux loading time and showing that the efflux in absolute values for both fractions was proportional to the intracellular concentration.

DISCUSSION

Choline Influx Choline ions were shown to penetrate very easily into cat ventricular cells. A modification by metabolism was ruled out (*a*) by the correspondence of the rise in intracellular choline content with the combined loss of Na and K; choline replaced mole for mole not only the extra- and in-

tracellular Na but also a substantial part of the intracellular K; (*b*) by a direct check of the identity of the radioactive material in the tissue as choline by paper chromatography. The radioactive material extracted from the tissues incubated in 137 mM choline Tyrode showed a distribution that was identical to that of choline added to a muscle immediately before the extraction. Of special importance was the observation of a uniform distribution in preparations containing both intra- and extracellular choline. Only in the experiments with 3.5 mM choline was there a small fraction (14% of the total) that showed a slightly smaller R_f value than choline. This amount is too small to account for the values of phase 2 or 3 in the choline efflux (Table IV; 120 min series), but might be responsible for the large value of phase 1 in these experiments. The reason why a fraction with a smaller R_f value was not seen in the 137 mM series could be found in the 10 times greater amount of choline accumulated by these preparations.

Choline influx in 137 mM choline Tyrode was estimated at 2.68–9.07 pmoles/cm^2·sec, a figure that closely resembles the sodium influx at rest in dog ventricular muscle (Conn and Wood, 1959; Langer, 1967), in rabbit atria (Carslake and Weatherall, 1962), and in frog ventricle (Keenan and Niedergerke, 1967). Choline ions, however, behave differently from Na in other aspects, and their behavior can better be compared to that of Li ions for the following reasons:

1. The intracellular choline concentration never tended to a steady state, but was continuously increasing while intracellular K decreased. From the data in Table I it can be calculated that the intracellular choline concentration in 137 mM choline rose to 92 mmoles/liter intracellular water after 1 hr and to 112 mmoles/liter at the end of the 2 hr incubation period. The increase in intracellular choline concentration in the 3.5 mM choline series, calculated in the same way, was practically linear with time and attained 18 mmole/liter after 2 hr, a concentration five times larger than the external concentration. The most plausible explanation for these results is to assume that choline ions are not actively pumped out, or, if they are, at a slow rate.

2. In 137 mM choline a pronounced net loss of intracellular K was observed. Similar net ion movements were observed in lithium Tyrode for the same tissue. As a consequence of the fall in equilibrium potential for K a decrease in resting potential might be predicted and was actually recorded by Page (1962 a). In two control experiments on cat ventricular muscles we have confirmed that the membrane potential after 1 hr in choline Tyrode may be lowered by as much as 30 mv.

Such a fall in resting potential will result in an increase of the intracellular Cl concentration. A rise in Cl content was actually observed in choline Tyrode. If Cl ions are distributed according to a Donnan equilibrium, their

intracellular concentration should be 8.7 mmole/liter intracellular water for a membrane potential of 75 mv. From the experimentally determined Cl content of 55.9 mmoles/kg wet wt a value of 8.7 mmoles/liter can be calculated if the extracellular space is assumed to be 354 ml/kg wet wt. When this value is taken as the extracellular space and the Cl content of 62.4 mmoles/kg wet wt in choline Tyrode one calculates an equilibrium potential of 46 mv for Cl ions under these conditions, a value which corresponds with the observed fall in resting potential. The weakness in this argument rests on the assumption of a rather large extracellular space. It was for this reason that different authors proposed a nonpassive behavior for Cl in heart muscle (Page, 1962 *b*; Lamb, 1960; Carmeliet and Janse, 1965).

The pronounced depolarization of cat ventricular muscle in choline Tyrode contrasts with the invariability or the increase of the membrane potential of sheep Purkinje fibers (Délèze, 1959, 1960; Hall, Hutter, and Noble, 1963). It should be noted, however, that guinea pig ventricle also depolarizes when Na is replaced by choline (Coraboeuf and Otsuka, 1956; Délèze, 1959). The difference might be due to a species factor or to the smaller surface: volume ratio of Purkinje cells.

Choline Efflux This could be separated into three components. The first phase corresponds to the extracellular space as measured by sucrose. The second and third phases represent intracellular choline.

Phase 2 exchanged very rapidly in both types of experiments (137 and 3.5 mM choline). Arguments were given in the section on results for saying that this phase was not extracellular. The outward movement corresponding to phase 2 cannot be regarded as an active transport. Its high rate of exchange compared to the influx, is not compatible with the fact that the intracellular choline concentration steadily increased. In quantitative terms choline efflux corresponding to phase 2 was two to five times larger than choline influx (compare Table I with Tables III and IV). A similar, but more pronounced discrepancy between influx and efflux was found for Na in the frog ventricle (Keenan and Niedergerke, 1967). Na influx as measured by these authors was only 2–3 pmoles/cm^2·sec while Na efflux amounted to 50–100 pmoles/cm^2·sec. Sodium efflux in mammalian cardiac Purkinje fibers at 37°C also yielded high values of 100–160 pmoles/cm^2·sec (Bosteels, 1969). The discrepancy between the Na influx and efflux as determined in the frog ventricle was tentatively explained by assuming a large proportion of Na "exchange diffusion" for Na efflux, while Na influx was measured as a net Na movement and represents therefore a true transmembrane flux (Keenan and Niedergerke, 1967).

The same phenomenon might explain the behavior of choline in our experiments. Choline influx was also measured as a net inward movement, while choline efflux was not: during the first minutes of choline efflux the

preparation can be regarded as being in a steady state; for later times intracellular choline was slightly increasing. Although no direct proof is given for the existence of exchange diffusion in the present experiments, this mechanism might also explain the increase in absolute value of the fast moving choline fraction, when influx was extended from 1 to 2 hr (3.5 mM choline Tyrode, Fig. 4) while the ratio of the fast over the slow fraction remained constant. According to Ussing's hypothesis (1949) exchange diffusion is proportional to the concentration as long as saturation of the carrier mechanism is not involved.

A second fraction of intracellular choline (phase 3) exchanged very slowly. The amount of choline present in this fraction increased as a function of the loading time. Its rate constant was not influenced by temperature (Table III) and seems to exclude any active transport mechanism.

The large difference in the rate of exchange of the two intracellular choline fractions might be explained by assuming two anatomically distinct intracellular compartments between which choline is poorly exchangeable. These compartments could be in parallel and/or in series.

With regard to a parallel or a parallel-series model one could speculate about the transverse tubular system and the sarcoplasm, as being the compartments responsible respectively for the fast and slow exchanging fractions or vice versa. The limited size of the transverse tubular system, however, is not compatible with the large volume of either of the two choline fractions.

With regard to a series model, one could assume that the rate of exchange between the intra- and extracellular media is determined not only by a surface resistance but also by the diffusibility in the intracellular medium (Harris, 1957; Ling, 1966). In order to accommodate the slowly exchanging fraction (25 mmole/kg wet wt after 120 min) one could imagine the adsorption of choline ions to anionic sites present in the cell matrix or in subcellular fractions. Our influx data, when plotted as a function of the square root of the time (Harris, 1957), are not contrary to this hypothesis. More experimental values are needed, however, especially between 30 and 120 min, in order to test adequately this possibility.

The uptake of choline by the muscle corresponded mole for mole to the combined loss of Na and K, while the cell volume stayed constant. This finding provides some difficulty for the adsorption hypothesis, unless one assumes that also Na and/or K ions are adsorbed to some extent. Although a direct proof of adsorption for Na or K in heart muscle has not been given, it should be stressed that Na efflux, as well as Li efflux, does not follow a simple exponential law (Carslake and Weatherall, 1962; Haas, Glitsch, and Trautwein, 1963; Carmeliet, 1964; Keenan and Niedergerke, 1967; Bosteels, 1969). More experimental evidence will be needed to elucidate the complex distribution of intracellular ions.

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