*ON THE STATE OF CREATINE IN HEART MUSCLE**

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Communicated July 17, 1961

It is well known[1-4] that a considerable fraction of the total creatine in heart muscle is present in such a form that upon extraction by conventional procedures, such as trichloroacetic or perchloric acid, it will react directly with the diacetyl reagent. It is conventionally called the free creatine fraction and amounts to about 7 mM per kg of rabbit heart muscle.[4] The concentration of free creatine in plasma is 0.03 mM per liter. There is therefore a steady-state difference in apparent concentration of 230 fold. Since creatine can be demonstrated to move across the muscle membrane, it would seem that there must either be a "pump" mechanism maintaining a steady-state concentration difference or the so-called free creatine must not all be actually in free solution in a compartment available for exchange with the extracellular fluids. The theoretical possibility that the membrane is virtually impermeable to creatine is discarded on the grounds that it is contrary to observation, and the possibility that there is a large source of creatine synthesis in the muscle cell is discarded also for lack of evidence for it.

In order to investigate further the state of creatine in muscle, studies have been made using isotopically labeled creatine with isolated perfused rabbit hearts. Creatine-1-C^{14} in tracer amounts has been incorporated in the perfusion fluid and the changes over time in the specific activity in the creatine phosphate and "free" creatine fractions in heart muscle measured. By such observations one can obtain information bearing upon the permeability of the cell membrane to creatine and upon the nature of the "free" creatine pool.

Methods.—Male rabbits about 3 kg body weight were anesthetized with sodium pentobarbital (50 mgm/kg) and thoracotomized under endotracheal ventilation with oxygen. After heparin (300 U.S.P. units/kg), the aorta was cannulated and perfusion of the coronaries with oxygenated saline begun as quickly as possible to avoid myocardial hypoxia. The perfusate at this stage was discarded. The heart was transferred to a temperature-controlled chamber (37°C) arranged for collection of the coronary venous outflow. After all blood was washed out, the venous outflow was returned to the reservoir system creating a closed circuit. The minimum volume used was 60 ml. The perfusion was at a pressure of 20 mm Hg maintained by finger-pump. The flow was about 25 ml per min.

The perfusion fluid was a phosphate buffered solution, NaCl 145.5, KCl 2.7, $CaCl_2$ 1.8, KH_2PO_4 0.17, Na_2HPO_4 1.48, and glucose 5.56 mM per liter. It was aerated with 100% oxygen. The initial pH was about 7.6. After a period of closed circuit perfusion, CO_2 and organic acids entered and the pH fell to 7.2–7.3.

The exchanges of creatine were studied in hearts beating spontaneously, during prolonged arrest, and also after a previously arrested heart was returned to a rhythmic contractile state. Arrest was induced by shifting to a calcium-free perfusate and in addition, introducing rapidly into the flowing perfusion fluid entering the coronary vessels 3.5 ml of a 0.20% solution of sodium citrate. The citrate rich perfusate was discarded, as was the first 50 ml of calcium-free perfusion fluid. The heart was maintained in asystole so long as the perfusion fluid was calcium-free. The beat was restored by shifting to perfusion with normal calcium level. The details of the ion effects upon arrest and recovery are complex and will be presented in another report.[5]

Creatine-1-C^{14} was added to the perfusion fluid at a concentration of 8.8 μM per 100 ml. The activity of the creatine was about 1.34 $\mu c/\mu M$. Since the entrance rate of creatine into the muscle cell is slow, it was essential to remove tracer-containing perfusate from the coronary

blood vessels and from the readily exchangeable extracellular space. Consequently, after perfusion with radioactive creatine solution, the heart was perfused with tracer-free solutions for the period of time found necessary in preliminary experiments for the venous effluent radioactivity to fall to a steady level, close to background. This required from 5 to 7 min of washout perfusion under different circumstances.

For analysis, the heart was frozen in liquid nitrogen, always after citrate arrest if the heart was not already in arrest. Previous studies[4] have demonstrated that creatine phosphate breakdown during freezing is prevented or diminished by prior calcium-lack arrest. The methods for creatine and creatine phosphate extraction and analysis have been described previously,[4] with the modification that 6% perchloric acid was used in place of trichloroacetic and neutralization with KOH allowed removal of the perchlorate. The extracts were passed through columns of Dowex-1 and Dowex-50 resins in a room at 2°C. The former retains creatine phosphate from neutral solutions but allows the creatine to pass. The creatine phosphate is eluted with ammonium formate solution, following the method of Hurlbert *et al.*[6] Dowex-50 retains creatine and allows creatine phosphate to pass with a water eluent. The radioactivity was measured with a liquid scintillation counter and specific activities calculated from the analytical concentrations and activities of creatine and creatine phosphate, respectively.

Results.—Table 1 presents the observed data from twenty-six experiments in which rabbit hearts were perfused with labeled creatine for periods from 5 to 90 min, followed by either 5 or 20 min of washout perfusion. It will be noted that the hearts tended to become edematous with longer perfusions as evidenced by a decrease in per cent dry weight, with some decrease (10%) in total creatine (per unit dry weight), and a definite decrease in the proportion of the total creatine that was present as the phosphate. These are not unexpected findings.

The more interesting observed facts relate to radioactivity present in the "free" creatine and creatine phosphate fractions, respectively. Here, contrary to what might have been predicted, the specific activity of the creatine phosphate fraction exceeds that in the "free" creatine moiety for the first 60 min of accumulation. The decline of the SA ratio after 60 min may be an artifact since the creatine phosphate concentration and fraction of total were both low and the dry weight values indicated considerable edema. The rate of rise of SA of "free"creatine from 60 to 90 min is apparently four times that in the preceding hour. The rate of rise of SA of creatine phosphate is also greater in the period 60 to 90 min than before, but the increase is very much less. It is possible that the occurrence of the edema may have resulted in some increase in trapped extracellular creatine which the standard washout perfusion of 5 min did not remove. A small difference in this quantity would create a large error, because the specific activity of the perfusate creatine was about 100 times that of the intracellular creatine, even at 90 min.

It will also be noted that when the time of washout perfusion is extended to 20 min, the observed specific activity of the creatine phosphate falls relatively little from the values obtained after 5 min of washout, while the SA of the "free" creatine declines to about 25 per cent of what it had been.

In Table 2 are shown the results of a series of experiments in which the tracer was presented to the heart muscle during periods of cardiac arrest, in comparison with the findings when the tracer was in the perfusion fluid only during a period of spontaneous beating after recovery from prolonged asystole. It may be noted that during asystole the incorporation of label in the creatine phosphate was definitely decreased, both in comparison with the incorporation in the same period of time with no prior arrest and after a period of arrest followed by recovery.

TABLE 1

CONCENTRATIONS AND SPECIFIC ACTIVITIES OF "FREE" CREATINE AND CREATINE PHOSPHATE IN PERFUSED RABBIT MYOCARDIUM

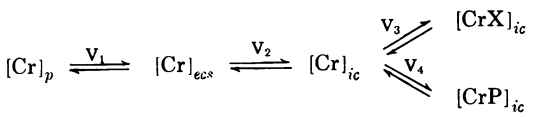
No. of rabbits	Perfusion with creatine-1-C^{14} (time in min)	Time of washout (min)	Dry wt (%)	Creatine μM/gm Dry Ventricular Muscle				Specific Activity		SA of Cr-P / SA of "Free" Cr
				"Free" Cr	Total Cr	Bound Cr-P	Bound (%)	"Free" creatine cpm/μM (range)	Creatine from Cr-P cpm/μM (range)	
4	5	5	14.3	30.04	66.45	36.41	54.8	173 (128–213)	276 (266–278)	1.59
2	10	5	14.9	29.85	68.47	38.63	56.1	286 (227–345)	461 (455–467)	1.61
2	15	5	14.6	30.92	65.27	34.35	52.6	391 (376–406)	696 (640–752)	1.78
5	30	7	14.5	34.50	65.80	31.30	47.5	650 (599–755)	968 (803–1,238)	1.49
3	60	7	13.7	33.21	62.29	29.08	46.7	1,141 (1,104–1,179)	1,415 (1,255–1,559)	1.24
4	90	7	11.9	33.97	60.92	26.95	44.2	3,302 (2,222–4,084)	2,440 (1,210–3,268)	0.72
3	15	20	14.8	26.41	61.60	35.19	57.1	96 (57–134)	510 (448–615)	5.31
3	30	20	13.2	24.81	55.27	30.46	55.0	183 (148–223)	772 (543–896)	4.22

TABLE 2

THE EFFECTS OF CARDIAC ARREST UPON THE INCORPORATION OF CREATINE-1-C^{14} INTO MYOCARDIAL CREATINE FRACTIONS

No. of rabbits	Perfusion with Creatine-1-C^{14} (time in min)			Time of washout (min)	Dry wt (%)	Creatine μM/gm Dry Ventricular Muscle				Specific Activity				SA of Cr-P / SA of "Free" Cr
	Normal beating	Arrest	Recovery			"Free" Cr	Total Cr	Bound Cr-P	Bound (%)	"Free" creatine cpm/μM	S.E.	Creatine from Cr-P cpm/μM	S.E.	
2	10	..	..	5	14.9	29.85	68.47	38.63	56.1	286 (227–345)	S.E.	461 (455–467)	S.E.	1.61
7	..	10	..	7	13.4	25.75	74.14	48.40	65.3	295	±45	173	±16	0.59
4	..	..	10	5	14.3	36.49	74.96	38.47	51.3	186	±30	259	±25	1.47

Discussion.—One can schematize the possible relationships between the various forms in which creatine exists in heart muscle and its environment, and the possibilities for its movement in position or in respect to form of chemical combination as follows:



In this expression the subscript *p* indicates plasma or perfusate, *ecs* the extracellular space, and *ic* the intracellular compartment. Cr refers to creatine, CrP creatine phosphate, and CrX a hypothetical creatine complex or state which will be considered later. On *a priori* grounds, it seems likely that the capillary wall offers no great barrier to movement and that V_1 is not a rate-limiting factor in the over-all transfer process. Therefore, $(Cr)_p$ probably is equal to $(Cr)_{ecs}$. However, the cell wall proper may or may not offer impediments to creatine diffusion. The relative magnitudes of V_2, V_3, and V_4 appear to be determining as to accumulation of the tracer. The facts presented in this paper bear upon these questions. There are several possibilities as to interpretation of the observed facts and they will be presented.

In terms of the scheme presented, the analytically determined "free" creatine comprises the sum of $(Cr)_{ic}$ and $(CrX)_{ic}$. The existence of CrX as a definite chemical entity has not been demonstrated, and its occurrence is being postulated to account for the facts (1) that the specific activity of the "free" creatine fraction in heart muscle is lower than that of the creatine phosphate and (2) that upon washout for 20 min., the specific activity of the "free" fraction falls very rapidly, indicating that some moiety of that fraction is able to leave the cell freely. The creatine phosphate specific activity fell comparatively less during the 20-min washout period.

An alternative possibility to the existence of a chemical entity CrX is that the Cr pool is not a unit but consists of pools in at least two compartments within the cell with high barriers to exchange between them. This hypothesis would appear to require the existence of low permittivity "membranes" within the cell separating such pool compartments in order to account for the fact that the "free" creatine specific activity rises so slowly during accumulation and falls so rapidly during washout. Such a multicompartment system is, of course, not impossible, but since such systems are not known to exist, it seems simpler to suppose that a fraction of the "free" creatine is somehow bound in such a way that it does not exchange rapidly with the true free creatine pool. Creatine is known to complex readily with other substances.[7]

If one accepts tentatively the postulates implicit in the scheme presented, it becomes possible to suggest that $(Cr)_{ic}$ may have a value equal to $(Cr)_{ecs}$ and that there may normally be no concentration gradient whatever for creatine across the cell wall. If $V_2 \ll V_1$, and $V_4 \gg V_3$, then the facts as observed in the tracer experiments can be harmonized with the scheme.

In the experiments performed, as noted in the description of methods, a period of 5 min of washout was used routinely to remove intravascular and readily diffusible

extravascular (presumably *ecs*) label. The experimental design was therefore not adequate to a precise quantitative evaluation of the several V values. Since the SA of the perfusion fluid creatine was about 100 times that of the CrP at 90 min, we cannot know the SA values of Cr_{ecs} and Cr_{ic} with any certainty after the washout period. A better design will be difficult to set up with an intact heart, but perhaps with thin strips of muscle one could measure the necessary variables. Nevertheless, certain facts in relation to rates are quite evident. The fact that the arrested heart shows an increase in label in the CrP which is a little less than half that found in beating hearts indicates that the reaction CrP $\rightleftharpoons$ Cr + P is proceeding during arrest, although at a slower rate. Since the oxidative metabolism of the heart is also greatly reduced by arrest, this result is not surprising. Since oxygen consumption measurements were not made in these experiments, it is not possible to say whether or not the CrP turnover was precisely proportionate to the oxidation changes. It is worth noting, however, that CrP turnover is not limited to a contraction-linked process. The increase in "free" creatine label was not less in the arrested than in the normal heart, or in one restored to beating after a 10-min period of arrest, indicating that its accumulation may be unrelated either to contractile process or to total oxidative metabolism. This deduction should be accepted with reserve, however, because the arrested heart was perfused at a lower pressure, since earlier observations showed that edema occurred quickly in perfused quiescent hearts, and the washout period was extended from the usual 5 min to 7 min, which was found necessary to bring the washout fluid to stable counts near background. Thus the conditions were altered sufficiently in the case of these seven hearts to make comparisons of absolute values of "free" creatine radioactivity dubious, but for the CrP and the SA ratios the values are undoubtedly valid, especially since extra washout time in the hearts perfused with labeled creatine during normal contraction produces exactly the opposite change in SA ratio from that found in this series.

Summary.—Studies on the rates of incorporation of creatine-1-C^{14} into the separated and analytically measured creatine phosphate and so-called "free" creatine fractions in perfused isolated rabbit hearts have shown that the specific activity of the creatine phosphate fraction rises more rapidly than does that of the "free" fraction. Upon washout the specific activity of the "free" fraction falls three times as rapidly as does that of the creatine phosphate fraction. Cardiac arrest slows but does not stop the incorporation of label in the creatine phosphate. The creatine phosphate breakdown and resynthesis is therefore not exclusively contraction-linked.

The kinetics of movement of label are incompatible with the assumption of a homogeneous "free" creatine pool. Reasons are presented for favoring the view that the fraction conventionally measured as free creatine consists of at least two chemical entities, one bound in such a way as to be freed during acid extraction. The actual concentration of free creatine in the cell need not be higher than that in the extracellular fluid to account for known facts, if one assumes the existence of a creatine complex which comprises most of the analytically determined "free" creatine.

* Aided by grant H-3212, U.S. Public Health Service.

† Established Investigator of the Minnesota Heart Association

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*A THEORY OF GASEOUS ANESTHETICS**

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Communicated by Joseph E. Mayer, July 5, 1961

In the course of an investigation of gas hydrates at low temperatures and their occurrence in the solar system,[1] it was noticed that many of the gases which form hydrates, such as ethylene, nitrous oxide, chloroform, and xenon, are also gaseous anesthetics.[2] A comparison of the pressure of anesthetic necessary to maintain a person in a given stage of anesthesia and the dissociation pressure of the hydrate at 0°C shows that there is indeed a correlation. The data are shown in Table 1. The ratio of anesthetic pressure to hydrate dissociation pressure is in the range 0.1 to 0.5, except for the low value of carbon dioxide and the high values of sulfur hexafluoride and xenon. The ratios are about the same for both Structure I hydrates (Gas·6 to 10 H_2O) and the Structure II hydrates (Gas·17 H_2O).

Taking into account the uncertainties of the data, the correlation can be considered good, with some of the agreement probably being fortuitous. This correlation is as good as the correlations obtained with the Meyer-Overton lipid theory of anesthesia.[5]

The gas hydrates: The gas hydrates are clathrate compounds of a gas encaged in a distorted ice matrix (for reviews, see refs. 3 and 17). These crystalline compounds occur in two forms, and the structures have been determined[12, 17–20]. Structure I has a cubic unit cell 12.0 Å on edge containing 46 water molecules and 8 cavities. The two smaller cavities are pentagonal dodecahedra each formed by an array of 20 water molecules giving a cavity of 3.95 Å which can encage molecules with diameters of 5.1 Å or less. The six larger cavities are tetrakaidecahedra (with two opposite hexagonal faces and 12 pentagonal faces) each formed by an array of 24 water molecules, giving a cavity of 4.3 Å radius which can encage molecules with diameters of 5.8 Å or less.

Structure II hydrates have a cubic unit cell of 17.4 Å on edge containing 136 water molecules and 24 cavities. The sixteen smaller cavities are distorted pentagonal dodecahedra each formed by an array of 20 water molecules giving a cavity of 3.91 Å radius which can encage molecules with diameters of 5.0 Å or less. The eight larger cavities are hexadecahedra each formed by an array of 28 water molecules giving a cavity 4.73 Å radius which can encage molecules with diameters of 6.7 Å or less.

Gases which form hydrates but are not anesthetics: There are a number of gases,