THE PHYSICO-CHEMISTRY OF CREATINE AND CREATININE

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While a vast amount of work has been done in recent years upon the biological relationship of creatine and creatinine,[1] the facts of a purely chemical nature have remained isolated in a number of widely scattered references, quite often appearing in papers the titles of which would not lead one to expect the contents.

Formerly, the lack of sources for creatine and creatinine precluded an exhaustive study of the chemical relationship of these compounds, but in recent years the discovery of a cheap source of supply of creatine (5), and improved methods for the transformation of creatine into creatinine (6) has led to extensive investigation.

It is the purpose of the present paper to make the facts relating to the chemistry of creatine and creatinine more readily available for the biological chemist, as well as other workers in the field.

OCCURRENCE AND PREPARATION

Creatine was discovered in 1834 by von Chevreul in meat extract (7). It has been found in the muscles of vertebrates in constant amounts (8), in the flesh of many animals (9), in the brain (10), in blood (11), and in urine under certain abnormal pathological conditions (12).

Creatinine occurs normally in urine and was discovered therein

[1] An excellent discussion of the metabolism of creatine and creatinine is given by Benedict and Osterberg (1) and subsequent papers of the same series in which the chemical relationship of the compounds involved are given consideration. Pioneer work in this field was done by Folin (2) and by Klercker (3). A very complete summary of the literature through 1922 with reference to creatine-creatinine metabolism is given by Hunter (4), and later through 1926 (69).

by Liebig (13). It has been found in a number of fluids and tissues for the most part of animal origin; in dogs' urine (11), in certain fish (14), in the muscles of mammalia (15), beef extract (16), crab extract (17) and in soils and grain seed (18).

Creatine may be synthesized from cyanamide and sarcosine. It is usually prepared from meat extractives and its preparation in this manner has reduced the price considerably thus making available quantities sufficient for large scale research.

Creatinine was formerly prepared exclusively from urine (19, 20, 21), a long, tedious and expensive process. It is now prepared from creatine, there being several procedures available. The method of Folin and Dennis (22) involves the heating of solid creatine in an autoclave, while that of Benedict (20) involves the intermediate formation of creatinine zinc chloride and subsequent decomposition of this salt with ammonia. Probably the most satisfactory method for all purposes is that of Edgar and Hinegardner (6) the principle of which depends upon the conversion of creatine into creatinine hydrochloride by treatment with hydrochloric acid and subsequent formation of creatinine from this salt by treatment with ammonia. Advantage is taken of the facts that ammonia will liberate creatinine from its salts; that creatinine is only moderately soluble in cold concentrated ammonia; that ammonium chloride is readily soluble under such conditions, and that ammonia has but little tendency to bring about the conversion of creatinine into creatine. A yield of 85 to 90 per cent may be expected, depending upon the technique. It is quite as well adapted to large as to small scale preparation. The product is quite pure, the actual degree depending upon the purity of the original creatine.

PURIFICATION AND PROPERTIES

Creatine may be purified by recrystallization from water at comparatively high temperatures. It is fairly insoluble in cold water and a good yield is obtained in this manner. Charcoal is employed to absorb foreign coloring matter and the final product is pure white, odorless and finely crystalline. The solid thus prepared contains one molecule of water of crystallization (23).

The purification of creatinine by crystallization presents certain difficulties. If water at a high temperature, or aqueous alcohol, is used as solvent there is always the danger that some creatine may be formed and as it is much less soluble in all ordinary solvents than creatinine, it is almost impossible to effect complete separation by crystallization. Undoubtedly the most satisfactory method is that of Edgar and Hinegardner (6) in which one part by weight of creatinine is dissolved in five parts of water previously heated to 65°C. as rapidly as possible; two volumes of acetone are immediately added and the mixture cooled in ice. After standing several hours the precipitated creatinine is filtered off, washed with acetone and dried. About 65 per cent of the product is recovered.

Creatine crystallizes in hard, colorless, bitter monoclinic prisms which lose their water of crystallization at 100°C., becoming then white and opaque.[2] It is soluble in 74 parts of water at 18°, becoming more soluble in hot water. It is only slightly soluble in alcohol and insoluble in ether. In aqueous solution its reaction is slightly alkaline (13). Its heat of combustion is 4240 calories per gram (25) and its decomposition temperature is 291 to 291.5°C. (corr.) (24), which is 12 to 15°C. lower than the value given by Mulliken (26).

Creatine upon hydrolysis yields methyl-hydantoic acid, urea, methyl-amino-acetic acid and carbon dioxide (27). It reduces boiling mercuric oxide to metallic mercury and the creatine is oxidized to methyl-guanidine and oxalic acid. Its reducing action is, however, far less marked that that of creatinine. Mercuric nitrate in neutral solution precipitates creatine; it is not precipitated by cadmium chloride or lead acetate. Creatine forms soluble normal salts with the mineral acids (28), and readily soluble double compounds with zinc chloride and cadmium chloride (29).

The homologues of creatine are prepared by the action of cyanamide upon the corresponding amino-acids in the presence

[2] According to Williams and Lasselle (24) four out of five persons to whom samples of creatine were submitted, pronounced the substance tasteless. Its crystals are characterized as being thin, tabular monoclinic prisms.

of ammonia (30). Upon treating creatine with absolute alcohol and dry hydrogen chloride, esterification of the carboxyl group is effected, rather than ring closure. In this manner esters were readily obtained with methyl-, ethyl- and n-butyl alcohol (31).

Creatinine crystallizes in anhydrous monoclinic prisms, soluble in 11.5 parts of water and 100 parts of absolute alcohol at 16°C. It is practically insoluble in ether and acetone. Its heat of combustion is 4986 calories per gram (25) and its decomposition temperature is given as 260° to 270°C.

Creatinine is a weak base being displaced by ammonia from its salts and forming soluble salts with the mineral acids (32). It is a much stronger reducing agent than creatine; mercuric oxide is reduced to metallic mercury, and an alkaline solution of copper hydroxide is reduced by creatinine. It forms an easily soluble crystalline compound with hydrochloric acid, and a number of double salts of which creatinine zinc chloride is a typical member (33). A number of the double compounds of creatinine are quite insoluble; viz., creatinine potassium-, rubidium- and cesium picrates, and are useful in isolating small quantities of creatinine (34). It is precipitated by mercuric nitrate and -chloride solutions (35). A number of the acyl- and alkyl-derivatives of creatinine have been prepared (36).

ANALYTICAL

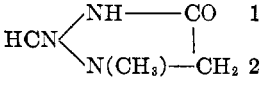
Creatine cannot be estimated quantitatively as such, but is usually converted into creatinine by evaporation with hydrochloric acid and thereby determined.[3] Creatinine is almost invariably determined colorimetrically, there being several reactions by which characteristic colorations may be brought about. Weyl's reaction (38) makes use of the addition of a few drops of sodium nitroprusside to an aqueous solution of creatinine in which a ruby-red color develops after making alkaline with sodium hydroxide. Salkowski's reaction (39) is brought about

[3] Creatine may be quantitatively converted into creatinine hydrochloride by evaporation with hydrochloric acid to dryness over a steam bath (37), or by passing gaseous hydrogen chloride through creatine solution at room temperature (6). If desired, the creatinine may be displaced by ammonia and hence recovered.

by the addition of excess acetic acid and warming the solution obtained in Weyl's procedure, a distinct yellow coloration being produced.

Jaffe's reaction (40), the development of a red color upon the addition of picric acid[4] to an aqueous solution of creatinine made alkaline with sodium hydroxide, is almost universally employed for the quantitative estimation of creatinine. The general method as outlined by Folin (42) with later improvements (43) consists in treating 20 cc. of creatinine picrate solution containing 0.5 mgm. creatinine per cubic centimeter (1.5119 grams salt per liter) with 25 cc. saturated picric acid solution and 10 cc. of sodium hydroxide solution (10 per cent). The standard so prepared is allowed to stand for seven minutes and is then compared in a colorimeter with the unknown creatinine solution. Care should be taken to have the two solutions at as nearly equal concentration as possible and treatment of the standard and unknown should be as nearly uniform, in general, as possible.[5]

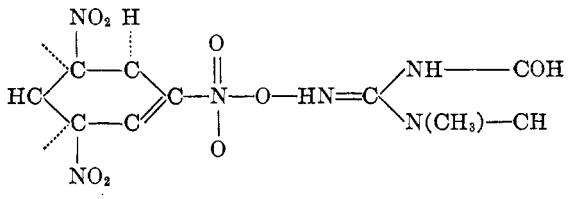
While Jaffe's reaction has been used for some time in the analysis of creatinine, it has been but recently that a study has been made of the chemistry involved (45). Thus it is probable that there is a keto-enol change within the creatinine molecule between positions 1 and 2,



[4] According to Benedict (41), commercial picric acid is quite unsuited for use in the determination of creatinine *per se*. It was found that many samples yielded a color with alkali alone. For best results the picric acid must be purified. Thus, to 400 grams of commercial picric acid 1 liter of pure benzene is added, heated to boiling and decanted through a filter. Heat the filtrate again in order to bring into solution such picric acid as may have crystallized out, allow to set over night. The crystals are washed twice with benzene and dried in the air. About 85 per cent recovery of acid is obtained.

[5] According to Morris (44) creatinine may be determined in the presence of acetone, acetoacetic acid or glucose. The creatinine is precipitated as the double salt of potassium and creatinine, the precipitated salt re-dissolved after washing, and the creatinine determined colorimetrically. As standard a solution of potassium creatinine picrate is employed. Precipitation is not complete and a correction factor must be applied.

and a change in the picric acid molecule involving the hydrogens in the meta positions and, probably, all three nitro groups, giving rise to a red tautomer which, for the present, is written



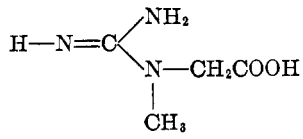
The broken lines indicate that the exact positions of these hydrogens and the disposition of the remaining valencies of the carbon atom are unknown.

According to Chapman (46), the red coloration developed in this reaction is due to the formation of the sodium salts of picramic acid (monoamino-dinitrophenol) and diamino-nitrophenol. Apparently the only foundation for this theory lies in the fact that a match was able to be obtained between the creatinine reaction product and a mixture of these substances.

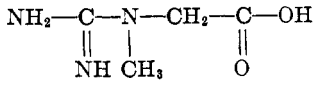
CHEMICAL RELATIONSHIP

While it is not within the scope of this paper to discuss fully the various theories relating to the origin of creatine and creatinine in the processes of metabolism, it is felt that a brief description of some of these would throw much light upon the chemical nature of the two compounds.

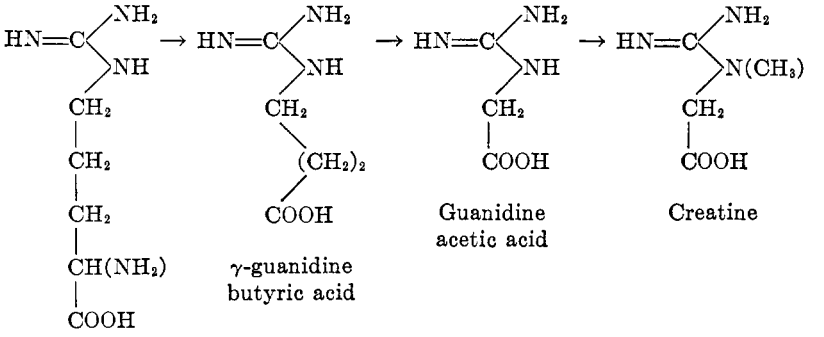
Creatine (Greek *kreas*, flesh) may be considered as methylguanidine-acetic acid,



or as methylglycocyamine,



It is thus related to arginine (δ-guanidine-α-amino-valerianic acid), from which it might be derived by methylation:

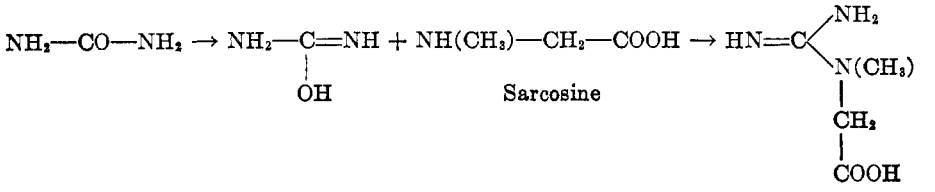


Arginine

On the other hand, it may be considered as a ureide of methyl glycocoll, which, with cyanamide, gives creatine. Thus,

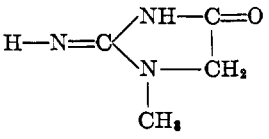
$$NH_2—CN + HN(CH_3)—CH_2—COOH \rightarrow NH_2—C(=NH)—N(CH_3)—CH_2—COOH$$

Considering cyanamide as an anhydride urea it may be prepared therefrom by the action of sodium; and cyanamide yields urea when treated with 50 per cent sulfuric acid. It would seem possible according to Mathews (47) that urea might give a similar synthesis with amino-acetic acid. Thus,

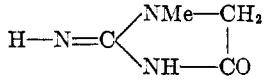


Creatine

Creatinine may be considered as methyl-guanidine-acetic acid anhydride,



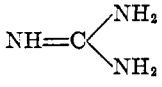
or as l-methylglycocyamidine. It may be synthesized by the action of guanidine carbonate,



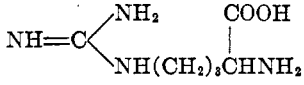
upon sarcosine at 140° to 160°C.

Its discovery as a constituent of soils and vegetable matter seems to point to the fact that it need not necessarily be considered as having origin exclusively in animal matter.

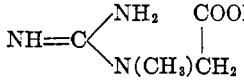
There is a very interesting structural relationship between arginine and guanidine on the one hand, and creatine and creatinine on the other. Thus, according to Shorey (18):



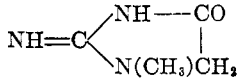
Guanidine



Arginine

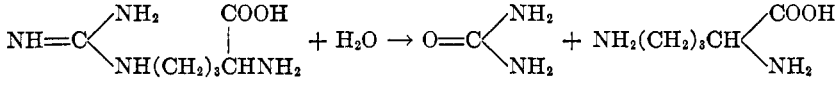


Creatine

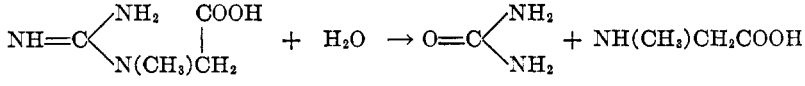


Creatinine

The close relationship existing between arginine and creatine, and thus creatinine, is shown by their behavior upon treatment with barium hydroxide:



Arginine Urea Ornithine



Creatine Urea Sarcosine

This relationship is also shown in the synthesis of the two compounds, creatine having been synthesized from sarcosine and

cyanamide (48), while that of arginine has been effected by the action of ornithine upon cyanamide (49). As a matter of fact, arginine has been found in the soil (50), and guanidine has been found in plants (51).

The purine bases bear a relationship to guanidine and hence to creatinine, and it is interesting to find that guanine, the only commonly occurring purine base not found in soils, gives guanidine upon oxidation; and further, that guanine is a constituent of organic fertilizers. This possible connection of creatinine with the purine bases and nucleic acids is, however, to be regarded as purely speculative.

PHYSICO-CHEMICAL

From the standpoint of physical chemistry, and perhaps biochemistry as well, the most important property of creatine and creatinine is the readiness with which they are transformed, the one into the other, under the influence of practically every aqueous medium—ammonia solution alone, to which reference has been made, being the only such medium directly inhibiting the mutual transformation. However, a very profound effect upon the extent and direction of the conversion is produced by the nature of the medium. Accordingly, it is necessary to consider the reaction in the light of these effects.

IN AQUEOUS SOLUTION

As early as 1847 Heintz (52) found that a precipitate of creatinine zinc chloride is produced upon cooling a solution of creatine which had been boiled with zinc chloride. Again, Wörner (53) obtained Weyl's reaction for creatinine by merely heating a solution of creatine to the boiling point, and further found that such a solution deposits crystals of creatinine picrate upon treatment with picric acid and allowing to stand. The complete conversion of creatine to creatinine in aqueous solution has been reported by Neubauer (54) when the solution is heated at the boiling point for several days in a closed tube, and the partial conversion by simply evaporating slowly upon the water-bath.

The reverse transformation is not so readily detected although

Dessaignes (55) showed in 1857 that creatinine was slowly converted into creatine, more rapidly by heating, under the influence of water alone. Later, Johnson (56) was able to convert creatinine almost completely into creatine by evaporating a solution of the former and removing the crystals of the latter from time to time as they were formed.

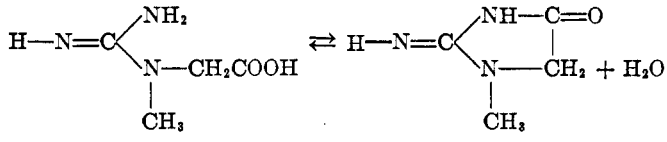
These facts, while purely qualitative, nevertheless indicated that the reaction was reversible. Considering the importance of the transformation in the theory of metabolism, it is surprising that the rigorous characterization of the equilibrium conditions

TABLE 1

Equilibrium constants at several temperatures

	25°C.	50°C.	70°C.	100°C.
K.....	0.54	1.02	1.59	2.89

in aqueous solution has but recently been accomplished. Thus in 1925, Edgar and Shiver investigated the reversible reaction (23),



Creatine Creatinine

and from the expression,

$$K = \frac{(\text{Creatinine})}{(\text{Creatine})}$$

obtained the results shown in table 1.

The results show conclusively that the reaction proceeds definitely to a condition of equilibrium in aqueous solution; that the equilibrium is markedly affected by temperature; and that the proportion of creatinine is increased with increasing temperature. Obviously, the statement occasionally appearing in the literature

that creatine can be quantitatively converted to creatinine by continued boiling with water alone is incorrect.

From the values of K at the several temperatures employed an equation has been derived from that of van't Hoff (the reaction isochore)

$$\frac{d \ln K}{d T} = \frac{\Delta H}{RT^2}$$

for calculating the equilibrium constant at any temperature. Solution of the above yields for ΔH, the heat absorbed in the reaction, the value 4963 calories. Integrating and converting to ordinary logarithms, this expression becomes

$$\log K = \frac{-\Delta H}{2.303\ T} + C$$

and by substitution of the appropriate values of ΔH, K and T, the mean value of C, the constant of integration, becomes 3.3652.

TABLE 2

Comparison of results

	25°C.	50°C.	70°C.	100°C.
K, calculated.....	0.541	1.024	1.604	2.830
K, determined.....	0.540	1.022	1.593	2.890

Thus the value of the equilibrium constant at any temperature may be calculated by the expression,

$$\log K = \frac{-1084}{T} + 3.3652$$

In table 2 is given a comparison of the results experimentally determined with those calculated from this equation.

Both creatine and creatinine are very weak bases and in pure aqueous solution are not appreciably dissociated, that is, the value of C_{OH^-} is always large as compared with the dissociation constants of the two bases; accordingly these values represent the equilibrium between the undissociated molecules. It is

apparent that the temperature markedly affects the rate at which equilibrium is established. Myers and Fine (57) state that the equilibrium is attained at 36°C. only after eleven months, while Hahn and Barkan (58) find $2\frac{1}{2}$ hours sufficient at 98°C. In the work cited above, the equilibrium had been established at 25°C. in 2460 hours, at 50°C. in 150 hours, at 70°C. in 70 hours and at 100°C. in 4 hours.

IN ACID SOLUTION

In acid solution of sufficient concentration creatine may be completely converted into creatinine. The destruction of the equilibrium state in acid solution is explained by Hahn and Barkan and by Edgar and Shiver on the assumption that the actual equilibrium is one between undissociated molecules, and that the apparent equilibrium is displaced by acids in the direction of the more dissociated base creatinine. Concerning the equilibrium conditions at intermediate hydrogen-ion concentrations no data at all are available before the work of Edgar and Shiver who have followed the progress of the reaction throughout the entire range.

Combining the above viewpoint with the simplifying assumptions employed in dealing with solutions of weak bases and their salts, the following expression has been derived for characterizing the effect of hydrogen-ion on the creatine-creatinine ratio:

$$\frac{m}{n} = \frac{K_3\left(\frac{K_w}{10^{-pH}} + K_1\right)}{\frac{K_w}{10^{-pH}} + K_2}$$

or more simply,

$$\frac{m}{n} = K_3\left(\frac{K_1 + [OH^-]}{K_2 + [OH^-]}\right)$$

in which n and m are the total concentrations of creatine and creatinine in all forms, K_1 and K_2 their ionization constants and K_3 the equilibrium constant for their molecular species (as previously determined).

From these equations certain qualitative conclusions may be drawn immediately. Thus when K_1 and K_2 are small compared with C_{OH^-},

$$\frac{m}{n} = K_3$$

and when K_1 and K_2 are large compared with C_{OH^-},

$$\frac{m}{n} = \frac{K_3 K_1}{K_2}$$

These two equations represent the limiting equilibrium conditions in alkaline and strongly acid solutions, respectively. For intermediate concentrations, quantitative values for K_1 and K_2 are necessary.

TABLE 3

Comparison of experimental and calculated results

TEMPERATURE	pH	m/n CALCULATED	m/n DETERMINED
°C.			
50	1.00	36.72	38.08
	2.00	18.98	31.00
	3.00	4.01	3.61
	4.00	1.33	1.47
	5.00	1.05	1.11
	6.00	1.03	1.08

By the employment of buffer solutions the effect of hydrogen-ion between pH 1.8 and 6.2 on the ratio m/n has been experimentally determined at 50°C. These results are shown in table 3, the observed values of m/n at round pH values being read from a graph. The values given in the third column of the table are those calculated by means of the expression given above. The values of K_1 and K_2 were those given by Hahn and Barkan at 18°C., 1.85×10^{-10} and 4.6×10^{-12}, respectively. It is apparent that the agreement is on the whole fairly good. However, when the newly determined values of K_1 and K_2 at temperatures more nearly equal that of the experiment are substituted the agreement is not so good, due possibly to the undetermined effect of tem-

perature upon the constants, or more probably, to the fact that the relationship is exponential.

For pure aqueous solutions C_{OH^-} is always very large as compared with K_1 and K_2, consequently the ratio m/n gives the true value for the equilibrium constant between molecular creatine and creatinine under such conditions. Any increase in C_{OH^-}, making the solution more alkaline, will not affect m/n, so that the same value for the equilibrium constant will be obtained in alkaline solutions as in aqueous solutions. In acid solution C_{OH^-} becomes negligible with respect to K_1 and K_2 and m/n reaches the limiting value, K_3K_1/K_2. Using the values above, the ratio $K_1/K_2 = 46$, thus nearly all the creatine will be converted into creatinine in such a solution, the actual fraction depending upon the value of K_3 which in turn depends upon the temperature as noted.

Even if the values utilized for K_1 and K_2 are in error, examination of the equation derived above shows that the same type of variation of m/n with pH must obtain in every case. Thus it will be noted that the apparent equilibrium shifts very slowly up to the region of about pH 4. In the region between this value and pH 1, the shift is very rapid, the ratio m/n showing at this latter point a conversion of 97.5 per cent of the creatine into creatinine. From pH 1 to infinite acid concentration the shift again becomes very slow. From neutrality, pH 7, throughout the entire range of alkalinity, to pH 14, the ratio m/n is unchanging as noted above, and under the conditions of the experiment corresponds to a conversion of creatine into creatinine of about 50.8 per cent.

The conversion of creatine into creatinine under the influence of strong acids has long been known, but it has been only recently that quantitative studies have been undertaken regarding the velocity of the reaction. Hahn and Barkan were first to follow the course of the reaction, but confined themselves to a normal concentration of hydrochloric acid and a temperature of 26°C. Under these conditions the transformation of creatine into creatinine proceeded to completion and followed the course of a simple monomolecular change. Later Hahn and Meyer (59) indicated

that the velocity of the reaction in buffered solutions increased rapidly fron pH 6 to pH 4.

A much more elaborate study of the kinetics of the reaction under various concentrations of hydrochloric acid and at several temperatures has been made by Edgar and Wakefield (60). A typical experiment is represented in table 4, with results for reaction-velocity constants calculated for a first order reaction.

The effect of temperature upon the velocity-constants may be calculated by substituting the values obtained at various temperatures in the Arrhenius equation, and solving for E. Thus

$$\frac{d \ln K}{d T} = \frac{E}{R T^2}$$

Calculation of E, the critical increment or heat of activation, leads to the value 20,000 calories which is for all practical pur-

TABLE 4

Reaction-velocity constants

Temperature 78°C. HCl 0.76 molar

	TIME					
	0 minutes	15 minutes	30 minutes	45 minutes	75 minutes	120 minutes
Conc. creatine, mol/l.....	0.0300	0.0250	0.0208	0.0171	0.0114	0.0066
Vel. const., K = 1/T ln a/(a − x).....		0.0122	0.0120	0.0124	0.0127	0.0124

Average K = 0.01235.

poses independent of the acid concentration and the temperature. Integrating this expression there is obtained the equation,

$$\ln K = \frac{E}{R T} + C$$

where C, the constant of integration, has a value depending upon the concentration of acid. Converting to ordinary logarithms this becomes

$$\log K = -\frac{4368}{T} + C$$

from which at 0.19 N HCl, C = 9.8496; at 0.38 N HCl, C = 10.1538, and at 0.76 N HCl, C = 10.5400. Table 5 gives a comparison between the calculated results and those experimentally determined, and indicates the range of conditions covered by the experiments.

It is apparent that the agreement between the determined and calculated constants is fairly satisfactory. If the values of C are plotted against the acid concentration, values of the constant for any acid concentration may be obtained by interpolation, and the approximate velocity constant at any temperature may thus be obtained. From the data it is shown that the velocity-con-

TABLE 5

Comparison of results

TEMPERATURE	CONC. HCl	INITIAL CONCENTRATION CREATININE	K DETERMINED	K CALCULATED
°C.	*mols/l*	*mols/l*		
25	0.38	0.00750	0.000034	0.0000314
57	0.38	0.0300	0.000777	0.000824
78	0.19	0.00447	0.00256	0.00254
100	0.38	0.0075	0.02816	0.02780
78	0.38	0.0075	0.00494	0.00512
100	0.19	0.00367	0.01367	0.01380
78	0.76	0.0300	0.01235	0.01245
78	0.76	0.0150	0.01256	0.01245

stants increase with increasing acid concentration, the slope of the velocity-constant-acid concentration curve passing through a minimum at about 0.4 N HCl. It would thus appear that the velocity-constants are proportional to the hydrogen-ion concentration, although an exact comparison could not be made from the available data.

According to Cannan and Shore (61) the data fail to take into account the relationship between the hydrogen-ion concentration and the velocities of the reactions under the conditions in which the reaction is reversible. They argue that if the equilibrium be determined by the ratio of the concentrations of the undissociated molecules of creatine and creatinine, the velocities of the two opposing reactions should be governed by the same factors.

That is, the velocity should be inversely proportional to the hydrogen-ion concentration on the acid side of the buffer range of K_b, the basic ionization constant of either reactant, and should be independent of the hydrogen-ion concentration on the alkaline side. However, the available data indicate that the velocities are proportional to the hydrogen-ion concentration in solutions of strong acids, and inversely proportional in strongly alkaline solutions.

In an attempt to explain these discrepancies, the writer undertook a series of kinetic investigations in buffered solutions between pH 1 and pH 10. The velocity expression is put in the following form:

$$k_1 + k_2 = \frac{1}{t} \ln \frac{Ka}{Ka - (K + 1)x}$$

in which k_1 is the monomolecular velocity constant for the hydration of creatinine, k_2 that for the dehydration of creatine, K the equilibrium constant for molecular creatine and creatinine and therefore equal to the ratio k_2/k_1, while a and x have their usual significance.

This expression may be utilized for the calculation of k_1 and k_2 provided K is known. By the employment of Edgar and Shiver's equations the value of K at 30°C. has been calculated as 0.6125. Making further use of these equations, Cannan and Shore relate K to C_{H^+} by an equation which, when k′ values are substituted for k_b values ($pk' = pk_w - pk_b$), takes the form:

$$K = 0.6125 \frac{k'' (k' + C_{H^+})}{k' (k'' + C_{H^+})}$$

This equation has been utilized for calculating K at the various pH values of the reaction mixtures. Upon substitution of these values of K into the previously noted expression, with the corresponding velocity data, a reasonable constancy of the term $k_1 + k_2$ within a single experiment is obtained.

The data show that in solution acid to about pH 3 the conversion of creatine to creatinine is one of the first order, and is substantially irreversible. Furthermore, in following the course of

the reaction it was found that the relation of velocities to pH exhibited a well defined optima at about pH 3. The data for a typical experiment on the acid side of pH 3 is given in table 6.

TABLE 6

Rate of dehydration of creatine at 30°C.

pH = 2: K = (Creatinine/Creatine) = 62.3: Conc. creatine 0.0106 M

t	a	x	$k_2 = 1/t \ln a/(a-x)$	$k_1 + k_2 = 1/t \ln Ka/[Ka - (K+1)x]$
25	100	1.97	80.0×10^{-5}	80.0×10^{-5}
75		5.58	76.4	76.4
125		9.12	76.4	76.4
170		12.25	76.8	76.8
385		24.33	72.5	73.1
865		46.18	71.5	72.9
1346		59.25	66.7	68.3
2017		76.92	72.7	75.2

TABLE 7

Rate of dehydration of creatine at 30°C.

pH = 3.77: K = (Creatinine/Creatine) = 5.673: Conc. creatine 0.0106 M

t	a	x	$k_2 = 1/t \ln a/(a-x)$	$k_1 + k_2 = 1/t \ln Ka/[Ka - (K+1)x]$
25	100	7.29	304.0×10^{-5}	350.0×10^{-5}
50		15.39	334.0	398.0
75		21.77	327.0	393.0
125		31.93	308.0	377.0
170		38.17	283.0	350.0
385		64.62	269.0	370.0
695		79.98	232.0	405.0
1130		83.33	159.0	347.0
1896		85.00	99.0	370.0

In table 7 there is given the data concerning the velocity relationship on the alkaline side of pH 3, and a comparison of the values for k_2 and $k_1 + k_2$.

Cannan and Shore state that their data are chiefly valuable in showing that while the equations of Edgar and Shiver accurately interpret the equilibrium data they do not adequately define the velocity data. That is, the velocity constants are related to C_{H^+} in a manner not apparent in the equilibrium constant, a rela-

tion which is exhibited in a retarding effect of both velocities alkaline to pH 3. The writers infer that since this is not reflected in a change in the equilibrium constants, the factors responsible must be of equal effect upon the two reactions, and further that this same factor is responsible for changes in both k_1 and k_2 within this range.

Considering the data from pH 2 to pH 10, it has been possible to derive equations based on Edgar and Shiver's treatment, but involving three empirical constants, which define the relationship of k_1 and k_2 to C_{H^+} within the range pH 2–pH 10. In more strongly acid or alkaline solutions these equations are invalid. The equations follow:

$$k_1 = \frac{A' k' [C' + (H^+)]}{[k' + (H^+)] [C + (H^+)]}, \quad k_2 = \frac{A'' k'' [C' + (H^+)]}{[k'' + (H^+)] [C + (H^+)]}$$

where $A' = 3.68 \times 10^{-3}$: $C' = 0.8 \times 10^{-6}$: $A'' = 2.25 \times 10^{-3}$; and $C'' = 1.9 \times 10^{-5}$. The calculated and observed values agree quite well within the range of pH indicated. It is pointed out that equations of this sort are of value solely in establishing an approximate summary of a mass of data, and it is particularly dangerous to attach any material significance to the empirical constants. However, Cannan and Shore are considerably nettled by finding that the value of C is identical with the dissociation constant of creatinine, and they state that it is difficult to see how the dissociation constant of creatinine can affect the intrinsic velocity of dehydration of creatine. The constants A and A′ are the values which k_1 and k_2 would have were they determined only by the concentration of undissociated creatine and creatinine respectively.

A determination of the relative rate of transformation of creatine into creatinine in muscle extract, brain extract and of pure creatine in Tyrode's solution used for making extracts has recently been made by Hammett (62). According to this work, the velocity constant calculated from the equation for a monomolecular reaction for the transformation of creatine into creatinine in Tyrode's solution buffered to neutrality at 38°C. was found to be 0.00058. That for muscle extract similarly buffered

and otherwise under the same conditions was 0.00119, while that for brain extract similarly treated was 0.00104. The rate of creatine transformation into creatinine is thus twice as great for extracts of brain and muscle tissues, which are approximately equal, as for that of creatine in buffered Tyrode's solution.

IN ALKALINE SOLUTION

Creatinine is at least partially converted into creatine in alkaline solution, the reaction having served as a method of preparation of creatine from creatinine according to Benedict. The change may be induced by any free base whatsoever. Hahn and Barkan succeeded in demonstrating the fact that the reaction in alkaline media was a reversible one, in spite of the fact that both creatine and creatinine undergo a progressive decomposition in such media. By a series of approximations they measured the equilibrium constant, K = (Creatine)/(Creatinine) at 26°C. in sodium hydroxide solutions ranging from N/1 to N/10. This value was found to be approximately 2.12, and it is interesting to compare this value with that of Edgar and Shiver at 25°C. in pure aqueous solution. The latter writers determined the reciprocal of Hahn and Barkans constant, and this value 0.54 agrees extremely well with that obtained above, 0.47. The fact that K remained sensibly constant throughout a tenfold increase in alkalinity is a good indication of the validity of Edgar and Shiver's equations which require that the constant remain the same throughout the entire alkaline range.

Hahn and Barkan further show that the rate of change of creatinine into creatine corresponds to that of a reversible monomolecular reaction. Substitution of the data into the characteristic expression for such a reaction yielded for $k_1 + k_2$ a constant, the value of which measured in N/1 NaOH in time units of 1 hour was found to be 0.079 to 0.084. The velocity of the two opposed reactions varied with the C_{OH^-} concentration, although a direct proportionality could not be established. Later Hahn and Fasold (63) found that the rate of transformation of creatine into creatinine progressively decreases as the alkalinity increases. This they explain by the assumption that it is the free creatine,

rather than creatine combined with base, that is involved in the conversion. Velocities predicted on this basis agree well with those observed, since as the concentration of free alkali is increased the quantity of free creatine arising from the hydrolytic decomposition of its alkali salt will decrease.

The rate of transformation of creatine into creatinine just on the alkaline side of neutrality is of considerable interest in any biological theory concerning the function of the two in metabolism. From the results of Cannan and Shore and those of Hahn and Meyer an interesting deduction may be made. Thus Hahn and Meyer found that at 38°C. in a solution of creatine of pH 7.01 a conversion of 1.32 per cent of the total creatine to creatinine was produced in 24 hours. This corresponds very closely with the creatinine in the adult urine as compared with the total body creatine. Thus, from this point of view, the origin of the creatinine in the urine is nothing other than the spontaneous dehydration of the creatine, and is governed by the active mass of creatine in the muscles, the temperature and the pH of the muscle. This is confirmed by the work of Cannan and Shore whose velocity constant for the conversion of creatine to creatinine at 30°C. and pH 7.2 is 0.000023, a value which becomes 0.000043 at 38°C. by application of the temperature correction of Edgar and Wakefield. This then corresponds to the dehydration of 1.03 per cent of the active mass of creatine per 24 hours, and is sufficient to account for the daily output of creatinine in the urine provided that the active mass of creatine in muscle is as great as 0.5 per cent. However, it now appears probable that only a small proportion of the creatine in living muscle is free, and it is very unlikely that the combined creatine is as readily converted into creatinine as the free creatine.

DISSOCIATION CONSTANTS

The various physico-chemical formulas characterizing the relationship of creatine and creatinine in various media are practically all concerned at one point or another with the ionization constants of the two substances. Considerable uncertainty has existed in the past concerning the ionization constants of these

compounds, Hahn and Barkan having reported at 17°C. the values 1.85×10^{-10} and 4.6×10^{-12} for creatinine and creatine respectively, while Wood (64) gives 3.57×10^{-11} and 1.81×10^{-11} at 40°C. Later work by McNally (65), Eadie and Hunter (66) and Cannan and Shore has led to the establishment of more concordant values. Several independent methods were employed in the determination of these values and the general agreement among them does much to enhance their value. In table 8 are listed the results obtained by the several investigators at the various temperatures employed.

TABLE 8

Basic ionization constants of creatine and creatinine

TEMPERATURE	CREATININE $K_b \times 10^{-10}$	AUTHOR	CREATINE $K_b \times 10^{-12}$
°C			
15	4.70	Cannan and Shore	
17	1.85	Hahn and Barkan	4.8
17		Cannan and Shore	2.8
20	6.40	Eadie and Hunter	9.6
25	7.00	McNally	
25	7.60	Cannan and Shore	5.5
30	1.50	Cannan and Shore	7.4
30	9.80	Cannan and Shore	7.4
40	10.10	McNally	
40	0.357	Wood	18.1

Thus certain differences exist between the values of different observers which cannot be explained as differences in temperature. The result for creatinine at 40°C. as reported by Wood is obviously incorrect, due as McNally points out, to the difficulty of carrying out measurements involving the rate of hydrolysis of methyl acetate at so high a temperature and at such low acid concentration. The first value for creatinine at 30°C. as given by Cannan and Shore is evidently a typographical error.

From the standpoint of its chemical behavior considerable interest attaches to the electrolytic dissociation of creatine. Its constitution indicates that it is an ampholyte, the conventional formula containing both an amino-group and carboxyl. In its

chemical behavior only the properties of a base are exhibited, and this but slightly as its ionization constant shows. Nevertheless in certain of its reactions it appears to be dissociated as an acid. Thus Hahn and Fasold found that creatine is more soluble in dilute sodium hydroxide than in water, 3.148 per cent in N/1 NaOH at 12°C. and 1.48 per cent in water, indicating a degree of hydrolysis of the creatine-sodium salt of 0.47, and therefore corresponding to an acid dissociation constant for creatine of $K_a = 5.2 \times 10^{-15}$. This is to be compared with its basic dissociation constant at 17°C, from which it is noted that creatine is about 1000 times weaker as an acid than as a base. Cannan and Shore find it difficult to justify on the grounds of organic chemistry the allocation of the first dissociation constant of creatine to the amino-group, and the assignment of purely negligible acid properties to the carboxyl group. They feel that a more likely interpretation would follow the application of Bjerrum's treatment of the amino-acids, the first constant ($k_1' = k_w/k_b$) becoming the acidic constant and the second (k_2'), the association constant of the basic group. Certain difficulties are inherent in such a view, and in any case, no clearer coordination of the dissociation constants with the physico-chemical data is obtained from this point of view.

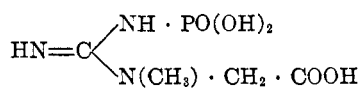
CONVERSION MECHANISM IN VIVO

The physico-chemical conditions governing the mutual conversion of creatine and creatinine *in vitro* have been fairly thoroughly established, as the foregoing indicates, while the direct application of the same principles to the transformation *in vivo* have not been eminently successful. The chief seat of creatine and creatinine in the body is the muscle and urine respectively, and the function of creatinine as an end product and derivative of creatine is accepted. This being the case the simplest application of the physico-chemical data would lead to the view that the mechanism *in vivo* is a simple dehydration of creatine, governed solely by the pH, the temperature and the creatine content of muscle. A necessary consequence of this view is that the creatine of the muscle be free, that is, present as such, otherwise

it may exist in such a state as to be incapable of direct conversion to creatinine.

The evidence has been accumulating for some time indicating that the greater portion of creatine in muscle is not present as such. Numerous investigators have postulated the existence of a creatine-containing complex, but until quite recently no such substance has been identified. In any case, if such a complex exists the data would seem to require that it be a combination of the loosest possible kind. It was, therefore, with greatest significance that Fiske and Subbarow (67) reported final success in isolating such a creatine complex in muscle.

According to this work and that later reported in confirmation by Eggleston and Eggleston (68) the compound consists of one molecule of creatine and one molecule of phosphoric acid, phosphocreatine according to the one and phosphagen according to the other. It is readily hydrolyzed in acid medium and has heretofore evaded discovery in that the usual conditions for the determination of phosphorus in muscle caused its destruction with the phosphorus appearing as inorganic phosphorus and the creatine as such. Fiske and Subbarow find its calcium salt to have the composition $C_4H_8O_5N_3Ca \cdot 4H_2O$, and suggest as its most probable structure,



They point out that its most characteristic chemical property, marked instability in acid solution, is characteristic of the few other compounds containing the group $-NH \cdot PO(OH)_2$. It is the first substance in which phosphorus is attached to nitrogen to be isolated from natural sources, and the instability of the phosphamic group makes it one of considerable biological importance.

Fiske and Subbarow have determined the second dissociation constant of phosphagen by titration of the calcium salt with acid, and find $k_2' = 2.5 \times 10^{-5}$, a value some 250 times as great as the corresponding constant of o-phosphoric acid. This is due to the unmasking of the carboxyl group and indicates that one of

the functions of phosphagen in muscle is to neutralize a considerable portion of the lactic acid formed during muscular contraction. Their data indicate that the third constant is considerably less than 10^{-7}, and subsequent calculations show that the hydrolysis of phosphagen liberates sufficient base to neutralize the lactic acid formed up to a concentration of about 0.23 per cent. This hydrolysis would then seem to be the principal factor in permitting contraction of muscle to a limited extent without fatigue.

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