LXV. THE NATURE OF THE SUGAR RESIDUE IN THE HEXOSEMONOPHOSPHORIC ACID OF MUSCLE.

BY JOHN PRYDE AND EDWARD THOMAS WATERS.

From the Physiology Institute, Welsh National School of Medicine, Cardiff.

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EMBDEN and Zimmermann [1924] have described the isolation from press-juice of rabbit muscle of a hexosediphosphoric acid which was obtained in the form of its neutral brucine salt. They experienced some difficulty in attempting its isolation owing to the presence of free phosphoric acid in their press-juice. Ultimately they eliminated the free phosphoric acid by employing a biological synthetic process involving the use of the fluoride ion which is known to further the synthesis of hexosephosphoric acid from carbohydrate and phosphates. Later Embden and Zimmermann [1927, 1], employing a somewhat different procedure and omitting the fermentative resynthesis, obtained from rabbit muscle pulp a hexosemonophosphoric acid. They were unable to detect the presence of a hexosediphosphoric acid.

In the present investigation it was desired to obtain a supply of the muscle hexosephosphoric acid (frequently referred to as "lactacidogen") in order to study the nature of the sugar residue present in this interesting intermediate substance. The two methods of Embden and Zimmermann were therefore both investigated as a means of obtaining one or other of the two hexosephosphoric acids, our immediate purpose being to obtain that which could be demonstrated to be a normal constituent of the muscle.

The earlier work of Embden and Zimmermann has been fully confirmed and we have obtained from rabbit muscle press-juice the neutral brucine salt of hexosediphosphoric acid identical in all respects with that obtainable from yeast fermentation and now shown by Robison and Morgan [1928] and by Morgan [1929] to be γ -fructose-1 : 6-diphosphoric acid. Similarly, employing the later methods of Embden and Zimmermann, we have likewise obtained only a hexosemonophosphoric acid and have been unable to detect the presence of any of the di-acid ester. It seemed possible that the different results obtained in the two processes of extraction might not be wholly ascribable to the omission of the fermentative resynthesis in the later method, but might in part be due to the other modifications introduced at the same time. It was therefore decided to check this point by employing the first method of extraction, omitting only the addition of sodium fluoride, glycogen and sodium bicarbonate, the reagents used in the fermentative resynthesis. There was

obtained only a hexosemonophosphate identical with the product obtained by the second method of extraction. No trace of diphosphoric acid was detected. The authors are therefore of opinion that the carbohydrate-phosphoric acid ester normally obtainable from resting muscle and regarded as "lactacidogen" consists of hexosemonophosphoric acid. The material used in the present investigation was therefore prepared from the mono-acid ester obtained by Embden and Zimmermann's second method of extraction.

Hexosemonophosphoric acid has been isolated not only from the muscles of rabbits but also from those of the goat and donkey. The yields obtained from the latter sources were lower than those obtained from the rabbit, details being given in the experimental section. This may in part be due to the enzymic breakdown of the compound owing to the necessarily longer time required for the preliminary extraction, but it is our opinion that the poorer yields cannot be wholly ascribed to this cause. The extraction was carried out with all possible speed and the cooling arrangements were as efficient as in the rabbit muscle extractions. It appears possible that the hexosemonophosphoric acid content of the muscles of the larger and more slowly moving animals may normally be less than that of the rapidly contracting muscles of the rabbit. During the course of the rabbit muscle extractions adenylic acid was obtained as has been recorded by Embden and Zimmermann [1927, 2], but this compound was obtained only in very small yield from the muscles of the larger animals. On the other hand, considerable quantities of inositol were obtained from the muscles of the goat and donkey, whilst it was not isolated from any of the rabbit muscle extracts. The best yield of hexosemonophosphoric acid obtained in the present work was 9 g. (37 g. of the neutral brucine salt) from 7 kg. of rabbit muscle, corresponding to 0.13 % of the weight of fresh muscle.

In the experimental section there is described the preparation of free hexosemonophosphoric acid *via* the barium salt, from the recrystallised brucine salt in which form it was first isolated. The acid showed $[\alpha]_{5461} + 33.6^\circ$, which is in good agreement with that quoted by Embden and Zimmermann for their preparation, namely $[\alpha]_D + 29.5^\circ$. Employing the Willstätter-Schudel hypiodite method of oxidation these workers found that the sugar constituent of their acid consisted of 91 % of aldose in one preparation and 93 % of aldose in another. Our preparations showed 90 % of aldose sugar. From the free acid a crystalline osazone was obtained without loss of the phosphoric acid grouping. It melted in the vicinity of $145\text{--}147^\circ$ with decomposition.

After a series of preliminary investigations which need not be detailed here it was decided that the nature of the sugar residue present in muscle hexosemonophosphoric acid could best be decided by oxidation to the corresponding hexonic acid and subsequent removal of the phosphoric acid group. The first step was effected with the use of bromine and the second with 10 % sulphuric acid in a sealed tube at 100° . There was finally obtained the calcium salt of a hexonic acid which was free from phosphorus and from reducing material. The free hexonic acid prepared from the calcium salt had $[\alpha]_{5461} + 10.9^\circ$, and

after heating for 1 hour at 65°–70° it showed $[\alpha]_{5461} + 24.0^\circ$. This change is characteristic of a hexonic acid undergoing lactonisation and these figures are in good agreement with those obtained for pure preparations of gluconic acid subjected to the same treatment. We are therefore of the opinion that the aldose of hexosemonophosphoric acid is *d*-glucose.

EXPERIMENTAL.

Isolation of hexosediphosphoric acid from rabbit muscle press-juice.

The first pair of rabbits used were not specially fed, but in the later experiments the rabbits were fed with generous supplies of oats for at least 3 days immediately before they were killed. Some were killed by a sharp blow on the back of the neck followed by immediate decapitation. A disadvantage of this method of killing is the resulting violent twitching of the muscles, and in the later experiments the animals were asphyxiated with coal gas until the corneal reflex failed and then decapitated. The pelt was removed as quickly as possible and the musculature was excised and minced in an ice-cold machine. The subsequent steps in the extraction were essentially those of Embden and Zimmermann [1924]. A Buchner oil press was used, and the juice was collected under pressures up to 150 kg./cm.² The cloth used was made of camel hair and was supplied by Messrs Premier Filterpress Co., Ltd., London. The yields obtained are indicated by the following typical results.

I. 2 rabbits, rather small and not specially fed. Juice obtained, 415 cc.; recrystallised brucine salt, 1.5 g.

II. 2 rabbits, fed on previous day with oats. Juice obtained, 500 cc.; recrystallised brucine salt, 2.5 g.

III. 2 rabbits, fed for 3 days with oats. Juice obtained, 540 cc.; recrystallised brucine salt, 3.5 g.

The brucine salt was twice recrystallised from aqueous methyl alcohol and dried *in vacuo* over sulphuric acid and finally at 56° over phosphorus pentoxide in a high vacuum. It gave satisfactory analyses and showed $[\alpha]_{5461}^{17^\circ} - 30.7^\circ$ (*c* = 1.01 %). For comparison purposes Mr W. J. T. Morgan, of the Lister Institute, London, kindly prepared a specimen of brucine hexosediphosphate from yeast fermentation and this showed in methyl alcohol $[\alpha]_{5461}^{18^\circ} - 30.7^\circ$, thus confirming the identity of the two products. When heated rapidly our twice recrystallised preparation melted with considerable charring between 170° and 180°.

Isolation of hexosemonophosphoric acid from the press-juice of rabbit muscle.

For reasons already given in the introduction it was decided to isolate from rabbit muscle and identify the hexosephosphoric acid or acids, using the same method as that used for the isolation of hexosediphosphoric acid, omitting only

the fermentative resynthesis involving the addition of glycogen and sodium fluoride in the presence of sodium bicarbonate.

The muscle juice from six normal rabbits was collected in as short a time as possible and in efficiently cooled vessels. With one exception, when the rabbit was killed by a sharp blow, all the animals were asphyxiated with coal gas. They had all been previously fed on oats for at least 3 days. The total volume of juice obtained was 1350 cc. At the stage when deposition of brucine hexosediphosphoric acid should have occurred no deposition of crystals was noted even after prolonged scratching and on allowing the solution to stand in the ice-chest overnight. The following day slow crystallisation began and after 2 days some clusters of small crystals separated from the solution. These were obtained in very small amount and were found to contain a pentose. They were subsequently proved to consist of the brucine salt of adenylic acid, to which reference will be made in a later communication. The solution was treated with several volumes of acetone in order to complete the deposition of the brucine salt of adenylic acid. The solid obtained was extracted with a small volume of anhydrous methyl alcohol and filtered. The filtrate was concentrated to dryness and again extracted with methyl alcohol and filtered. The solution was again taken to dryness and the crystalline residue was dried to constant weight. It had $P = 2.83\%$ (calc. for brucine hexosemonophosphate 2.96% and for the hexosediphosphate 3.21%), and showed $[\alpha]_{5461} - 19.4^\circ$ in methyl alcohol. Our later preparations of brucine hexosemonophosphate showed in the same solvent $[\alpha]_{5461} - 20.3^\circ$. In this experiment therefore there was obtained no evidence of the presence of a hexosediphosphoric acid and it is concluded that the latter is either entirely absent or present only in very small amounts in normal muscle press-juice.

Isolation of hexosemonophosphoric acid from rabbit, goat and donkey muscle.

The method of extraction was that already referred to as Embden and Zimmermann's second method, with minor modifications which need not be detailed.

Embden and Zimmermann [1927, 1] report an average yield of 11 g. of unrecrystallised brucine hexosemonophosphate from twelve rabbits yielding 6 kg. of muscle. The best yield obtained in the present work was 23 g. of brucine hexosemonophosphate from twelve rabbits yielding 7 kg. of muscle, whilst in this case the basic lead acetate fraction gave a further yield of 14 g. of the brucine salt. That our yields are materially higher than those recorded by Embden and Zimmermann is doubtless in part due to the fact that we subjected all protein residues to high pressure extraction, using the Buchner press.

After one recrystallisation brucine hexosemonophosphate gave in water $[\alpha]_{5461} - 29.9^\circ$ and in anhydrous methyl alcohol $- 20.3^\circ$. The recrystallised salt after drying *in vacuo* over phosphorus pentoxide showed signs of softening at 145° , melted at 155° and decomposed at $158-160^\circ$.

In order to obtain larger supplies of hexosemonophosphoric acid extracts were made from the minced muscles of larger animals, namely two goats and one donkey. The yields were disappointingly small considering the size of the animals. The animals were fed for some days on oats and hay and were killed with a "humane killer" and in no case was any muscle twitching observed after the death of the animal. The extractions were carried out in a manner identical with that used in the case of the rabbits and the operations were expeditiously carried through and the bulky solutions were efficiently cooled with ice. The protein precipitates were filtered on large stoneware filters, using muslin to give added support to the filter-papers. The work was continued without a break until the deproteinised filtrates had been freed from mercury and excess hydrogen sulphide and then almost neutralised with 33 % sodium hydroxide. The results may be summarised as follows.

Goat I ♀. All muscles minced and in acid bath in 25 minutes. Temp. rose for few minutes to 10°, but was quickly reduced to 2°. Weight of muscle, 6 kg.

Via normal lead acetate precipitate, 3.3 g. of brucine salt and 1.3 g. inositol.

Via basic lead acetate precipitate, 8.8 g. brucine salt and 1.8 g. inositol.

Goat II ♀. All muscles minced and in bath in 20 minutes. Temp. rose for few minutes to 10°, but solution was quickly cooled to 2°.

Weight of muscle, 10.5 kg.

Via normal lead acetate precipitate, 1.4 g. brucine salt and 0.7 g. inositol.

Via basic lead acetate precipitate, 3.0 g. brucine salt and 1.3 g. inositol.

Donkey ♂. All muscles minced and in acid bath in 50 minutes. Cooling arrangements efficient. Temp. quickly reduced to about 3° or 4°. Weight of muscle, 30.2 kg.

Via normal lead acetate precipitate, 3.4 g. brucine salt and 0.2 g. inositol.

Via basic lead acetate precipitate, 2.1 g. brucine salt and 4.5 g. inositol.

The brucine salts after recrystallisation gave the same optical rotation and melting point as those obtained from rabbit's muscle. Further, the free hexosemonophosphoric acid prepared *via* the barium salt from the mixed brucine salts obtained from rabbit, goat and donkey gave the same optical rotation.

A point which has been referred to in the introduction is the absence of adenylic acid and the presence of inositol in these later extractions. Using Bial's reagent it was established that the pentose-containing constituent was present only in very small amounts and it was found impossible to isolate any adenylic acid either from the goats or from the donkey. On the other hand, there was obtained, from the aqueous-acetone solutions from which adenylic acid was deposited in the case of the rabbit muscle extractions, a white crystalline compound which was recrystallised from hot water, in which it was readily soluble. It gave a negative result on testing with Molisch's reagent, did not reduce Fehling's solution, contained no nitrogen or pentose constituent and possessed a definitely sweet taste. It was found to be optically inactive, which indicated with the previous tests that this crystalline compound was *i*-inositol. This was confirmed by a melting point, the dried substance melting at 224–225°

with preliminary softening, and also by a positive Sherer's test. After two recrystallisations from water the substance still retained a slight trace of organically combined phosphorus (indicated with Bell-Doisy reagents).

Preparation of barium hexosemonophosphate.

Barium hexosemonophosphate was prepared from the recrystallised brucine salt by the method adopted by Embden and Zimmermann. The barium salt was precipitated as an amorphous solid by adding a saturated solution of barium acetate in 80 % methyl alcohol to a 10 % aqueous solution of the brucine salt. After standing for a few hours in the cold the salt was collected on a small Büchner funnel, washed with 80 % methyl alcohol, and finally with pure methyl alcohol. The product was then thoroughly dried over phosphorus pentoxide in a vacuum. It was ground in a mortar with a little water when most of the barium salt dissolved, leaving a less soluble yellowish residue. The barium salt was then reprecipitated with methyl alcohol and treated as before. This treatment was repeated at least once, sometimes twice; with all samples of the barium salt prepared. In this way the final product was freed from brucine and also from the more insoluble constituent with which it is initially associated. In one preparation it was found advantageous to warm for some time with a little norite. Again, in another preparation a considerable quantity of the less soluble material was separated from the barium salt by extracting with warm water. But it is essential that the precipitated product be thoroughly dried before a further aqueous extract is made, otherwise it is impossible to obtain the barium hexosemonophosphate in a state of purity.

Embden and Zimmermann record having obtained evidence of iron and phosphorus in one of the less soluble yellowish residues already referred to. The authors obtained negative results in testing for iron, except in one sample when a very faint blue colour was developed with potassium ferrocyanide, after thoroughly digesting the material with hot concentrated sulphuric acid and 30 % hydrogen peroxide. The brucine used in this instance, however, was itself found to contain traces of iron and it is therefore not thought that the iron found in the residue from the barium hexosemonophosphate has necessarily any biological significance. Repeated extraction of the less soluble material with water left the residues progressively poorer in phosphorus. Further, barium salts (especially those prepared from brucine salts obtained *via* the basic lead acetate precipitates) contaminated with this extraneous material gave low analytical figures and the free hexosemonophosphoric acid subsequently obtained showed a lower specific rotation and gave lower reduction values. These syrupy residues, which on drying form a glass, are therefore not of the nature of sugar phosphates and were not further investigated.

Hexosemonophosphoric acid.

The optical rotation of the free hexosemonophosphoric acid was determined in the following manner. A weighed amount of the dried barium salt was dissolved in water and the barium precipitated by the addition of a slight excess of sulphuric acid. The barium sulphate was centrifuged off, and the clear supernatant solution decanted into a suitable measuring flask. The barium sulphate precipitate was then washed twice with small quantities of water and after centrifuging these were added to the solution, which was finally made up to a definite volume. The free acid, liberated in the above manner from carefully dried barium hexosemonophosphate (Ba = 34.5 %, P = 7.8 %, calc. for $C_6H_{11}O_5(PO_4Ba)$, Ba = 34.7 %, P = 7.85 %), gave $[\alpha]_{5461}^{17^\circ} + 33.0^\circ$ in water ($c = 0.770$).

The hexosemonophosphoric acid obtained in the same manner from the mixed extracts of rabbit, goat, and donkey muscle, gave $[\alpha]_{5461} + 33.6^\circ$ in water ($c = 2.93$). As indicated above, specimens of barium hexosemonophosphate which gave low analytical figures, gave rise to a free acid with lower specific rotation and, further, correspondingly low copper reduction values. Thus one preparation of barium hexosemonophosphate gave a specific rotation for the free acid of $[\alpha]_{5461} + 23.1$ (calc. from the weight of barium salt originally taken). On regrinding some of the barium salt with water a small amount of insoluble material remained. After reprecipitation with methyl alcohol the salt was carefully dried as before. The barium salt obtained still had a low phosphorus content (P = 7.25 % calc. 7.85), but the rotation was higher than the previous value, being $[\alpha]_{5461} + 26.5^\circ$.

The copper-reduction value of hexosemonophosphoric acid was then determined, employing the method described by Bertrand. The values obtained were compared with those of glucose.

$$\text{Ratio } \frac{\text{Hexosemonophosphoric acid (calc. as hexose)}}{\text{Glucose}} = 0.74.$$

Formation of barium methylhexosidemonophosphate.

About 2.1 g. of barium hexosemonophosphate was dissolved as quickly as possible in 55 cc. of anhydrous methyl alcohol containing 1 % hydrochloric acid. Polarimetric observations were made on the solution and the observed rotations in a 1 dm. tube are recorded below. After the first 8 hours the solution was maintained at 25° in a well-stoppered flask. A short time after the first observation was made barium chloride began to separate out and it was then necessary to filter the solution immediately before observing the optical rotation. The solution still reduced Fehling's solution 74 hours after it was prepared. Tested after 97 hours the solution showed no reduction with Fehling's solution. Observed rotations $[\alpha]_{5461}$: $+0.41^\circ$ (8 hrs.), 0.29° (12 hrs.), 0.38° ($32\frac{1}{2}$ hrs.), 0.51° ($73\frac{1}{2}$ hrs.), 0.57° ($98\frac{1}{2}$ hrs.), 0.59° ($121\frac{1}{2}$ hrs.), 0.61° (146 hrs.), 0.64° ($168\frac{1}{2}$ hrs.). It will be noticed that initially there is a fall in

the observed rotation followed by an increase, which gradually approaches a maximum. These changes are evidently due to the different rates of formation of the α - and β -isomers of the methylhexosidemonophosphate.

An attempt was made to isolate the barium methylhexosidemonophosphate and then to dephosphate this compound with a preparation of bone enzyme, using the method described by Morgan [1927] and applied by him to the investigation of hexosediphosphoric acid from yeast. But the different solubilities of the corresponding salts of the hexosemonophosphoric acid make this method unsuitable, especially with small amounts of material.

An osazone was prepared from the hexosemonophosphoric acid without liberation of free phosphoric acid. 0.3 g. of the barium salt was treated with a slight excess of sulphuric acid and after removing the precipitated barium sulphate the solution was heated for half an hour in a boiling water-bath with 0.5 g. of phenylhydrazine hydrochloride and 1 g. of sodium acetate. Crystallisation commenced immediately the solution was cooled. Viewed under the microscope the crystals presented the appearance of clusters of flat needles. They began to soften at 140° and melted at 145°–147° with decomposition.

Willstätter-Schudel oxidation.

Employing the technique described by Goebel [1927] for the Willstätter-Schudel oxidation with hypiodite, hexosemonophosphoric acid gave values indicating that 90 % of the hexose in this acid is of an aldose nature.

After oxidation by this method, the solution still reduced Fehling's solution. This is interpreted as indicating the presence of some 10 % of a ketosemonophosphate admixed with 90 % of aldosemonophosphate.

Preparation of hexonic acid from hexosemonophosphoric acid.

About 0.8 g. of hexosemonophosphoric acid dissolved in 23 cc. of water was treated with 0.75 g. of bromine. After standing for 16 hours at air temperature a portion of the solution, after aerating to free from bromine, still reduced Fehling's solution. The solution was still slightly reducing to Fehling's solution after 9 hours in a water-bath kept at 30°. A micro-determination of sugar was then made employing the method of Shaffer and Hartman. Later determinations, after the solution had been maintained at 30° for a further period of 11 hours, gave a reduction value only slightly less than that obtained previously. From these determinations about 0.04 g. (calc. as glucose) remained unoxidised. It may be noted here that only slight traces of free phosphoric acid could be detected in the solution after the above oxidation. The solution was aerated to free from bromine and then sufficient hydrobromic acid added to give a 14 % solution of the acid. This solution was heated in a sealed tube at 85° for 20 hours. On opening the tube, free phosphoric acid was found to be present in small amount only. Sulphuric acid was then added sufficient to give a 10 % solution of this acid, and then after resealing the solution was

heated for 7 hours at 100°. The resulting perfectly clear solution contained considerable amounts of free phosphoric acid. It was neutralised with silver oxide, and the filtrate subsequently obtained was freed from silver with hydrogen sulphide. After aeration the solution was found to be free from phosphorus, non-reducing, and optically active in the dextro-sense. The solution was heated to 75°, calcium carbonate added, and the solution well stirred for several minutes. After cooling, the solution was filtered and concentrated to dryness at 45°. The solid residue was then extracted with a small volume of hot water and filtered. The filtrate still contained calcium sulphate which was precipitated by the addition of an equal volume of absolute alcohol. After standing for some days the solution was filtered and concentrated under reduced pressure. Calcium sulphate was still present in minute quantities, and the solution was again precipitated by adding several volumes of alcohol. The precipitate was collected and extracted with about 10 cc. of water. The small amount of insoluble material was centrifuged off and the clear supernatant solution again precipitated with several volumes of alcohol. The perfectly white product was collected and dried overnight in an evacuated desiccator containing phosphorus pentoxide. The dried material gave a calcium content of 9.2 % (micro-determination) (calc. for $(C_6H_{11}O_7)_2Ca$, 9.3 %).

0.118 g. of the calcium salt was dissolved in water and one drop of concentrated hydrochloric acid added to liberate the free hexonic acid. The volume was made up to 10 cc. thus giving a 1.06 % solution of the free hexonic acid.

This solution gave $[\alpha]_{5461}^{18^\circ} + 10.9^\circ$.

After the solution had been maintained at 50° for 1½ hours the rotation increased, and on cooling gave $[\alpha]_{5461}^{21^\circ} + 14.6^\circ$.

On the addition of a small drop of concentrated hydrochloric acid the rotation dropped immediately to its previous value. The solution was reheated for an hour at 65°–70° and then gave $[\alpha]_{5461}^{68^\circ} + 26.8^\circ$.

The temperature of the jacketed tube was then speedily reduced to 24°. The solution then showed $[\alpha]_{5461}^{24^\circ} + 24.0^\circ$.

A reading taken 15 minutes later showed identically the same value. These results are in good agreement with those recorded by Tollens [1914] for gluconic acid. The calcium salt has $[\alpha]_D + 10^\circ$ and prepared directly from the calcium salt the equilibrium mixture of free acid and lactone has $[\alpha]_D + 23.4^\circ$ after the solution has been heated for some time.

SUMMARY.

(1) The hexosephosphoric acid of normal muscle press-juice from rabbit, goat and donkey is a monophosphoric acid. The diphosphoric acid, identical with that of yeast fermentation, is only obtained when the fermentative resynthesis using sodium fluoride is employed.

(2) The carbohydrate residue of muscle hexosemonophosphoric acid consists of 90 % of aldose, identified as *d*-glucose, and 10 % of ketose.

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