CLI. THE METABOLISM OF CREATINE

II. THE CONVERSION OF ARGININE INTO CREATINE IN THE ISOLATED RABBIT HEART

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THE use of the whole animal for the study of the metabolism of creatine has a number of disadvantages. A large body of evidence exists which indicates that quite considerable quantities of creatinine may be injected into an animal, or administered parenterally, without the subsequent appearance of any extra creatine in the urine. Furthermore, the recent work of Benedict & Behre [1937] and of Gaebler [1937] appears to show conclusively that there is no creatinine in the blood of many animals which excrete creatinine, or at least that the upper limit of creatinine concentration in the plasma is so low that it is obvious, from what is known concerning the creatinine clearance, that the creatinine in the urine must originate in the kidney. It follows that it should be possible to produce profound changes in the amounts and proportions of creatine and creatinine which are excreted by an animal merely by altering the activity of the kidney. In addition, a very large number of the known hormones is capable of influencing the excretion of one or both of creatine and creatinine, and finally, most of the possible precursors of creatine have alternative paths of metabolism, so that there is little possibility of demonstrating a quantitative conversion of precursor into creatine and thus meeting the criticism that any extra production of creatine observed may be due to a stimulant rather than to a participant action of the putative precursor. It follows that such a wealth of alternative hypotheses is available for the interpretation of the results of experimental intervention in the creatine metabolism of the whole animal that a clear-cut solution to any of the fundamental problems of creatine metabolism is hardly likely to be obtained by means of such intervention.

For this reason we have attempted the investigation of the creatine metabolism of an isolated organ. We have chosen the rabbit heart since the organ is of sufficient size for reasonably accurate work, and yet will remain in good condition after a considerable period of perfusion with a simple Ringer-Locke solution, if the proper precautions are taken. Further, the heart has the advantage that its spontaneous activity provides a simple measure of its condition. And, lastly, it is an example of a muscular tissue and is therefore of the class of tissue in which it might be expected that creatine synthesis would be manifest.

Methods

(1) *Perfusion.* It is necessary to be able to determine the total creatinine of the perfusate with precision, as inspection of Tables XIII and XIV in the appendix to this paper will show. It will be seen that the total creatinine of the perfusate may comprise from 3.4% (perfusion 90: Table XIII) to 20.5% (perfusion 64: Table XIV) of the total creatinine of the heart plus perfusate, and it is clear that data for the heart alone would be useless.

In order to be able to determine the total creatinine of the perfusate accurately we have used a closed-circuit perfusion apparatus which is shown diagrammatically in Fig. 1. We were fortunate to obtain the use, from Sir Henry Dale, of the apparatus used by Bodo & Marks [1927] in their investigation of the effect of insulin on sugar utilization of the isolated perfused heart. We have modified the apparatus in detail, in so far as the heating system, water-jacket and heart cannula are concerned, and have added a flowmeter.

Procedure. The apparatus is set up and 60 ml. of perfusate are placed in the upper reservoir. The flowmeter and cannula are filled and all bubbles are removed by appropriate manipulation. The oxygen supply is turned on and the fluid is allowed to circulate at the rate of 12–16 ml./min. until the temperature of the fluid in the heart cannula is steady. This circulation ensures (a) that any particles detached from the glass-wool plug in the upper reservoir shall not enter the heart once they have been washed through the cannula, (b) that the whole of the perfusion fluid shall be oxygenated and (c) that the perfusion temperature shall be steady from the beginning of the perfusion. The rabbit is killed by a blow on the neck and the heart is exposed by one of us. The other meanwhile stops the circulation of the perfusion fluid, fixes the heart cannula in a clamp and starts a slow flow of the fluid into a 25 ml. graduated cylinder fitted with a funnel. The heart is excised and the aorta is tied on the cannula. The perfusate is allowed to flow freely into the heart, and that which escapes is collected in the cylinder. As soon as it is judged that the heart is reasonably free from blood, the cannula is transferred to the heart vessel and the circulation of the perfusion fluid is begun. The volume of fluid in the cylinder is measured and, if necessary, enough of the perfusion fluid (at the perfusion temperature) is added to the reservoir to make the total volume 50–60 ml.

The times of the death of the animal and the placing of the heart in the perfusion circuit are noted, the latter time being taken as the beginning of the perfusion. At 5 min. and again at 15 min. after this time the heart rate is taken, the temperature of the perfusion fluid noted and the flowmeter read. At 20 min. 10 ml. of the perfusate are removed from the upper reservoir with a pipette and expelled into a 25 ml. centrifuge tube containing 2 ml. 25 % trichloroacetic acid. 10 ml. fresh perfusate, at the perfusion temperature, are added to the upper reservoir. The volume of the perfusate is thus kept constant throughout the perfusion. This procedure is repeated exactly for six periods of 20 min.

After the final sample has been taken, the heart rate, the perfusion temperature and the flow are quickly noted, and the perfusion is stopped. The heart is removed and freed of excess moisture by gentle squeezing between hardened filter papers. Fat and connective tissue are removed as completely as possible and the heart is cut into two approximately equal parts, care being taken to include nearly equal portions of both ventricles in each part. One half of the heart is placed in a tared stoppered bottle, weighed and put into an oven to dry at 110°. The other half is placed in a tared 50 ml. centrifuge tube containing 10 ml. 10 % trichloroacetic acid, weighed and finely minced with scissors which are finally washed with 10 ml. distilled water. This is set aside for extraction and determination of the total creatinine of the heart.

Fig. 1. Closed-circuit perfusion apparatus.

The perfusion samples are centrifuged and the clear supernatant liquid is decanted into clean dry 25 ml. beakers from which aliquots are taken for the analyses.

Since the analysis of separate samples of the perfusate has not yielded results of any particular significance, a shorter procedure has been adopted in recent perfusions. The samples of the first five periods are pooled by running them into a 100 ml. centrifuge tube containing 10 ml. 25 % trichloroacetic acid. The sixth sample is run into a 25 ml. centrifuge tube containing 2 ml. 25 % trichloroacetic acid. At the end of the perfusion the tubes are centrifuged, and aliquots of the protein-free fluid are taken for the analyses.

(2) *Control hearts.* In order to provide data on the dry weight and total creatinine of the normal, unperfused heart, another rabbit is killed during the perfusion and its heart is removed, washed in Ringer-Locke solution until free of blood, and treated in exactly the same way as the perfused heart.

(3) *Extraction of the heart muscle.* The finely minced heart is extracted three times with 20 ml. 5 % trichloroacetic acid. The minced muscle is kept in the acid for 1 hr. or more, and the supernatant liquid is filtered through hardened filter-paper into a 100 ml. volumetric flask. The particles of tissue remaining on the filter-paper are picked off with a glass rod and returned to the mass of the tissue in the centrifuge tube. The tissue is then vigorously ground for a few minutes with the glass rod, and the tissue is dispersed, and the rod thoroughly washed, by the addition, first of 10 ml. 10 % trichloroacetic acid and then of 10 ml. distilled water. After the final extraction, the tissue is transferred to the filter-paper and the tube and tissue are washed with 10 ml. of the acid, then with several small portions of distilled water. The extract is made up to volume with distilled water, and aliquots of this are used for the determination of the total creatinine of the heart.

The efficiency of this method of extraction was tested by extracting a number of hearts five times. The fluid from the last two extractions was filtered into a 50 ml. volumetric flask and made up to volume with distilled water. 20 ml. samples were used for the estimation. The results of these extractions are

Table I. *Recovery of total creatinine from three and from five extractions of the heart tissue*

Heart	Total creatinine (mg.)		% of total creatinine in 4-5
	Extracts 1-3	Extracts 4-5	
1	4.043	0.036	0.88
2	5.285	0.065	1.21
3	4.153	0.055	1.31
4	5.029	0.064	1.25
5	3.169	0.026	0.81
6	4.689	0.030	0.63

given in Table I. This seemed to afford satisfactory evidence that we were recovering at least 98 % of the creatinine, preformed and in the form of creatine, in the heart tissue.

(4) *Determination of total creatinine.* Duplicate 5 ml. aliquots of the protein-free fluid from the perfusates and triplicate 1 ml. aliquots of the heart extracts are taken for the estimation of total creatinine by the method described in Part I [Fisher & Wilhelmi, 1937]. In addition the total creatinine in 15-25 ml. of the heart extracts has been determined by the Folin open-flask method, as described by Peters & Van Slyke [1932]. Certain observations are presented

below which we have made on (a) the recovery by the micro-method of creatinine added to perfusates and heart extracts, (b) the effects of glycocyamine and arginine on the micro-method, (c) the effects of glycocyamine on the estimation of the total creatinine of heart extracts by the macro-method and (d) the correspondence between the two methods when applied to extracts of control hearts and of glucose- and arginine-perfused hearts.

(a) Table II illustrates the recovery of creatinine from perfusates and heart extracts. The figures demonstrate that the recovery is to all intents and purposes quantitative. The probable error of an estimation is $\pm 1\gamma$, or 5 %

Table II. *Recovery of added creatinine*(Figures expressed in γ)

Creatinine of sample	Creatinine added	Creatinine found	Recovery	Percentage recovery
Heart extracts:				
20.7	20.0	43.9	23.2	116
13.7	20.0	35.4	21.7	109
12.6	20.0	33.5	20.9	105
14.6	20.0	37.5	22.9	115
47.7	10.0	57.2	9.5	95
47.7	20.0	65.6	17.9	90
37.9	20.0	51.0	13.1	66
Perfusates:				
15.5	20.0	29.8	14.3	72
19.0	20.0	36.7	17.7	89
30.5	8.6	39.0	8.5	99
13.2	20.0	32.6	19.4	97
14.4	20.0	29.7	15.3	77
33.8	10.0	43.2	9.4	94
33.8	20.0	53.0	19.0	95

of the creatinine added in most of the above experiments, and it is not surprising therefore that an occasional recovery depending on the difference between two sets of estimations should differ from the ideal by more than 10 %.

(b) Table III a shows the effect of glycocyamine on the estimation of the total creatinine of heart extracts. In these experiments the conversion of creatine into creatinine was carried out in the presence of the glycocyamine, since it was to be expected that glycocyamidine formed as a result of heating in

Table IIIa. *Effects of glycocyamine on the micro-method*

Total creatinine present γ	Glycocyamine added mg.	Total creatinine found γ	Difference γ
30.0	0.5	40.25	10.25
30.0	0.5	36.47	6.47
30.0	1.0	44.42	14.42
30.0	1.0	49.39	19.39
30.0	1.5	67.97	37.97
30.0	1.5	59.61	29.61

acid solution would have more marked effects on the estimation than glycocyamine itself, and in consequence, the effects of any glycocyamine present on the estimation of the total creatinine in the perfusates could be properly examined only if the glycocyamine underwent the conversion procedure. These data indicate that an amount of extra creatinine equal to 1.3–2.5 % by weight of the glycocyamine present appears under the conditions of the micro-method.

Table III *b* demonstrates the absence of effect of arginine on the estimation of total creatinine by the micro-method.

Table III *b*. *Effects of arginine on the micro-method.*

Arginine present γ	Total creatinine (γ)		% recovery
	Theoretical	Observed	
675	16.87	16.94	100
701	16.87	14.98	89
546	16.87	14.78	88
690	16.87	17.31	103

(c) Table IV shows the effect of glycocyamine on the estimation of total creatinine by the macro-method. The figures indicate that an amount of

Table IV. *Effect of glycocyamine on the direct estimation of creatinine.*

Total creatinine (mg.)		
Heart extract	Heart extract plus 1 mg. glycocyamine	Difference
0.35	0.43	0.08
0.55	0.64	0.09
0.76	0.88	0.12
0.76	0.92	0.16
1.01	1.12	0.11
	Mean	0.11

apparent extra creatinine appears which is equal to 11% by weight of the glycocyamine present. The micro-method, therefore, appears to be much more specific than the macro-method with respect to glycocyamine.

(d) In three series of hearts determinations of the total creatinine were made by both the macro- and the micro-methods. The mean difference in the amounts found by the two methods was (1) for 17 control hearts, $+1.32 \pm 0.93 \gamma/\text{ml.}$; (2) for 10 glucose-perfused hearts, $-0.61 \pm 1.73 \gamma/\text{ml.}$; (3) for 10 arginine-perfused hearts, $+0.73 \pm 0.67 \gamma/\text{ml.}$

It is obvious that there is a good correspondence between the two methods in all three groups of hearts, and that there is particularly good correspondence in the group of arginine-perfused hearts. The possibility that glycocyamine may accumulate in arginine-perfused hearts will have to be taken into consideration later in this paper. We believe that the correspondence demonstrated above, in conjunction with the demonstration under headings (b) and (c) respectively that the micro-method is relatively much less sensitive to glycocyamine than the macro-method, provides definite evidence against the presence in the heart extracts of any significant amounts of glycocyamine.

(5) *Determination of arginine.* The colorimetric method of Jorpes & Thorén [1932], based on the Sakaguchi reaction, is used for the estimation of arginine. Our procedure for obtaining a coloured solution from the arginine samples is identical with that of Jorpes & Thorén, but the colorimetric comparison is carried out in a Leitz colorimeter (one stage, with built-in illumination) using neutral grey solution as the standard, with a colour filter (Leitz S.F. 6) in the eyepiece. "Absolute" colorimetry, using neutral grey, is a type of photometric comparison, and eliminates the difficulty due to the rapid fading of the colour in standard arginine solutions which Jorpes & Thorén mention and which we have confirmed.

In our experiments the colorimetric estimation is usually completed within 4 min. of the development of the colour.

The relation between concentration of arginine and colour developed was determined by making a large series of observations on arginine solutions containing 12–84 γ arginine in 5 ml. The depth of column of neutral grey solution required to give a colour match against a 10 mm. depth of column of the arginine solution was determined in each instance. The results of these observations are given in Table V. When the points are plotted on a double logarithmic

Table V. *Relation between arginine content of sample and neutral grey reading*

Arginine γ	Neutral grey reading (mm.)				
12	3.52	3.75	3.78	3.83	3.87
24	7.28	7.46	7.48	7.60	7.94
36	10.45	10.81	11.04	11.17	11.43
48	14.35	14.59	14.72	15.35	15.38
60	17.06	17.58	17.66	18.07	18.58
72	20.65	20.66	20.70	20.75	21.38
84	23.33	23.44	23.66	23.88	24.27

Fig. 2. Calibration chart for arginine estimation.

scale they lie on a straight line. Fig. 2 is an accurately drawn calibration chart, derived from the data of Table V, and may be used in making arginine determinations by the method described. The relation between neutral grey reading and arginine concentration is:

$$Y = 3.636x^{0.949},$$

where Y is the concentration of arginine in γ , and x is the reading of neutral grey in mm. when the unknown is set at 10 mm. depth of solution. The error of an individual estimation is $\pm 4.5\%$.

5 ml. samples of the heart extracts and 0.5 ml. samples of the protein-free fluid from the perfusate, made up to 5 ml. with distilled water, are taken for the estimations. The arginine content of the fresh perfusate is checked by estimations in triplicate on 0.5 ml. samples of the solution, made by running 10 ml. of fresh perfusion fluid (at the perfusion temperature) into a 25 ml. centrifuge tube containing 2 ml. 25% trichloroacetic acid. We have satisfied ourselves, by making determinations on known solutions of arginine, that trichloroacetic acid, in the concentrations used in both the perfusates and the heart extracts, has no effect on the accuracy of the estimation of arginine.

(6) *Composition of the perfusion fluids.* The perfusate used is a modified Ringer-Locke solution having the following composition: NaCl 0.90%, CaCl_2 0.02%, KCl 0.01%, NaHCO_3 0.02% and glucose 0.20%. Stock solutions of the various constituents, except glucose, are kept continuously in cold store, and the perfusate was freshly made up from them immediately before each perfusion, the glucose being added when the solution was made up to volume.

In the perfusions with arginine the perfusate contained approximately 20 mg./100 ml. pure *D*-arginine carbonate (Fraenkel and Landau, Berlin). The amounts added were carefully weighed out and, in the perfusions in which arginine was estimated, were checked by determinations on samples of the fresh perfusate.

In our early perfusions the concentration of glucose in the perfusion fluid was 0.10%, the concentration usually recommended and, in these conditions, 10 out of 11 hearts failed before the end of 2 hr. Better maintenance of the condition of the heart was achieved by increasing the glucose concentration to 0.20%; in over 70 subsequent perfusions only two hearts have failed for no immediately apparent reason, and in each instance clot or glass wool was found at the aortic end of the coronary arteries.

(7) *Control perfusions.* The total effects of the perfusion procedure, the method of sampling, the conversion of creatine into creatinine and the colorimetric estimation on the determination of known amounts of creatine and arginine were examined in a series of "blank" perfusions. No heart was placed in the apparatus, but 50 ml. of perfusate to which known amounts of creatine and arginine were added were kept constantly in circulation for 2 hr., the rate of flow through the cannula being 15–16 ml./min. The perfusion procedure, the conversion of creatine into creatinine in the samples and the colorimetric estimations were carried out exactly as described above for the heart perfusions, and in addition determinations were made on samples of the fresh perfusate to check the theoretical concentrations of arginine and creatine. The results of two control perfusions by the "long method" and three by the "short method" already described are collected in Table VI. It is apparent from these data that no significant difference exists between the two methods, and that no serious or consistent errors in the determination of total creatinine or of arginine arise from either method.

Table VI. *Control perfusions (negative differences indicate apparent disappearance)*

All quantities in mg.						
Procedure	Creatine added	Creatine found	Difference	Arginine added	Arginine found	Difference
Long	0.395	0.379	– 0.016	—	—	—
Long	0.431	0.414	– 0.017	15.355	15.146	– 0.209
Short	0.301	0.382	+ 0.081	13.080	13.104	+ 0.024
Short	0.367	0.356	– 0.011	16.500	16.968	+ 0.468
Short	0.401	0.409	+ 0.008	16.036	16.260	+ 0.224
	Mean difference		+ 0.009			+ 0.127

(8) *Material.* Only healthy male rabbits were used. The animals were obtained partly from a local dealer, and partly from the Department of Pathology. The stock was mixed, having representatives, pure and mongrel, of several different strains commonly fancied by breeders in this country. Information in the literature about the age and weight of rabbits at puberty is scanty, and in any case precise figures would not apply to a mixed stock. We have concluded, from the information available to us, that on the average puberty is attained about 6 months of age, at which time the body weight is 1.8–2.0 kg. In order therefore to obtain one group of animals predominantly immature, and another group of animals predominantly mature, we have confined our data to animals weighing less than 1.8 kg. and more than 2.0 kg.

RESULTS

The results of 48 observations on control hearts are presented in Fig. 3. The full circles represent hearts from animals weighing less than 1.8 kg. and the hollow circles represent hearts from animals weighing more than 2.0 kg. The differences in the relation between total creatinine and heart dry weight in the

Fig. 3. Control observations.
(For explanation, see text.)

Fig. 4. Relation of heart dry weight
to body weight.

two groups strike the eye immediately. It is obvious that the slopes of the regression lines differ considerably, and that the scatters of the points in the two groups are very different. The regression line for the lower group does not differ significantly from a line through the origin, whereas that for the upper group does. We are therefore constrained to believe that comparisons between control and perfused hearts cannot profitably be made on the simple basis of content of total creatinine per unit of dry weight, and that the regression lines shown must be taken as the basis for comparison.

The use of the two groups of animals was based on the expectation of a pubertal change in creatine metabolism. Various investigations, such as those of Light & Warren [1934] and Buadze [1933], into creatinuria in boys, and those of Schrire & Zwarenstein [1932, 1, 2; 1933; 1934] into the differing effects of

pre- and post-pubertal castration on the creatinine excretion of rabbits, have established that pubertal changes in the metabolism of creatine and creatinine do occur. The discontinuity in the dry weight-total creatinine relation shown in Fig. 3 has therefore a physiological as well as a statistical basis. A third type of evidence in support of the reality of this discontinuity may be adduced from the response of the perfused heart to the addition of arginine, and we shall discuss this later. Meanwhile we wish to emphasize the fact that the discontinuity is not a statistical artefact. Some further insight into its significance can be gained from consideration of Fig. 4, in which the relation between heart dry weight and body weight is represented. The broken horizontal line in this figure is at the level of 0.8 g. dry weight, i.e. the heart dry weight at which the two regression lines on Fig. 3 intersect. It will be seen that the most probable body weight in any large group of rabbits from the population from which our supply was drawn, in which the heart dry weight is close to 0.8 g., is approximately 1.6 kg., but that heart dry weights in this neighbourhood are observed in animals weighing between 1.2 and 2.2 kg. That is, we can say that, although there is very considerable morphological variability in our animals, the discontinuity occurs in a position which is not inconsistent with the view that it is connected with puberty. In view of the variability of the animals it would have been desirable to exclude a larger group than that comprised between 1.8 and 2.0 kg. body weight, since it is almost certain that some mature animals must be included in the lower group and some immature ones in the upper group. But, since certainty of the mutual exclusiveness of the two groups from the point of view of sexual maturity could hardly be obtained without limiting the body weight to less than 1.0 kg. and more than 2.5 kg., we have preferred the probable increase in variance due to overlap of the immature and mature groups to the decrease in the range of observations which would result from more rigorous restriction of the groups. In fact, if all our observations between 1.5 and 1.8 kg. and all those between 2.0 and 2.3 kg. are excluded, the positions of the regression lines are not changed significantly and the point of intersection remains at 0.8 g. dry weight. But the number of observations in each group is halved and the reliability of the estimates of the slopes of the regression lines is impaired. This statistical experiment shows that the probable dilution of each of our groups is not sufficient to modify the characteristic relations obtaining within each group.

Further evidence in favour of the reality of this discontinuity is provided by consideration of the 22 glucose perfusions. In Fig. 5 the full and hollow circles have the same significance as in Fig. 3. The continuous lines are the regression lines for the two groups of glucose-perfused hearts, and the broken lines are the regression lines for the controls. It will be seen that the glucose-perfused hearts also exhibit the discontinuity found in the controls. Furthermore, the regression lines for glucose-perfused and control hearts do not differ significantly in either group. This is a matter of some importance, since it appears to indicate that neither synthesis nor destruction of creatine occurs in the surviving heart in the absence of added precursor, and it is of value in another way since it indicates that the control hearts provide as good a control on the effects of perfusion with arginine as do the glucose perfusions.

In order to obtain the best estimate of the total creatinine to be found in the heart not perfused with arginine we have therefore pooled the control and glucose perfusion data. The regression lines of the two groups of pooled data are:

$$Y = 7.43x + 0.14 \text{ (animals below 1.8 kg.)},$$

$$Y = 4.01x + 3.19 \text{ (animals above 2.0 kg.)},$$

where Y = total creatinine in mg. and x = heart dry weight in g.

The difference in the slopes of the two lines is 3.42 ± 0.88 mg. creatinine per g. dry weight, and there is less than one chance in a thousand that such a difference would be observed between two random samples of the size of those used here if the samples were drawn from the same population. In other words, the difference in the slopes is highly significant, and we may take it that in so far as the total creatinine and heart dry weight relation is concerned the pre- and post-pubertal animals are drawn from different populations.

Fig. 5. Observations on glucose-perfused hearts. (For explanation see text.)

Fig. 6. Observations on arginine-perfused hearts. (For explanation see text.)

The data for the arginine-perfused hearts (25 perfusions) are presented in Fig. 6, together with the regression lines (continuous) and the regression lines for the pooled control data (broken), and it will be seen that, although the data are far from ideal in their distribution, there is no doubt that arginine brings about an increase in the total creatinine of the hearts in the upper group. The difference in the relation between the two groups of arginine-perfused hearts and the corresponding controls is yet another instance of a change in character of the creatine metabolism of the heart at puberty, and we shall offer further evidence that the difference apparent in Fig. 6 is a real one.

The difference between the slopes of the upper arginine and control lines is 2.09 ± 0.88 , i.e. the difference is a significant one, and it is possible to calculate from the two lines that:

$$\Delta C = (Y_a - Y_c) = 2.09x - 1.53,$$

where Y_a and Y_c are the amounts of total creatinine to be expected in an arginine-perfused and in a control or glucose-perfused heart respectively, and x is the heart dry weight in g. ΔC is the most probable value for the total creatinine appearing during the arginine perfusion.

Since the most probable mode of action of arginine is that it itself should be converted into creatine, we have examined the disappearance of arginine from the perfusion system in a number of the arginine perfusions. The estimation of the disappearance presents some difficulties. The estimation of the arginine disappearance from the perfusate is simple, but it is difficult to interpret the

figures obtained for the arginine content of the heart. In the first place, we have found that control and glucose-perfused hearts contain a certain amount of apparent arginine when the estimations are made by the colorimetric method described. This apparent arginine bears no obvious relation to the size of the heart, and its concentration is of the order of magnitude to be expected on the basis of the creatine content of the samples taken for analysis. A few experiments on the effect of conversion of the creatine of the samples into creatinine on the apparent arginine have shown that the decrease corresponds with that expected on the basis of the intensity of the Sakaguchi reaction given by creatinine. The evidence appears to justify us in concluding that there is no appreciable amount of arginine pre-existing in the heart. Table VII summarizes the available data on the apparent arginine content of control and glucose-perfused hearts, and we have estimated the true arginine content of arginine-perfused hearts by subtracting the mean of all the figures in this table from the total arginine found.

Table VII. *Apparent preformed arginine content of rabbit hearts*

Controls			Glucose-perfused		
No.	Dry wt. g.	Arginine mg.	No.	Dry wt. g.	Arginine mg.
44 C	0.37	0.23	106	0.44	0.39
			104	0.50	0.32
48 C	0.71	0.24			
45 C	0.74	0.27			
51 C	0.80	0.29			
58 C	0.89	0.34			
			86	0.98	0.35
49 C	1.03	0.34			
57 C	1.04	0.32	82	1.10	0.35
			78	1.10	0.19
60 C	1.19	0.32			
62 C	1.30	0.26			
59 C	1.38	0.38			
61 C	1.39	0.29			
			83	1.43	0.19
			92	1.49	0.31
			93	1.51	0.24
47	1.95	0.26			
	Mean	0.30		Mean	0.29

Further, during the perfusion water passes from the perfusate into the heart. If arginine diffuses into the heart in amount corresponding to the water which has passed in, the passage of arginine into the heart will not affect the significance of the value for the arginine disappearance obtained by analysis of the perfusate. But if more or less arginine is present in the heart than corresponds to the extra water, the arginine disappearance estimated from the perfusate will be correspondingly too great or too small. Fortunately, as the data presented in Fig. 7 show, an excellent estimate of the expected water content of a heart of known dry weight can readily be obtained, and the difference between this estimate and the observed water content of the heart gives the value of the extra water in g. We have not made any correction for the conversion of g. into ml. at the perfusion temperature, since the correction is infinitesimal compared with the error of the estimation of the extra water. The extra water, reckoned in ml., is multiplied by the arginine content of 1 ml. of the final sample of perfusate,

and from the product is subtracted the arginine content of the heart (less the apparent arginine). The result of this procedure gives a correction which has to be applied to the estimated arginine disappearance from the perfusate.

Fig. 7. Control observations on the relation between heart dry weight and heart water content.
Regression: $y = 2.56x - 0.67$.

This procedure has been applied to 14 perfusions, in ten of which animals in the upper group were used. In the remaining four, young animals were used. The results, together with the steps in deriving the correction, are collected in Table VIII.

Table VIII. *Correction of arginine disappearances*

Per- fusion no.	Heart dry wt. g. (1)	Extra water g. (2)	Arginine in extra water mg. (3)	Pre- formed arginine mg. (4)	Total account- able arginine mg. (5)	Arginine in heart mg. (6)	(5)-(6) mg. (7)	Arginine disap- pearing from perfusate mg. (8)	(8) + (7) mg.
68	1.37	2.19	0.27	0.29	0.56	0.45	0.11	1.74	1.85
76	1.42	2.10	0.27	0.29	0.56	0.53	0.03	2.42	2.45
80	1.16	1.55	0.20	0.29	0.49	0.50	-0.01	1.44	1.43
107	1.51	2.55	0.38	0.29	0.67	0.68	-0.01	2.50	2.49
95	1.36	1.85	0.28	0.29	0.57	0.60	-0.03	1.75	1.72
96	1.37	1.85	0.27	0.29	0.57	0.44	0.13	1.67	1.80
97	1.29	2.22	0.34	0.29	0.64	0.50	0.14	1.65	1.79
99	1.32	1.82	0.27	0.29	0.56	0.33	0.23	1.92	2.15
100	1.40	1.36	0.20	0.29	0.49	0.48	0.01	2.36	2.37
101	1.18	2.32	0.31	0.29	0.60	0.37	0.23	1.57	1.80
87	0.40	0.51	0.08	0.29	0.38	0.67	-0.29	0.19	-0.10
103	0.39	0.26	0.04	0.29	0.33	1.04	-0.71	0.42	-0.29
105	0.45	0.81	0.13	0.29	0.42	1.01	-0.59	0.48	-0.11
108	0.80	1.42	0.21	0.29	0.50	1.11	-0.61	1.24	+0.63

Two interesting points emerge. In the first place, the correction is not of any great importance in perfusions in the upper group, and it is unlikely that the errors inherent in its estimation can affect seriously the observed relation between arginine disappearing and total creatinine appearing. Secondly, the hearts from

young animals contain proportionately very large amounts of arginine. This observation is consonant with the view that the small hearts do not utilize the arginine which diffuses into them from the perfusates, whereas the large hearts do. In fact Table IX appears to show that, in the small hearts, not only is all the water of the heart in diffusion equilibrium with the perfusate, but a variable amount of arginine is adsorbed on to the heart solids as well.

Table IX. *The arginine content of arginine-perfused young rabbit hearts, in relation to the arginine content of the perfusate*

(The two figures given for the arginine content represent the total arginine found less 0.19 and 0.39 mg. respectively, these being the extremes of the values found for preformed apparent arginine of the heart.)

No.	Heart dry wt. g.	Heart water g.	Heart arginine mg.	mg. arginine per 100 g. heart water	Perfusate: mg. arginine per 100 ml.
87	0.40	2.21	0.28-0.48	13-22	15
103	0.38	1.92	0.65-0.85	34-44	16
105	0.45	2.63	0.62-0.82	23-31	16
108	0.78	4.13	0.72-0.92	17-22	15

In Table X estimates are collected of the extra creatinine appearing in the arginine-perfused heart made (a) by applying the relation derived from the heart dry weight-total creatinine regressions, i.e. by calculating ΔC , (b) by calculating the amount of creatinine corresponding to the uncorrected arginine disappearance and (c) by making the same calculation for the corrected arginine disappearance. It is clear that the ratio of the amount of creatinine calculated by either (b) or (c) to the amount calculated by (a) is remarkably close to unity for the hearts in the upper group, and that the major effect of the correction is to bring the more aberrant ratios nearer to unity. In the lower group no ratio can be calculated since the expected extra creatinine is zero. But the mean disappearance of arginine in four experiments is only 0.03 mg. (see Table VIII). Even if fortune had not favoured us with such a satisfying figure for the mean disappearance in the lower group, it would have been obvious that the changes in arginine content of the system in the lower group are different in character from those in the upper group, and that the results of estimations of arginine

Table X. *Relation of calculated creatinine appearance in arginine perfusions to the disappearance of arginine*

Perfusion no.	Creatinine equivalent of uncorrected arginine dis- appearance ΔA	Creatinine equivalent of corrected arginine dis- appearance $\Delta A'$	Creatinine appearance calculated from regression ΔC	$\Delta A/\Delta C$	$\Delta A'/\Delta C$
68	1.13	1.21	1.32	0.86	0.92
76	1.57	1.59	1.43	1.10	1.11
80	0.94	0.93	0.88	1.07	1.06
95	1.13	1.12	1.30	0.87	0.86
96	1.09	1.17	1.32	0.83	0.89
97	1.07	1.16	1.16	0.92	1.00
99	1.25	1.39	1.22	1.02	1.14
100	1.53	1.54	1.39	1.10	1.11
101	1.02	1.16	0.93	1.10	1.25
107	1.62	1.63	1.62	1.00	1.01

The mean value of $\Delta A/\Delta C$ is 0.99 ± 0.03 .

The mean value of $\Delta A'/\Delta C$ is 1.04 ± 0.04 .

disappearance are in full accord with expectations based on the comparison of total creatinine-heart dry weight regression lines. We are in some necessity to emphasize the validity of our method of estimating the most probable value of the extra creatinine from regression lines, since, so far as we are aware, such a method has not previously been utilized for metabolic studies, except in the instance of earlier investigations by one of us into the source of carbon for purine synthesis in birds [Fisher, 1935, 1, 2].

We believe that these results show conclusively that the adult rabbit heart is capable of converting arginine quantitatively into creatine. In view of the quantitative character of this conversion, as demonstrated in Table X, the results obtained when known amounts of glycocyamine were added to heart extracts (Tables III *a* and IV) and the close correspondence observed between estimations of the total creatinine of arginine-perfused hearts by the micro- and macro-methods render it very unlikely that we have been observing a conversion of arginine into glycocyamine. For example, an appearance of 1.30 mg. extra creatinine (Table X, perfusion 95) corresponds to an increase over the normal value of 0.13 mg. creatinine in the 20 ml. samples taken for estimation by the macro-method. From Table IV it will be seen that at least 1 mg. of glycocyamine would have to be present in the sample to account for the apparent increase in creatinine, or 10 mg. in the whole heart, an amount considerably in excess of the equivalent amount of arginine which has disappeared (1.75 mg.). Further, since the micro-method is only one-fifth as sensitive to the same amount of glycocyamine as is the macro-method, an amount of glycocyamine sufficient to produce 1.30 mg. apparent extra creatinine by the macro-method would estimate as only 0.26 mg. apparent extra creatinine by the micro-method, a difference of over 1 mg. From this it could be expected that, if glycocyamine were really responsible for the observed increases in total creatinine, there would be a systematic difference of the order of 5 γ /ml. between the estimation of total creatinine by the two methods. Since the observed difference in arginine-perfused hearts is $+0.73 \pm 0.67 \gamma$ /ml., this condition is obviously not fulfilled.

We also have evidence which excludes the other possibility, namely that the increase in total creatinine is due to an increase in preformed creatinine. Preformed creatinine was estimated in three instances, one control heart (62 C), and two arginine-perfused hearts, one from an animal in the low body-weight group (108), and one from an animal in the high group (107). The preformed creatinine of control 62 was 0.21 mg. or 2.14 % of the total creatinine. That of No. 108 was 0.22 mg. or 4.04 % of the total creatinine of the system heart plus perfusate, and that of No. 107 was 0.32 mg., or 2.98 % of the total creatinine of the system. In the perfusions, the preformed creatinine was evenly divided between the heart and the perfusate. From these data it is apparent that the amount of preformed creatinine is far too low to account for the extra total creatinine, and that the proportion of the total creatinine which is preformed does not appear to be affected by arginine perfusion. The hypothesis that arginine is converted into creatinine can therefore be dismissed on two counts, and the conclusion that these experiments comprise a demonstration of the synthesis of creatine from arginine is well established.

DISCUSSION

Our finding of a quantitative conversion of arginine into creatine in the isolated rabbit heart is at variance with the conclusions of Rose [1935] and of Hunter [1928]. Rose says: "Arginine is still regarded by some as the source of

creatine or creatinine or both", and Hunter is of the opinion that "so large a body of almost purely negative evidence [concerning the formation of creatine from arginine] leads one rather forcibly to suspect that, if creatine is related to arginine at all, its mother substance must be, not the free amino-acid, but the still-combined arginine of the muscle or other protein".

Despite these pronouncements, we believe that, having regard to the limitations of the experimental methods employed by other workers, our results are not at variance with the evidence on this point which is to be found in the literature.

Since it is clear that arginine will be required in the organism for protein synthesis, and for the maintenance of the mechanism for urea synthesis [Krebs & Henseleit, 1932, 1, 2], and that it is likely to be lost also by reason of the action of arginase and deaminases, it appears that only a small proportion of injected or ingested arginine is likely to be available for creatine synthesis. Indeed, the early experiments of Thompson [1905; 1906] on dogs appear to show that 50–96 % of ingested arginine is promptly excreted as urea even when the amount given is as high as 0.5–1.6 g. per kg. per diem. Kiech *et al.* [1931] have shown that the white rat behaves very similarly and that 79 % of ingested arginine is recovered in 5 hr. as urea when the dose is 1.1 g. of arginine per kg. It follows that very high dosage with arginine may well be necessary to provide arginine in such excess of the organism's capacity to catabolize it as to permit of an appreciable synthesis of creatine, and the only simple criterion of the provision of this excess would appear to be the demonstration of the excretion unchanged of an appreciable fraction of the administered arginine. Using this criterion, no single investigation into the effect of arginine on creatine excretion is satisfactory, since arginine excretion has never been studied in such an investigation. Furthermore, even if this criterion were satisfied, there is the further difficulty that an approach to quantitative recovery of ingested creatine is obtained only when massive doses are used, and the percentage recovery falls off rapidly as the dose diminishes [see Hunter, 1928, p. 140]. Thus, if we accept a figure of 20 % of ingested arginine as available for conversion into creatine and 30 % for the recovery in the urine of the creatine formed, it follows that even a quantitative conversion of the available arginine would result in the recovery as creatine of only 7 % of the administered arginine. In addition, a recovery of this magnitude could be expected only if the dose of arginine was at least 1 g. per kg.

There is yet another caveat to enter against the facile interpretation of the effects of arginine administration upon creatine excretion. Scull & Rose [1930] have shown clearly that the increase in the arginine content of growing white rats placed on an arginine-low diet may be two or three times as great as their arginine intake during the experimental period, and it is legitimate to speculate that the administration of arginine in excess of the normal amount in the diet might well diminish this synthetic activity, with the result that, unless the arginine dose were massive, the total arginine available for metabolic processes might not be significantly increased by arginine administration. We believe therefore that, having regard to the difficulties which we have shown to exist in the recovery of ingested arginine as creatine, the published results of investigations into this problem are consonant with the interrelation of these substances in metabolism. The results of the published investigations into the effect of arginine administration on the excretion of creatine and creatinine are shown in condensed form in Table XI.

It will be observed that in the two species, the rabbit and the dog, in which a considerable number of observations have been made, the positive observations

Table XI. *The recorded effects of arginine administration on the total creatinine of the urine.*

Animal	No. of exps.	Average length days	Arginine dose g. per kg.	Effect	Authors
Duck	15	2	0.22-0.59	+	Thompson [1917]
Rabbit	2	3	0.45-0.90	-	Jaffe [1906]
	4	1	1.25-2.27	+	Shapiro & Zwarenstein [1932]
	8	2	1.00	+	Thompson [1917]
Dog	3	1	0.08-0.12	-*	Lieben & Laszlo [1926]
	1	35	0.06-0.12	-	Grant <i>et al.</i> [1929]
	2	2	0.29	-	Baumann & Hines [1918]
	14	2	0.22-0.31	+	Thompson [1917]
Pig	6	1	0.14-0.17	+	Gross & Steenbock [1921]
Man	2	35	0.02	-	Hyde & Rose [1929]

* Phlorhidzinized dogs.

outnumber the negative. Three of the six negative observations on the dog were made on phlorhidzinized dogs. One of the remaining three was made using an extremely low dose. On the other hand, out of 16 observations on animals receiving more than 0.2 g. of arginine per kg. 14 yielded positive results. The only other negative observations were those of Hyde & Rose on man, and here the dosage was lower than any other recorded.

One further objection may be raised here to Hyde & Rose's work, which will apply also to the single negative observation recorded by Grant *et al.* on the dog. In both these experiments arginine was fed daily for a considerable period of time, and the objection may be raised that Edlbacher & Schuler [1932] have produced evidence of an increase in arginase activity of skeletal muscle as a result of the administration of arginine to guinea-pigs, and they are of the opinion that the total arginase activity of the organism may possibly be increased by such means; it follows that prolonged administration of arginine may well defeat its own object, in that the rate of destruction of arginine may rise as the administration continues.

Some of Thompson's [1917] experiments are interesting in this connexion. He noticed that a repetition of the administration of arginine to dogs usually failed to cause any increase in creatine output, or produced far less increase than the initial dose. On the other hand, repetition of arginine administration was almost or completely without influence on the increase in the creatine output of ducks. In view of the well-known fact that the arginase content of birds is very small in comparison with that of mammals, this observation would seem to support Edlbacher's opinion concerning the effect of arginine administration on arginase content of the organism.

It appears to be justifiable to summarize Table XI by saying that 47 observations on four species have yielded positive results and two observations of an unexceptionable character have yielded negative results, whilst three others which are questionable by virtue of the length of the experiment and the small size of the dose have also yielded negative results. The total is made up by five negative results each of which is questionable in view of the small size of the dose. On this ground we feel confident in concluding that the published evidence is consonant with the view that arginine can be converted into creatine in the vertebrate organism. But such evidence is no proof of such a conversion, since it is still possible to adduce as alternative explanations of the increase in creatine output following arginine administration not only all those hypotheses

adumbrated in the introduction to this paper but also such additional ones as the elution of creatine from some site of adsorption in the tissues, the arginine acting as an eluant by virtue of the guanidine grouping which it has in common with creatine, and also the inhibition of renal reabsorption of creatine (a process which has never been formally demonstrated, which is, however, extremely likely to occur).

There are two other investigations which bear on the work which we have reported. Baumann & Marker [1915] failed to obtain any increase in creatine when they added arginine to the defibrinated blood perfusing the isolated hind-limb of a dog. Adequate data for the calculation of the total creatine are not presented, i.e. the creatine content of 1 g. of dry muscle before and after perfusion is given, but there are no figures for the total weight of muscle on the perfused limb, and no controls on the uniformity of the creatine contents of different samples of muscle from the same limb. Furthermore no glucose or other nutrient was added to the perfusate to maintain the condition of the limb, although the perfusions lasted for 75 and 110 min. respectively. Despite these defects the indication is that in both perfusions slight increases in creatine did occur.

Thomas [1920] also reported a failure to observe any conversion of arginine into creatine in perfused limbs, but no experimental details are given. Two other investigations have been reported in which the effect of the perfusion of the isolated heart on the total creatinine has been examined. Weber [1908] carried out one perfusion of a dog heart and three perfusions of cat hearts in which the total creatinine of the heart and the perfusate was determined. No conclusions germane to the present work can be drawn from these experiments. Herrmann *et al.* [1934] and Decherd *et al.* [1934] perfused rabbit hearts with Ringer-Locke solution (0.1 % glucose) in a closed-circuit type of apparatus. In the first group of experiments no provision was made for oxygenation of the perfusate, and the provisions which were made in the second group (oxygen lift) appear to have been inadequate, since 250 ml. of perfusate which had not been previously oxygenated were placed in the reservoir at the beginning of the perfusion. No determinations of the total creatinine of the perfusate were made, and it seems that the amount present in the perfusate may have exceeded 20 % of the total. We have frequently found as much as 10 % of the total in our perfusates, when the total volume has been in the neighbourhood of 100 ml. Decherd *et al.* [1934] report four perfusions in which arginine was used in a concentration which is not stated. The results are expressed in terms of mg. total creatinine per 100 g. of heart and are compared with the corresponding figures from ten control hearts, a procedure which we have shown to be fallacious. The sex and size of the animals used are not stated. We do not believe that any of these investigations provide reason for modification of our view that creatine can arise from arginine in vertebrate metabolism, and we believe that our demonstration of a quantitative conversion is decisive in the establishment of this conclusion. Other experiments have therefore been commenced in which we hope to verify this quantitative conversion by other means.

We do not wish to discuss in detail at present the curious relation established in male rabbits between total creatinine and heart dry weight. However, it is of interest to note that Fig. 6 seems to show that, on perfusion with arginine, the creatine content of post-pubertal hearts tends to come into line with that for pre-pubertal ones. Since the regression for the pre-pubertal control plus glucose-perfused hearts passes practically through the origin, i.e. the total creatinine in these hearts is directly proportional to the dry weight, the following tentative

hypothesis would fit the facts: before puberty the creatine content increases in direct proportion to the increase in solids in the heart because the synthesis is limited only by the accumulation of creatine, and there is, naturally, a limiting concentration of creatine, which is that attained in these hearts; post-pubertally some other factor, probably hormonal, inhibits synthesis to such an extent that the limiting concentration is not reached, with the consequence that the more the heart grows post-pubertally the further its creatine content falls below that to be expected on the basis of the pre-pubertal relation. With the additional assumption that the inhibiting factor has its origin outside the heart tissue, the increase in creatine content of post-pubertal hearts when arginine is added to the perfusate is easily explained: the inhibiting factor is diluted during perfusion, and then, since the creatine concentration is below the limiting value, synthesis can occur until that level is approached. As it is immediately apparent that this hypothesis provides a basis for a number of investigations, work on the relation between total creatinine content and heart dry weight has already been begun on the white rat, in the hope of demonstrating the discontinuous relation which is fundamental to this hypothesis. Further work on the failure to demonstrate creatine synthesis in young animals is also planned.

SUMMARY

The isolated male rabbit heart, perfused for 2 hr. with a modified Ringer-Locke solution, exhibits no change in total creatinine content. When arginine is added to the perfusate, the total creatinine of hearts from post-pubertal animals increases to an extent which corresponds almost precisely with the amount of arginine which disappears. No increase in total creatinine is observed in these conditions in hearts from pre-pubertal animals.

Evidence is adduced to show that the increase in total creatinine is due solely to an increase in creatine.

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APPENDIX

Table XII. *Total creatinine of control hearts*

No.	Body wt. kg.	Heart dry wt. g.	Heart wet wt. g.	Total creatinine mg.
39 C	0.99	0.32	1.61	2.58
40 C	0.99	0.35	1.77	2.74
44 C	0.86	0.37	1.79	3.06
50 C	0.98	0.41	2.04	3.31
10 C	1.21	0.64	2.44	4.65
24 C	1.23	0.77	3.59	6.16
11 C	1.24	0.84	3.73	5.70
3 C	1.30	0.50	2.60	3.37
48 C	1.42	0.66	3.05	5.81
42 C	1.44	0.60	2.83	4.95
46 C	1.52	0.71	3.18	5.78
34 C	1.62	0.66	3.09	5.60
23 C	1.63	0.79	3.56	5.98
45 C	1.63	0.74	3.24	5.59
51 C	1.64	0.80	3.44	5.86
58 C	1.73	0.90	4.05	7.25
38 C	1.74	0.70	3.24	5.16
9 C	1.76	0.86	4.51	6.12
35 C	1.79	0.63	2.92	4.18

Table XII (*cont.*)

No.	Body wt. kg.	Heart dry wt. g.	Heart wet wt. g.	Total creatinine mg.
12 C	2.04	1.16	4.44	7.49
28 C	2.07	0.97	3.96	6.20
49 C	2.08	1.03	4.77	7.75
6 C	2.08	1.37	4.48	8.73
43 C	2.12	0.86	3.94	6.98
52 C	2.42	1.13	5.01	7.21
21 C	2.14	1.40	5.29	9.77
55 C	2.16	0.88	4.10	6.14
29 C	2.18	1.05	4.20	6.56
53 C	2.20	1.10	4.60	7.75
4 C	2.22	1.43	6.60	9.32
30 C	2.22	0.94	4.37	7.30
57 C	2.25	1.04	4.42	6.60
60 C	2.28	1.19	4.64	6.91
37 C	2.43	1.32	5.56	8.63
61 C	2.41	1.39	5.69	9.50
41 C	2.48	1.29	5.10	8.39
13 C	2.49	1.36	5.83	8.27
59 C	2.54	1.38	5.76	9.91
20 C	2.62	1.49	5.53	8.33
17 C	2.73	1.55	5.78	8.10
16 C	2.94	1.27	5.37	7.90
22 C	2.94	1.55	6.30	9.49
26 C	2.96	1.19	4.96	7.67
47 C	2.98	1.95	7.36	11.22
8 C	3.01	1.42	5.57	7.90
5 C	3.11	1.59	6.86	10.17
62 C	2.57	1.30	5.50	9.80

Table XIII. *Total creatinine of glucose-perfused hearts*

No.	Body wt. kg.	Heart dry wt. g.	Heart wet wt. g.	Total creatinine (mg.)	
				Heart	Perfusate
106	1.00	0.44	3.05	2.99	0.34
104	0.95	0.50	3.25	3.17	0.36
88	1.64	0.68	3.79	5.12	0.19
19	1.13	0.66	3.65	4.48	0.39
14	1.58	0.75	4.51	5.84	0.28
13	2.40	1.07	6.39	7.97	0.59
33	2.44	0.98	6.19	6.66	0.23
42	2.03	0.85	5.21	5.95	0.53
43	2.09	1.07	5.92	7.01	0.53
44	2.04	0.97	5.97	7.35	0.49
46	2.95	1.40	8.24	7.56	0.32
47	2.11	1.20	5.64	5.68	0.67
53	2.82	1.29	7.00	8.55	0.36
78	2.14	1.10	6.27	7.88	0.42
69	2.27	0.92	5.68	6.91	0.31
81	2.74	1.54	8.44	8.33	0.78
82	2.10	1.10	5.88	7.62	0.30
83	2.95	1.43	8.56	9.63	0.36
86	2.15	0.98	5.81	8.00	0.32
90	2.68	1.40	6.77	9.05	0.32
92	2.61	1.49	7.31	8.78	0.47
93	3.00	1.51	8.09	7.97	1.44

Table XIV. *Total creatinine of arginine-perfused hearts*

No.	Body wt. kg.	Heart dry wt. g.	Heart wet wt. g.	Total creatinine (mg.)	
				Heart	Perfusate
16	1.11	0.50	3.21	3.98	0.37
60	1.77	0.71	3.89	4.78	0.29
66	1.60	0.57	3.98	4.79	0.52
87	1.05	0.40	2.62	3.27	0.31
103	0.80	0.38	2.31	3.07	0.42
105	1.00	0.45	3.08	2.72	0.40
108	1.38	0.80	4.93	4.47	0.98
56	3.03	1.37	8.33	10.31	0.97
57	2.16	0.85	4.98	5.62	0.39
58	2.75	1.08	5.87	9.88	0.37
59	3.29	1.81	8.93	12.21	0.52
62	2.71	1.16	6.52	7.55	0.62
64	2.25	1.11	6.25	6.67	1.72
65	2.42	1.03	6.25	7.20	0.41
68	2.83	1.37	7.74	8.63	0.34
79	2.73	1.71	9.12	11.64	0.75
76	2.72	1.42	7.83	9.76	0.49
80	2.36	1.16	6.34	8.82	0.55
95	2.86	1.36	7.36	8.70	0.83
96	2.30	1.37	7.38	9.05	0.67
97	2.42	1.29	7.48	7.82	0.61
99	2.72	1.32	7.20	8.99	0.62
100	2.46	1.40	7.01	9.23	0.96
101	2.43	1.18	7.20	8.61	0.82
107	2.54	1.51	8.60	9.24	1.49