Precursors of creatine

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PRECURSORS OF CREATINE.

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BY

John Israel Marker, B.Sc. 1913.
ACKNOWLEDGMENT

The writer desires to express his gratitude to Dr. L. Baumann at whose suggestion and under whose supervision the following work was carried out. Thanks are also due to Dr. J.T. McClintock for his suggestions and help in perfecting the technique of perfusion, and also for the use of apparatus and supplies which he so freely permitted.
Precursors of Creatine.

Creatine is of interest because of the assumed biological relation to its anhydride creatinine, a never failing constituent of mammalian urine, and also because creatine forms from 1.2 to 2% of dry muscle substance. The origin, role and fate of creatine in the animal organism has been the study of experimental investigation, but little direct work has been done as to its immediate precursors.

The search for possible precursors has always led to a consideration of substances which like creatine, contain the guanidine nucleus as arginine and glycocyamine. Or it has led to substances which contained the methyl amino groups as choline, betaine and sarcosine. Van Hoogenhuyze and Verploegh (1) and Jaffe (2) found no increase of creatinine in the urine after the subcutaneous injection of arginine nor after feeding proteins containing the amino acid. Thompson (3) found that the bulk (73 to 96%) of the arginine nitrogen administered appeared as urea in the urine. Inouye (4) has observed that when arginine is added to liver in vitro or perfused through the organ an increase in creatine results. Riesser (5) working on rabbits found that a 10 to 15% increase occurred in muscle creatine after the injection of choline and a 6 to 11% increase after the injection of betaine.

The muscles of cats and dogs were selected for the following experiments. In the present investigations the
substances were perfused through the muscle tissue or brought into contact with the hashed muscle in vitro. Muscle was used as a possible site of creatine formation because of its comparatively high creatine content. Methyl ureido acetic acid and arginine carbonate have been studied as possible precursors of creatine. Arginine is an amino acid which is present in most body proteins. Methyl ureido acetic acid has not been found in the animal organism but the work of Ackermann (7) has shown that its anhydride methyl hydantoin, is a putrefaction product of creatinine. If this process were reversible, it would involve a reaction of ammonia with the keto oxygen and the formation of creatine from methyl ureido acetic acid, with the elimination of water.

$$\text{NH}_2\text{C:O} + \text{NH}_3 \rightarrow \text{NH}_3\text{N-CH}_2\text{COOH} \quad \text{C:OH} + \text{H}_2\text{O}$$

Methyl ureido acetic acid. Methyl guanidino acetic acid.

The conversion of arginine into creatine would be the result of oxidation, deamination and methylation.

$$\text{NH}_2\text{C:NH} \rightarrow \text{HN-CH}_2\text{-CH}_2\text{-CH(NH}_2\text{)COOH} \quad \text{C:NH} + \text{H}_2\text{O}$$

(alpha amino- delta guanidino valerianic acid. Arginine) Methyl guanidino acetic acid.

The arginine was obtained by means of the method of Kossel and Kutscher (8) from edestin, the globulin derived from
hemp seed. The methyl ureido acetic acid was prepared from sarcosine and urea (9).

The arginine was employed as the carbonate and the methyl ureido acetic acid as the sodium or ammonium salt.

Muscle creatine was determined by the method of Baumann (10) and the creatine of the blood and perfusates was determined by an adaptation of the muscle creatine method, as follows:

25 cc of blood was heated for three hours under the reflux condenser with 62.5 cc of 5 N sulphuric acid and then filtered and made up to volume in a 150 cc volumetric flask. 10 cc of this solution was pipetted into a 50 cc volumetric flask and exactly neutralized (litmus paper) with 2.5 N sodium hydroxide. Thirty cubic centimeters of a saturated picric acid solution and water to the mark are then added. 25 cc of the clear filtrate is employed for the colorimetric determination according to Folin (11) using 5 cc of creatinine solution (equivalent to 0.1 milligrams of creatinine) as a standard and adding 1.25 cc of 2.5 N alkali to develop the color.

**EXPERIMENTAL**

First the effect of blood-muscle mixtures on arginine and methyl ureido acetic acid at 37.5°C. for approximately 24 hours, was studied. The blood and muscle was obtained from a dog killed by bleeding just prior to the experiment.

Flask A contained 25 cc of defibrinated blood, 25 gm muscle and 10 cc of toluol and was analyzed immediately.

Flask B contained 25 cc of blood, 25 gm muscle and 10 cc of toluol. It was analyzed after 23 hours.
Flask C contained 25 cc of blood, 25 gm of muscle, 10 cc of toluol and 1 gm of arginine carbonate.

Flask D contained 25 cc of blood, 25 gm of muscle, 10 cc of toluol and 1 gm of methyl ureido acetic acid neutralized with sodium hydroxide.

Flasks B, C and D were kept in the incubator for 23 hours at a temperature of 37.5° C after which they were analyzed.

The absence of bacterial growth in these flasks was demonstrated by smears and cultures.

<table>
<thead>
<tr>
<th>Flask</th>
<th>Creatine</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>64.37 mgm</td>
</tr>
<tr>
<td>B</td>
<td>65.83 mgm</td>
</tr>
<tr>
<td>C</td>
<td>65.57 mgm</td>
</tr>
<tr>
<td>D</td>
<td>111.57 mgm - 50 mgm = 61.57</td>
</tr>
</tbody>
</table>

A previous trial showed that 1 gram of methyl ureido acetic acid when subjected to the process employed for the determination of creatine in muscles gave rise to a color equivalent to 50 mgm of creatine. This amount was subtracted from the total creatine in the experiment with the acid. These analyses show no increase of creatine in the presence of the methyl ureido acetic acid or arginine carbonate.

The injection experiment was tried but once and only with methyl ureido acetic acid. It was not entirely satisfactory as the animal (cat) died just as the injection of the neutralized acid was completed. Time of injection was 50 minutes. Before the injection the semimem-
branousus, semitendinosus and gastrocnemius muscles were
removed from the right leg. A cannula was then placed
in the left femoral vein and 65 cc of a 0.9% sodium
chloride solution containing 2 gram of methyl ureido
acetic acid neutralized with ammonia was injected. When
the animal died similar muscles were removed from the left
side for analysis. Creatine and water determination were
made on each. The following results were obtained for 50
gram of muscle.

<table>
<thead>
<tr>
<th>Water content</th>
<th>Creatine content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right limb</td>
<td></td>
</tr>
<tr>
<td>before inj.</td>
<td>73% 180 mgm</td>
</tr>
<tr>
<td>Left limb</td>
<td></td>
</tr>
<tr>
<td>after inj.</td>
<td>72% 197 mgm</td>
</tr>
</tbody>
</table>

The water was determined by noting the loss after
drying to constant weight in vacuo, over sulphuric acid
and phosphorus pentoxide, at room temperature.

If these values be expressed in terms of dry muscle
substance then one gram of muscle before injection con­
tained 13.3 milligrams and after injection it contained
14 milligrams of creatine or an increase of 0.7 milli­
grams per gram of dry muscle. This slight increase may
be due to the presence of the substance employed, which
as stated above gives a color when treated with picric
acid and alkali, hence no significance can be attached to
this result.

Perfusion of a limb seemed to offer the best physiol­
ogical conditions for the determining the formation of
creatine in the muscle from other substances hence an
apparatus was constructed for this purpose.

This apparatus is patterned after one which Brodie (12)
described for perfusing various organs. The essential part is a pump (P) mechanically driven and two chambers, a receiving and a compressing chamber (R and C). In the tube which joins the two chambers to the pump, two valves (V and V') are placed to prevent back flow and to maintain the pressure. The perfusate is aerated by bubbling oxygen through the fluid in the receiving chamber. A mercury manometer connected to the compression chamber registers the pressure under which the perfusate is forced through the limb. A connection (rubber tubing) between the "arterial" tube and the receiving chamber permits shunting the perfusate thus regulating the pressure in the compression bottle. The rate of flow through this tube is regulated by a screw clamp. The pump is a 2 oz hard rubber syringe. The height of the excursion of the piston is regulated by a sliding contact between the drive and piston rod. When the set blocks (B and B') are approximated the stroke of the piston is as long as the diameter of the drive wheel, when separated the length of stroke is diminished. The volume of fluid pumped is thus roughly regulated by the adjustment of these blocks. The apparatus is designed for perfusing the hind leg of a dog but by adjustment can be made to work equally well on cats or similar small animals. The drive wheel is run by a small motor and by means of a system of cone pullies made to revolve about 80 times per minute. The temperature of the perfusate is kept constant by immersion of the two chambers in a water bath at about 40°C.

The perfusion technique was carried out as follows:
The dog was anaesthetized and prepared for operation. The femoral vessels of one leg and the carotid artery are exposed. The animal is exsanguinated, after which the blood is defibrinated and filtered through cheese clothe. A measured quantity of the blood is taken and to it the substance, dissolved in a known quantity of 0.9% sodium chloride, is added.

After bleeding the dog a tourniquet is placed around the limb above the point of exposure of the femoral vessels. This prevents loss of fluid through the collateral circulation of the hip and thigh. The femoral vessels are tied off and the cannulae inserted. Preliminary to the blood perfusion the vessels are washed with Ringer's solution which removes any blood which might clot and give rise to thrombi interfering with perfusion. The perfusate is then placed in the receiving bottle at once and the apparatus started. The volume of the perfusate in our experiments was usually 150 cc but a much smaller quantity may be employed. An incandescent lamp placed under the limb and a reflector over it helped to maintain the temperature of the limb constant.

Experiment Q.
The right hind leg of a dog weighing 5 kilos was perfused for 1.5 hours with a mixture of 100 cc of his own defibrinated blood and 50 cc of 0.9% sodium chloride solution containing 2 gm of methyl ureido acetic acid neutralized with ammonia. The pressure fluctuated between 80 and 100 mm. At the end of the perfusion 95 cc of the perfusate was recovered. The semimembranosus, gastrocnemius and tibialis anticus muscles were removed from both sides and hashed immediately. 50 gram samples were taken for creatine determination and from
l to 2 gram samples for the determination of the water content. Creatine was also determined in the defibrinated blood and the perfusate.

RESULTS. In 50 grams of perfused muscle containing 74.98% of water 108 milligrams of creatine were found. One gram of dry muscle substance therefore contains 8.59 milligrams of creatine. In 50 grams of non-perfused muscle containing 70.98% of water 124 milligrams of creatine were found. One gram of dry muscle substance contains 8.54 milligrams of creatine. Twenty five cubic centimeters defibrinated blood contains 2.43 mgm of creatine and twenty five cc of the perfusate contains 8.59 mgm of creatine. This increase in the perfusate is due to the methyl hydantoin formed by the heating the methyl ureido acetic acid, with 5 N sulphuric acid.

Experiment R

A dog weighing seven kilos was selected and prepared as above for perfusion of the right hind leg. The perfusion was started with 150 cc of solution consisting of 125 cc of defibrinated blood and 25 cc of 0.9% sodium chloride containing 2 grams of arginine carbonate, and maintained for one hour and fifty minutes after which 91 cc of the perfusate was recovered. For analysis the hamstring group, the gastrocnemius and tibialis anticus muscles were taken. The blood and perfusates were also analyzed as in Q.

RESULTS. Fifty grams of the perfused muscle contained 92.99 milligrams of creatine and was 72.57% water. This gives a creatine content of 6.72 milligrams to 1 gram of the dry muscle. Fifty grams of the non-perfused muscle contained 116 milligrams of creatine and 64.26% of water. One gram of the dry muscle substance contains 6.49 milligrams of creatine. Twenty five cc of the defibrinated blood of this animal contained 3.37 milligrams of creatine while the same amount of the perfusate contained 2.82 milligrams of creatine. This shows a slight increase in muscle creatine and a diminution of creatine in the perfusate.

Experiment S

The right hind leg of a dog weighing 19 kilos was perfused one hour and fifteen minutes with 150 cc of his own blood and 30 cc of 0.9% sodium chloride which contained two grams of arginine carbonate and 1.56 grams of acid potassium phosphate. The pressure during the perfusion was from 120 to 140 mm. At the end of the perfusion there was 200 cc of fluid recovered. For analysis the muscles of the leg were all removed. Determinations were made the same as in Q.

RESULTS. In fifty grams of perfused muscle containing 75.82% water 131.9 milligrams of creatine were found. One gram of dry muscle substance contained 10.91 milligrams of creatine. Fifty grams of non-perfused muscle containing 75.36% of water contained 138.3 milligrams of creatine and one gram of the dry substance 11.22 milligrams of creatine. 25 cc of the perfusate contained 3.05 milligrams of creatine and there were 3.37 milligrams in the defibrinated blood.
Conclusion. Methyl ureido acetic acid and arginine carbonate under the experimental conditions employed viz: autolysis perfusion and injection of muscle, do not give rise to an appreciable increase in muscle creatine.

Literature references.

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BIOGRAPHY.

J.I. Marker was born at Liscomb, Marshall County Iowa. After attending the public schools and graduating from the High School at Liscomb in 1905, he entered Mount Morris College, Mount Morris Illinois. Here he continued his preparatory work and later did the first year of collegiate work. In the fall of 1909 he entered the State University of Iowa in the Sophomore class L.A. After finishing the work of the Sophomore year and taking two years of the Medical course he was granted the degree of Bachelor of Science at the end of the Summer Session 1913. The present work was started then and continued to the present time.

June 9 1915.