XXXVII. ON AMINO-ACIDS. PART II. HYDROXYGLUTAMIC ACID.

By HENRY DRYSDALE DAKIN.

REPORT TO THE MEDICAL RESEARCH COMMITTEE.

(Received November 7th, 1919.)

THE object of the following communication is to record the results of experiments made in continuation of work already published in this *Journal* [Dakin, 1918]. In the previous communication it was shown that the products of hydrolysis of a protein, *e.g.* caseinogen, might be extracted with partially miscible solvents, such as butyl alcohol, so that the feebly ionised monamino-acids and proline were substantially removed¹, while the strong acids and bases remained behind. Evidence was presented of the occurrence among the acids, in addition to glutamic and aspartic acids, of a new amino-acid which was regarded as β -hydroxyglutamic acid from a study of its composition and reactions. The present paper contains the results of the further study of this acid together with experiments on its synthesis and that of allied substances. For convenience, the subject-matter is divided into the following sections:

I. On the synthesis of inactive β -hydroxyglutamic acid and allied substances.

II. The *p*-nitrophenylosazones of malic semi-aldehyde and tartronic semi-aldehyde.

III. Optical activity of natural β -hydroxyglutamic acid.

IV. Alkaloid salts of β -hydroxyglutamic acid and other amino-acids.

V. Identification of β -hydroxyglutamic acid among the products of hydrolysis of glutenin and gliadin.

VI. The fate of β -hydroxyglutamic acid in the diabetic organism.

I. ON THE SYNTHESIS OF INACTIVE β -HYDROXYGLUTAMIC ACID AND ALLIED SUBSTANCES.

At first sight it appeared that the synthesis of inactive β -hydroxyglutamic acid would be a relatively simple problem capable of solution by many methods,

¹ It is perhaps worth recording the fact that if the amino-acid aqueous solution is saturated with potassium carbonate and then extracted with ordinary ethyl alcohol, the monamino-acids are found in the alcoholic extract as potassium carbamates and may be precipitated by acetic acid.

but, at any rate in the writer's experience, this has not proved to be the case. The first method that was tried consisted in brominating the acetyl derivative of β -hydroxyglutaric anhydride described by Blaise [1903] and then acting on the product with ammonia:

The acetyl derivative is obtained by the action of acetic anhydride on β -hydroxyglutaric acid, prepared by the reduction of acetonedicarboxylic acid. But while the bromination apparently went smoothly, the action of ammonia removed the halogen without introducing an amino-group, probably through the formation of a trimethylene derivative. An analogous reaction was observed by Perkin and Tattersall [1905] in the case of α -bromoglutaric acid.

A more promising method presented itself in the reduction of α -isonitroso-acetonedicarboxylic ester:

A very large number of experiments were made with this substance, using almost every known reducing agent that had the smallest prospect of success, but the reaction did not proceed smoothly. Using sodium or sodium amalgam in neutral or acid solution, it was an easy matter to reduce the ester and to obtain an acid which, in many ways, resembled the natural hydroxyglutamic acid and had approximately the same composition. But a variable proportion of the product when tested with nitrous acid was found not to be an amino-acid nor was it a hydroxypyrrolidonecarboxylic acid for it did not give an amino-acid on warming with hydrochloric acid. The separation of the products of reaction proved very difficult and has not been satisfactorily accomplished. It appeared that complicated condensations followed the production, as the first stage of the reaction, of α -amino-acetonedicarboxylic ester, such as are known to occur with amino-acetoacetic ester. It therefore seemed desirable to limit the reactivity of the carbonyl group. The introduction of an acetyl group attached to oxygen, by means of acetyl chloride in pyridine solution, proved of no avail. Later the observation was made that ethoxyglutaconic ester, obtained by the action of orthoformic ester on acetonedicarboxylic ester, on reduction with sodium amalgam in the presence of excess of carbon dioxide

gave β -hydroxyglutaric acid instead of β -ethoxyglutaric acid as might be expected:

It therefore appeared possible that α -isonitroso-acetonedicarboxylic ester might be converted into isonitroso- β -ethoxyglutaconic ester (II) and that the latter might be successfully reduced to hydroxyglutamic acid. Orthoformic ester was found to react vigorously with isonitroso-acetonedicarboxylic ester but the product, which cannot be distilled, sometimes gave results on analysis indicative of the ethoxy ester, but often, after simple manipulations and careful drying, nothing but unchanged nitroso-acetonedicarboxylic ester was obtained. It was clear that the ethoxy group was very easily removed and indeed on reversing the order of the reactions and starting with ethoxyglutaconic ester (I) it was found that nitrous acid could convert it into nitroso-acetonedicarboxylic ester (III) and when present in excess into hydroxy-isoxazoledicarboxylic ethyl ester (IV):

As is well known from Claisen's researches, the initial reaction between a β -ketonic ester and orthoformic ester in the presence of a suitable catalyst involves the formation of a γ -diethoxy ester which usually parts with a molecule of alcohol to give an ethoxy derivative of an olefinic acid, but which in turn may be reconverted into the original ketonic ester by simple hydrolysis:

Accordingly the reaction between orthoformic ester and isonitroso-acetonedicarboxylic ester was effected at low temperatures, and in order to limit decomposition of the product, it was reduced with sodium amalgam after a minimum of manipulation. The results were rather more satisfactory than those obtained by the direct reduction of isonitroso-acetonedicarboxylic ester, and small amounts of an amino-acid were eventually isolated which bore a very close resemblance to natural β -hydroxyglutamic acid. However, the final stage in the preceding synthesis does not proceed as smoothly as might be desired, and as the amino-acid to be finally isolated resembles the natural acid in being extremely soluble and difficult to crystallise or characterise by

derivatives, and, moreover, readily passes over into a pyrrolidone derivative with loss of water, it appeared desirable to attempt some other mode of synthesis, as a confirmation.

The next method to be tried was based upon Strecker's well-known synthesis of amino-acids from aldehydes by way of the amino-nitriles. For this purpose it was necessary to prepare the hitherto unknown semi-aldehyde of malic acid, *i.e.* β -hydroxy- γ -aldehydobutyric acid. This was successfully accomplished in the following way— γ -diethoxyacetoacetic ester (I) obtained as previously described by Dudley and the writer [1914], was reduced with sodium amalgam to γ -diethoxy- β -hydroxybutyric acid (II). The latter substance, on careful hydrolysis with dilute sulphuric acid, gave malic semi-aldehyde (III):

Unfortunately, the malic semi-aldehyde, when subjected to Strecker's reaction substantially under the same conditions as those used by Fischer in the synthesis of serine from glycollic aldehyde, gave only a negligible trace of an amino-acid. From 20 g. of the aldehyde, only enough amino-acid was obtained to observe its qualitative reactions, which resembled those of the natural acid, and to furnish about 0.2 g. of its silver salt. Attempts to improve the yield were unavailing and the method, which seems often to prove unsatisfactory in the case of hydroxyaldehydes, was abandoned.

More satisfactory results were obtained with a synthesis starting with glutamic acid. Glutamic acid (I) was converted in α -uraminoglutaric acid (II)

by the action of potassium cyanate, and hydantoin-propionic acid (III) was obtained from the latter by warming with hydrochloric acid, as already described [Dakin, 1910]. The action of bromine on hydantoin-propionic acid was then studied. It was found that on warming hydantoin-propionic acid dissolved in excess of glacial acetic acid with a molecular proportion of bromine, the latter quickly disappeared and that much more than half of the bromine was obtained as hydrobromic acid. The products of the reaction reduced permanganate solution freely and could absorb further amounts of bromine, so that it was clear that unsaturated products had been formed. On brominating concentrated solutions in glacial acetic acid, it was found that less of the bromine appeared as hydrobromic acid and a crystalline mixture of substances was separated which appeared to be made up of the unsaturated acid together with its hydrobromic acid addition product. The latter could be readily separated in the form of colourless cubes by repeated crystallisation from hydrobromic acid solution and from its reactions and composition is regarded as hydantoin- β -bromopropionic acid (V). It is reduced by zinc dust to hydantoin-propionic acid and, like most β -bromo-compounds of glutaric acid, is very unstable towards alkali. The unsaturated acid was obtained by boiling the mixture just referred to with zinc dust, separating it as zinc salt and then decomposing the latter with hydrogen sulphide. It is a sulphur yellow substance, reducing permanganate freely, and is convertible into hydantoin- β -bromopropionic acid with hydrobromic acid. It absorbs bromine slowly in the cold when dissolved in acetic acid but rapidly at 100°. This substance, to which is assigned the formula (IV), is somewhat difficult to name satisfactorily but may be regarded as *hydantoin- β . γ -propenylic acid*. Its relation to glutaric acid is clear and the possibility of *cis* and *trans* isomers is also evident. It is not improbable that it is formed by the removal of hydrobromic acid from a bromohydantoin-propionic acid in which the bromine is in the γ -position.

Additional evidence as to the constitution of hydantoin- β -bromopropionic acid and its formation by the addition of hydrobromic acid to hydantoin- β . γ -propenylic acid is furnished by the following facts. In the first place, the yield of this substance is increased by brominating hydantoin-propionic acid in the presence of excess of hydrobromic acid. Secondly, although the hydantoin-propionic acid is strongly optically active, the β -bromo-derivative is entirely inactive although containing an asymmetric carbon atom. This result is readily interpreted if the reaction takes place as indicated, for the intermediate unsaturated acid (IV) has lost its asymmetry. Lastly, if the bromination of hydantoin-propionic acid is carried on in fuming hydrochloric acid solution, hydantoin- β -chloropropionic acid (VI) is readily obtained.

A close analogy to the first stage of the reaction under discussion is furnished by the action of bromine upon *p*-hydroxybenzylhydantoin which was found by Johnson and Hoffman [1912] to give the unsaturated 3·5-dibromo-4-hydroxybenzalhydantoin. Gabriel [1906] also observed a similar

change in the formation of "pyvureidic acid" on brominating hydantoin-acetic acid.

The conversion of hydantoin- β -bromopropionic acid into β -hydroxyglutamic acid, a reaction involving the opening of the hydantoin ring by hydrolysis and replacement of the halogen by hydroxyl, is not as easy as might be wished. On warming the acid with excess of barium hydroxide, a clear solution is at first obtained, but in a few moments an insoluble barium salt separates out. This salt is a mixture which contains progressively less nitrogen as the amount of barium hydroxide and time of heating are increased and is mainly made up of barium salts of acids containing two or possibly three carbon atoms together with barium carbonate. On prolonged boiling with excess of barium hydroxide traces of an amino-acid are formed, but the bulk of the substance undergoes complete decomposition. On boiling with pyridine or other bases, the halogen of hydantoin- β -bromopropionic acid is readily removed, but carbon dioxide is evolved and neutral products result. The instability of the five carbon chain of hydantoin- β -bromopropionic acid towards alkali is not surprising in view of the ease with which β -bromoglutaric acid is known to give vinylacetic acid when warmed with soda [Wislicenus, 1899; Ssamenov, 1899].

Better results were obtained on simple boiling of hydantoin- β -bromopropionic acid with water. Hydrobromic acid is slowly split off, and among other complex products a crystalline substance is easily separated in about 20 % yield, which is an unsaturated acid, isomeric with the hydantoin- β . γ -propenylic acid already described. Like the latter substance, it is a bright sulphur yellow compound and absorbs hydrobromic acid and bromine on warming. This substance appears to be hydantoinacrylic acid as represented by formula (VII). On prolonged treatment of this substance with hot barium hydroxide solution, the hydantoin ring is opened with removal of ammonia and carbon dioxide, while the elements of water are taken up with formation of β -hydroxyglutamic acid. The opening of the hydantoin ring by alkaline hydrolysis is of course a common reaction, as is the formation of a β -hydroxy-acid from an unsaturated acid by treatment with alkali, *e.g.* glutaconic acid gives β -hydroxyglutaric acid and fumaric acid yields malic acid. The yield of amino-acid is disappointing, amounting to about 20 % of the hydantoinacrylic acid taken. Calculated on the glutamic acid used as the starting material, the yield is not more than about 2 %. An alternative method of converting hydantoin- β -bromopropionic acid into hydroxyglutamic acid consisted in hydrolysing the former with fuming hydrochloric or hydrobromic acid in a sealed tube at 130°, and subsequently removing the excess of acid and boiling the product with lime water to replace the halogen by hydroxyl. The results were not much more satisfactory than those just described.

The synthetic inactive β -hydroxyglutamic acid isolated as described in the experimental portion of the paper could not be obtained in large enough amounts to permit of a detailed study, but the following points of resemblance

to the natural optically active acid were noted and no dissimilarities were observed.

The acid is extremely soluble in water and crystallises with great difficulty. It is insoluble in alcohol and most organic solvents. The whole of its nitrogen is in the NH_2 form and can be liberated by nitrous acid in van Slyke's apparatus. On heating to 105° , almost the whole of the nitrogen goes over into the (NH) form, through formation of a hydroxypyrrolidonecarboxylic acid with loss of water, and no longer reacts with nitrous acid. The natural acid shows precisely the same behaviour and the ease with which it parts with water accounts for the lack of a sharp melting point. The copper salt, like that of the natural acid, is very soluble in water, insoluble in alcohol. The acid titrates to litmus as a monobasic acid and forms neutral salts with the alkali metals containing one equivalent of base and alkaline salts containing two equivalents. The *silver salt* is a white granular precipitate almost insoluble in water, contains two equivalents of silver and resembles the salt of the natural acid closely. The acid is saturated and does not promptly reduce permanganate in the cold in alkaline solution.

On treating a few milligrams of the substance with diazobenzenesulphonic acid and then adding sodium hydroxide, a cherry red colour develops (or orange red if the solution is very dilute) which becomes intensely deep on warming¹. If sodium carbonate be used as alkali, the colour tone in the cold is orange red which deepens to cherry red on warming. The natural acid, separated from its crystalline strychnine salt, shows the same reaction. Lastly, on gently warming a few milligrams of the acid dissolved in a drop or two of water with β -naphthol and concentrated sulphuric acid, a brilliantly fluorescent yellowish green solution results². The same reaction is shown by the natural acid and by malic acid.

Since β -hydroxyglutamic acid contains two dissimilar asymmetric carbon atoms it may occur in four active forms and at least two inactive forms. It has not been possible to obtain sufficient of the inactive synthetic acid for resolution into active forms so that the space relationships of both active and synthetic acid await further investigation.

In order to save unnecessary duplication by other workers, it may be of some value to indicate a number of other attempted syntheses of β -hydroxyglutamic acid which were tried without success, though of course it is quite

¹ Most amino-acids on *boiling* with diazobenzenesulphonic acid and caustic soda develop a reddish colour, but the reaction is trivial compared with the above. Rosenthaler [1912] has described an analogous reaction for malic acid.

² In the original description of the natural acid it was stated that it gave a violet red colour with resorcinol and sulphuric acid. This is an error and is apparently connected with the use of silver nitrate in separating the acid. Nitric acid gives a similar reaction, but preparations of the amino-acid containing so little nitric acid that no reaction could be obtained with the brucine test still gave a positive reaction. When no silver nitrate was used in the separation of the acid, the characteristic reaction with resorcinol was not obtained. Possibly it is due to a trace of some oxidation product.

possible that some other of them might be successful if more suitable conditions were found than those employed by the writer:

(a) Bromination of benzoylglutamic acid, with the hope of obtaining a benzoylamino-glutaconic acid by removal of hydrobromic acid.

(b) Reduction of *o*-toluidine-azo-acetonedicarboxylic ethyl ester [cp. Bülow and Göller, 1911].

(c) Reduction of various hydrazine derivatives of acetonedicarboxylic ester.

(d) Condensation of oxymethylenehippuric ester with malonic ester.

(e) Condensation of dichloroacetal with malonic ester followed by reactions analogous to Leuch's synthesis of serine.

(f) Reduction of isonitrosoglutaconic ester.

(g) Reduction of hydroxyisoxazoledicarboxylic ethyl ester.

The isolation of β -hydroxyglutamic acid from the products of hydrolysis of proteins makes it desirable to know more of related hydroxy-amino-dicarboxylic acids. Reference was made in a previous paper [Dakin, 1918] to Skraup's statement as to the occurrence of hydroxyaspartic acid in caseinogen. It was there stated that "As Fischer has pointed out Skraup's experiments are not described in a form capable of repetition and no evidence other than the analysis of a copper salt is adduced to show that the minute amount of substance obtained by Skraup was a dicarboxylic acid. The value of this analysis for determining the basicity of the acid is small, since no carbon or hydrogen determinations were made on the free acid." Almost immediately after Skraup's announcement, Neuberg stated that he had obtained hydroxyaspartic acid in small amount by the action of nitrous acid on diaminosuccinic acid. Erlenmeyer and Bade [1904] state that they synthesised the same substance by reducing oxalylhippuric ester, but no account of the process or product has yet appeared, so far as we have been able to ascertain. The writer has endeavoured to synthesise hydroxyaspartic acid and has found that it is formed by the action of ammonia on barium chloromalate, a reaction which is similar to one partially investigated by Lossen. A detailed account of the acid, which has entirely different properties from those described by Skraup and by Neuberg, will form the subject of a separate communication.

It is a curious fact that the amino-acid gives a so-called abnormal copper salt, resembling iso-serine in this respect, as was shown by Fischer. The presence of a hydroxyl group contiguous to a carboxyl group is undoubtedly responsible for the fact that the copper salt contained three equivalents of copper instead of two. The structural similarity between iso-serine which combines with two equivalents of copper and hydroxyaspartic acid is clear from the following formulae:

The formation of this abnormal copper salt of synthetic hydroxyaspartic acid is clearly of value in further questioning the identity of Skraup's acid which was considered to be a hydroxyaspartic acid largely on the basis of the analysis of a copper salt which was believed to possess a normal composition.

EXPERIMENTAL.

In the following section, the experiments leading to the synthesis of β -hydroxyglutamic acid from hydantoin-propionic acid will be considered first, since they have given the most satisfactory results. The preparation of malic semi-aldehyde and its derivatives will then be described. Only a brief outline of experiments on the reduction of isonitroso-acetonedicarboxylic ester and its condensation products with orthoformic ester will be included since they cannot be regarded as satisfactorily completed. The melting points recorded are all "uncorrected" observations, unless otherwise indicated.

Hydantoin-propionic acid. The preparation and properties of this substance have already been described by the writer [1910]. Certain minor changes in its preparation have proved advantageous as regards the yield. Glutamic acid, 29.4 g., is heated on the water-bath with about 100 cc. of water, and strong sodium hydroxide solution is gradually added until the whole of the acid is dissolved and the reaction of the solution is approximately neutral to litmus. To the solution of mono-sodium glutamate thus obtained 20 g. of freshly prepared potassium cyanate (1.2 mols.) is added and the mixture allowed to evaporate slowly over the water-bath for about an hour. The solution is then made acid to Congo-red with hydrochloric acid and an additional 30 cc. of the concentrated acid is added. The solution is then heated in a covered dish on the water-bath for two or three hours in order to convert the uramino-acid first formed in the alkaline solution into hydantoin-propionic acid. The mixture is next transferred to a capacious flask and evaporated to dryness under diminished pressure. The residue is then extracted with boiling alcohol, thus dissolving the acid and leaving sodium and potassium chlorides undissolved. The alcoholic solution is at once diluted with water and the alcohol removed by evaporation. Hydantoin-propionic acid slowly crystallises out of the concentrated solution in a yield fully equal to the weight of glutamic acid taken. The direct evaporation of the alcoholic solution without dilution with water should be avoided, as in this case an uncrystallisable syrup frequently results, possibly owing to ester formation.

Bromination of hydantoin-propionic acid. The first point to be investigated concerned the proportion of hydrobromic acid formed on acting upon hydantoin-propionic acid (1 mol.) with bromine (1 mol.) in glacial acetic acid solution. The experiments were conducted in small sealed tubes which, after heating at 90° until all the bromine had disappeared, were opened under water, acidified with nitric acid and the hydrobromic acid estimated volumetrically with standard silver nitrate solution. Appropriate blanks were carried out and the necessary corrections made. In experiments employing

0.2 g. of the acid and an equivalent amount of bromine in 1 cc. of glacial acetic acid, it was found that hydrobromic acid was liberated equivalent to from 1.5–1.78 molecular proportions. Slightly higher results were obtained on increasing the dilution with acetic acid and lower results if the solvent were reduced. It was therefore clear that a large proportion of any bromine entering the hydantoin-propionic acid molecule was removed as hydrobromic acid, but, as was subsequently found, the unsaturated products formed can again take up hydrobromic acid under suitable conditions.

On brominating larger quantities of the hydantoin-propionic acid dissolved in six parts of glacial acetic acid, as above described, and then mixing the resulting solution with water and crystallising after evaporation, a light yellow substance was obtained melting indefinitely around 210–220°. Under the microscope it was clearly seen to be a mixture and contained about 35 % carbon, 3 % hydrogen and about 20 % of bromine. This mixture furnished hydantoin- β -bromopropionic acid on repeated crystallisation from hydrobromic acid solution, while on reduction with zinc dust it gave hydantoin- β , γ -propenylic acid, as described later. The yield of crystalline mixture obtained amounted to about half the weight of acid brominated.

Hydantoin- β -bromopropionic acid. The preceding experiments clearly indicated that the bromine derivative of hydantoin-propionic acid was almost surely formed by the secondary addition of hydrobromic acid to an unsaturated intermediate product (hydantoin- β , γ -propenylic acid). It was therefore probable that it could best be prepared in a medium already containing preformed hydrobromic acid. Five parts of aqueous hydrobromic acid (50 %) were tried with satisfactory results, but the best yields were obtained as follows.

Hydantoin-propionic acid, 17.2 g., is boiled with 30 cc. of glacial acetic acid in a strong flask and while the mixture is still fairly hot, 35 cc. of glacial acetic acid previously saturated with hydrobromic acid at 0° are added and the mixture at once cooled under the tap. The acid will now readily dissolve and remain in solution. Bromine, 16 g., is added and the flask is then closed and gently warmed at about 60°–70° on a water-bath. In the course of an hour or two, the whole of the bromine will disappear and the mixture is then allowed to stand in a cool place for a day or two. After a time, a crop of white crystals separates and is removed by filtration, using an asbestos pad on a porcelain funnel. On concentrating the mother liquor under reduced pressure, further crops may be obtained leaving a thick syrup as mother liquor. The crystals are recrystallised from boiling 30 % hydrobromic acid and separate as perfectly white cubes and rhombic prisms melting at 228–230° with effervescence and turning deep brown in colour. If crystallised from water, slight decomposition takes place, giving a yellowish product. The yield amounts to 9–11 g. The substance reacts as a monobasic acid to litmus. On titrating 0.25 g. with *N*/10 sodium hydroxide 10 cc. were required, equivalent to a molecular weight of 250, compared with a calculated value of 251.

Analysis. 0.1253 g.; 0.1334 CO₂; 0.0309 H₂O,
0.20 g.; 0.0221 N (Kjeldahl),
0.10 g.; 0.0753 AgBr.

	C	H	N	Br
Calculated for C ₆ H ₇ O ₄ N ₂ Br	28.7	2.78	11.1	31.8
Found 	29.0	2.74	11.0	32.0

Hydantoin- β -bromopropionic acid is readily soluble in hot water, sparingly in cold water, but dissolves more easily in acetic acid. It is sparingly soluble in ether or chloroform. On ignition, the vapours do not give the pyrrole reaction with a pine splinter, but if the substance is mixed either with sodium carbonate or zinc dust before ignition, an intense reaction is obtained. The whole of the bromine is readily removed on warming for a few minutes with strong sodium hydroxide solution, but it is decidedly more stable towards ammonia. On heating with excess of pyridine at 100°, carbon dioxide is split off together with the bromine, and a neutral product, melting at over 270°, results. Its reaction towards boiling water is described in a subsequent section and the action of barium hydroxide has already been referred to. The neutral salts are not particularly unstable, for a small amount of the acid, digested on the water-bath for half an hour with a suspension of chalk in water, lost only about a third of its bromine.

Hydantoin- β -bromopropionic acid (1 part) was reconverted into hydantoin-propionic acid by warming it on the water-bath with granulated zinc (3 parts) and 50 % acetic acid (10 parts). The solution was evaporated to dryness, dissolved in water and the zinc removed with hydrogen sulphide. On concentrating the filtrate, the hydantoin-propionic acid readily crystallised.

Hydantoin- β , γ -propenylic acid. This acid was separated from the mixture of crystalline substances resulting from the bromination in acetic acid solution of hydantoin-propionic acid, as described in the preceding section. The mixture (5 g.) was boiled for one hour with zinc dust (20 g.) and water (400 cc.). The bulk of the hydantoin- β -bromopropionic acid is completely decomposed by this treatment and the remainder reduced to the very soluble hydantoin-propionic acid, while the unsaturated acid is substantially unaffected. On filtering and concentrating the filtrate to small bulk, a deep yellow zinc salt, moderately soluble in water but insoluble in alcohol, crystallises out. This was filtered off, dissolved in hot water, the zinc removed with hydrogen sulphide and the filtrate again concentrated. About 0.7 g. of a sulphur yellow compound, crystallising in fine hexagonal prisms, was obtained. It is readily soluble in hot water and fairly soluble even in cold water. It melts at 222–223° without darkening and gives a solid sublimate. It gives a strong cherry red colour with diazobenzenesulphonic acid in the presence of sodium hydroxide, thus resembling glutaconic acid, of which it may be regarded as a derivative. On warming with silver nitrate and ammonia, very little or no reduction takes place. On treatment with β -naphthol and excess of concentrated sulphuric acid it gives a clear yellowish green solution in the cold, turning suddenly violet red on gentle warming. The pyrrole reaction is positive. The acid re-

duces permanganate freely in sodium carbonate solution, and absorbs bromine or hydrobromic acid in acetic acid slowly in the cold but readily at about 80°.

Analysis. 0.1598 g.; 0.2468 CO₂; 0.0512 H₂O.

	C	H
Calculated for C ₆ H ₆ O ₄ N ₂ ...	42.3	3.53
Found	42.1	3.55

Hydantoin-β-chloropropionic acid. This compound was obtained by warming in a sealed tube at 95° hydantoin-propionic acid (8.6 g.) dissolved in fuming hydrochloric acid (40 cc.) and bromine (8.0 g.). The whole of the bromine disappeared in less than an hour. When the tube was kept in a cold place for a couple of days about 3 g. of crystals separated, and further small amounts of less pure material were obtained on concentrating the mother liquor under reduced pressure. The substance was purified by recrystallisation from 20 % hydrochloric acid, in which it is readily soluble when hot but sparingly soluble in the cold. It crystallises in rectangular plates which are without colour when pure. On recrystallising from water they become yellowish. The reactions of the substance are identical with those of the corresponding bromine compound already described. Using litmus paper as indicator, the substance reacts as a monobasic acid—0.2 g. required 9.77 cc. of *N*/10 sodium hydroxide equivalent to a molecular weight of 206—the theoretical value being 206.5.

Analysis. 0.1299 g.; 0.1660 CO₂; 0.0381 H₂O,
0.20 g.; 0.0266 N (Kjeldahl),
0.10 g.; 0.0682 AgCl.

	C	H	N	Cl
Calculated for C ₆ H ₇ O ₄ N ₂ Cl	34.9	3.39	13.5	17.2
Found	34.9	3.26	13.3	16.9

Hydantoinacrylic acid. Hydantoin-β-bromopropionic acid (10 g.) was boiled with water (200 cc.) under a reflux condenser for three or four hours. The solution was then concentrated to about 50 cc. and allowed to crystallise. The yellow crystals thus separated were again boiled for a short time with sufficient fresh distilled water to dissolve them and allowed to crystallise again. On diluting the first mother liquor, again boiling and concentrating to small bulk, a little more of the crystalline product may be obtained. The total yield amounts to 15–20 % of the theoretical. It should be tested for the absence of bromine and if any is found it should be again boiled with water, or alternatively it may be dissolved in excess of warm sodium hydroxide and then precipitated with hydrochloric acid. The substance when pure is sparingly soluble in cold water and only moderately so in hot water. It crystallises in rosettes of sulphur yellow needles melting at 256–258° with effervescence. It is very sparingly soluble in acetic acid, alcohol or ethyl acetate. It does not react with phenylhydrazine acetate but reduces ammoniacal silver solutions on boiling. It does not reduce mercuric chloride. Bromine and hydrobromic acid in acetic acid solution are absorbed slowly when cold, but readily on warming. Diazobenzenesulphonic acid gives a strong cherry red reaction with

a trace of the acid dissolved in sodium hydroxide. The substance reacts as a monobasic acid to litmus—0.1427 g. required 8.8 cc. *N*/10 sodium hydroxide, though the end point was not perfectly sharp. This titration is equivalent to a molecular weight of 162 compared with a calculated value of 170.

Analysis. 0.1405 g.; 0.2179 CO₂; 0.0480 H₂O,
0.1425 g.; 0.0248 N (Kjeldahl).

	C	H	N
Calculated for C ₆ H ₆ O ₄ N ₂ ...	42.3	3.53	16.5
Found	42.1	3.81	16.7

β-Hydroxyglutamic acid. Hydantoinacrylic acid (2 g.) was boiled under a reflux condenser with recrystallised barium hydroxide (20 g.) and water (60 cc.). At the top of the condenser a small absorption bulb was placed containing standard sulphuric acid and a little alizarin as indicator. Whenever the contents of the bulb became alkaline owing to absorption of ammonia, 1 cc. more normal acid was added. In this way it is possible to follow the course of the hydrolysis and to stop the heating after ammonia has been liberated equivalent to half the nitrogen of the hydantoinacrylic acid. From 5–6 hours were usually sufficient to complete the reaction and more prolonged boiling is disadvantageous. The solution in the flask, which was turbid from separation of barium carbonate but was no longer yellow, was made acid to Congo-red with sulphuric acid and filtered from barium sulphate. The solution was then concentrated to about 20 cc. on the water-bath and while still acid to Congo-red silver nitrate (5 g.) added. The small precipitate containing traces of silver chloride was filtered off and the silver salt of the amino-acid was precipitated from the filtrate as previously described for the natural acid [Dakin, 1918]. It is important to use pure alkali especially free from silica. The silver salt mixed with excess of silver oxide was filtered off and thoroughly well washed with cold water. It was then suspended in water (20 cc.) and completely decomposed with hydrogen sulphide. The filtrate from the silver sulphide was then concentrated to about 1 cc. in volume at a temperature of not over 50°. Absolute alcohol in excess was then added to the syrup with stirring and the sticky mass of amino-acid thoroughly well washed with alcohol. It was then dissolved in a few drops of cold water, in which it dissolves with the greatest ease, and reprecipitated with alcohol. The acid separates out as a white granular powder which is apt to be sticky if much water is present. It is dried *in vacuo* over phosphorus pentoxide at room temperature since it very readily passes over to hydroxypyrrolidonecarboxylic acid on heating. It was not found possible to crystallise the acid satisfactorily and its solutions when allowed to evaporate *in vacuo* gave a brittle glassy mass. The yield of amino-acid from 2 g. of hydantoinacrylic acid varied from 0.3–0.4 g.

Like the natural acid, the synthetic product gives the pyrrole reaction on heating, which is intensified on igniting with zinc dust. On coupling with diazobenzenesulphonic acid in sodium carbonate solution an orange red colour is obtained, while with sodium hydroxide as alkali, a deep cherry red is

obtained which is intensified on heating. The reaction is sensitive to about 1 : 1000. On warming with β -naphthol and excess of concentrated sulphuric acid a greenish-yellow fluorescent solution is obtained similar to that given by malic acid (Pinerua). Its conversion through loss of water on heating to 105° into a hydroxypyrrolidonecarboxylic acid is also characteristic.

As stated earlier, a small amount of the amino-acid may be obtained by the direct decomposition of hydantoin- β -bromopropionic acid with barium hydroxide, but by far the larger part of the acid undergoes profound decomposition with rupture of the carbon chain and the yield of amino-acid is extremely small. Better results may be obtained by hydrolysing the hydantoin- β -bromopropionic acid (5 g.) by heating in a sealed tube for four to five hours with five parts of fuming hydrochloric or hydrobromic acid. The contents of the tube are evaporated under reduced pressure, milk of lime is then added until the acid reaction to Congo-red is almost but not quite abolished and the diluted mixture (200 cc.) is then boiled for a couple of hours. Excess of lime (5 g.) is then added and boiling resumed until ammonia is no longer evolved. Excess of lime is removed by a current of carbon dioxide and the filtrate containing the calcium salt of the amino-acid is concentrated at low temperature to a volume of about 10 cc. The calcium salt is then precipitated by alcohol, leaving the bulk of the calcium chloride or bromide in solution. The white, very soluble calcium salt is dissolved in water, and the calcium removed exactly with sodium oxalate. Residual traces of chlorides and bromides are removed with silver nitrate after acidifying with a little sulphuric acid and then the amino-acid is precipitated as silver salt as previously described and the latter decomposed with hydrogen sulphide. The concentrated aqueous solution of the amino-acid is twice precipitated with alcohol as described in the preceding paragraph. The acid thus obtained appears to be identical with that from hydantoinacrylic acid.

In some cases, an additional precipitation of the acid was effected by precipitation of its aqueous solution with mercuric acetate in feebly acid or neutral solution. The white mercury precipitate was washed with water, then with alcohol and finally decomposed with hydrogen sulphide in the usual way. Occasionally this procedure is useful for removing traces of inorganic impurities.

For analysis, the acid was dried at room temperature over phosphorus pentoxide at 2 mm. pressure.

Analysis. 0.1979 g.; 0.2670 CO_2 ; 0.1008 H_2O ,
0.1022 g.; 0.1399 CO_2 ; 0.0483 H_2O ,
0.0100 g.; 1.46 cc. N at 17° and 752 mm. (van Slyke).

		C	H	N
Calculated for $\text{C}_5\text{H}_9\text{O}_5\text{N}$	...	36.8	5.52	8.59
Found		36.8	5.66	8.34
		37.3	5.57	

On heating the acid either under normal or reduced pressure, water is evolved

freely in the neighbourhood of 100° and the substance is converted mainly into hydroxypyrrolidonecarboxylic acid. Thus on heating 46.2 mg. of the acid for one hour at $105-107^{\circ}$, the product on treatment with nitrous acid gave only 0.48 cc. nitrogen, equivalent to 0.58 % of nitrogen in the NH_2 form, indicating that about 93 % of the acid had been decomposed. An analysis of such a dried product is appended:

Analysis. 0.1168 g.; 0.1783 CO_2 ; 0.0542 H_2O .

	C	H
Calculated for $\text{C}_5\text{H}_7\text{O}_4\text{N}$...	41.3	4.9
Found	41.7	5.02

The copper salt of *i*- β -hydroxyglutamic acid is a clear blue-green salt which is very soluble in water but insoluble in alcohol. It resembles the copper salt of the natural active acid except that it appears a shade less blue and more green. It was prepared in the usual way by digesting an aqueous solution of the acid with excess of well-washed copper hydroxide. After filtering off the excess of copper oxide, the solution should be evaporated at a low temperature, as prolonged heating is apt to cause the separation of a little copper oxide. The concentrated aqueous solution of the copper salt was precipitated with alcohol and dried first over sulphuric acid and then in the oven at 100° .

Analysis. 0.1681 g.; 0.1591 CO_2 ; 0.0497 H_2O ; 0.0566 CuO ,
0.0200 g.; 2.1 cc. N at 13° and 752 mm. (van Slyke).

	C	H	N	Cu
Calculated for $\text{C}_5\text{H}_7\text{O}_5\text{NCu}$...	26.7	3.12	6.23	28.3
Found	26.0	3.28	6.12	27.7

The calcium salts of *i*- β -hydroxyglutamic acid are very soluble in water but insoluble in alcohol. They are precipitated as white powders on adding alcohol to their aqueous solutions. The acid calcium salt was obtained by warming the acid with calcium carbonate in water and was found to contain 11.3 % calcium (calculated 11.0). The "neutral" salt which is strongly alkaline in reaction towards litmus, was obtained by using excess of milk of lime and precipitating with alcohol. It contained 20.0 % calcium (calculated 19.9). It is decomposed, but incompletely, by passing carbon dioxide into its aqueous solution.

The strychnine salt of *i*- β -hydroxyglutamic acid was prepared by warming a concentrated solution of the acid with twice its weight of strychnine and adding methyl alcohol (10 parts). When all the base had dissolved, the water and alcohol were removed under diminished pressure and the sticky residue was crystallised from ten parts of boiling butyl alcohol. On standing, rosettes of needles separated out, which were readily soluble in water or methyl alcohol, but less so in ethyl or butyl alcohol.

Analysis. 0.1192 g.; 0.2750 CO_2 ; 0.0666 H_2O .

	C	H
Calculated for $\text{C}_{26}\text{H}_{31}\text{O}_7\text{N}_3$...	62.8	6.24
Found	62.9	6.21

Malic semi-aldehyde.

Malic semi-aldehyde or β -hydroxy- γ -aldehydobutyric acid was obtained by the hydrolysis of its acetal, γ -diethoxy- β -hydroxybutyric acid, which was in turn obtained by the reduction of γ -diethoxyacetoacetic ester already prepared by Dudley and the writer [1914].

γ -Diethoxy- β -hydroxybutyric acid. Ethyl γ -diethoxyacetoacetate (21.8 g.) together with alcohol (20 cc.) were mixed with 180 cc. of water. The mixture was surrounded by an ice bath and thoroughly agitated with a mechanical stirrer. 4 % sodium amalgam (200 g.) was added fairly rapidly and but little gas was evolved until the reduction was complete at the end of about seven hours. The mixture was allowed to stand overnight and then saturated with carbon dioxide and evaporated almost to dryness under reduced pressure. In the earlier experiments the sodium salt of the hydroxy-acid was extracted by hot methyl alcohol in which it is freely soluble, but later this step was avoided as follows: 25 % sulphuric acid was added to the thick residue until the mixture was acid to Congo-red and an oily layer separated out. The oil was at once extracted with ether and the aqueous layer again shaken with ether. The ether extract was washed with a few drops of water, dried over sodium sulphate and evaporated. The oil which weighed about 14 g. after drying over sulphuric acid was practically pure as judged by analysis. On distilling the acid under reduced pressure, there is a good deal of decomposition; about half of it is obtained as a clear oil boiling at 120° under 5 mm. pressure.

Analysis. 0.1558 g.; 0.2860 CO₂; 0.1186 H₂O,
0.1285 g. (distilled); 0.2341 CO₂; 0.0935 H₂O.

			C	H
Calculated for C ₈ H ₁₆ O ₆	...	...	50.0	8.33
Found	...	...	50.1	8.45
"	...	...	49.7	8.08

The acid is a sour smelling oil suggestive of butyric and hydroxybutyric acids. It mixes readily with a little water but is precipitated on diluting, finally again dissolving in about six parts of cold water. It does not decolorise bromine even after distillation and is readily soluble in ether and chloroform, and to a noticeable extent in petroleum ether. The salts are almost all very soluble and do not crystallise easily. The *sodium salt* soluble in methyl alcohol has already been referred to. The *zinc salt* is very soluble and is not precipitated by alcohol. When its solutions are evaporated in a desiccator, it is obtained as clear brittle semi-crystalline flakes which melt at a low temperature. On adding silver nitrate to a 3 % solution of the sodium salt, no precipitation occurred in the cold but, on warming, prompt reduction took place. The acid when titrated with alkali, using phenolphthalein as indicator, gave the following result: 0.2850 g. neutralised 15.0 cc. *N*/10 sodium hydroxide, giving a molecular weight of 190 compared with a calculated value of 192.

Malic semi-aldehyde. γ -Diethoxy- β -hydroxybutyric acid (15 g.) prepared as just described was gently boiled with 50 cc. of 0.2 *N* sulphuric acid for

about an hour. After a few minutes, the whole of the oil dissolved. An amount of barium hydroxide exactly equivalent to the sulphuric acid was then added and the barium sulphate removed by filtration. The filtrate was evaporated under reduced pressure at not over 45° , when a clear syrup, readily soluble in water and alcohol but sparingly soluble in ether or chloroform, was left behind. It was impossible to distil the substance without decomposition and it did not crystallise. The yield of dry syrup was 8 g. It is inadvisable to treat the substance with strong alcohol for any length of time since either acetal or ester formation takes place easily. A portion was however dissolved in alcohol, filtered from a trace of inorganic impurities, at once diluted with water and then concentrated. It was finally dried over phosphorus pentoxide *in vacuo* and analysed with the following result:

Analysis. 0.1454 g.; 0.2185 CO_2 ; 0.0721 H_2O .

		C	H
Calculated for $\text{C}_4\text{H}_6\text{O}_4$	...	40.7	5.09
Found		41.0	5.51

The complete purity of the product can hardly be anticipated since it could be neither distilled nor crystallised, but the results indicate no great amount of impurity. On titrating with alkali, using phenolphthalein as indicator, 0.1560 g. required 12.8 cc. *N*/10 sodium hydroxide, equivalent to a molecular weight of 122 compared with a calculated value of 118.

Malic semi-aldehyde reduces ammoniacal silver solutions on warming and instantly in the cold in the presence of sodium hydroxide. It gives a strong reaction with Schiff's reagent, especially if the reagent is dilute and not excessively acid. Bromine water oxidises the aldehyde freely, and on warming with excess of sodium hydroxide it gives inactive malic acid which was identified through its lead salt. It reacts with phenylhydrazines, but with the exception of *p*-nitrophenylhydrazine the osazones formed are not very attractive. On mixing the aldehyde in aqueous solution with semicarbazide hydrochloride, preferably without addition of alkali, a good yield of *semicarbazone* is obtained crystallising in prisms melting at 211° with effervescence.

Analysis. 0.1287 g.; 0.1630 CO_2 ; 0.0580 H_2O .

		C	H
Calculated for $\text{C}_5\text{H}_9\text{O}_4\text{N}_3$	...	34.3	5.14
Found		34.5	5.00

Action of ammonia and hydrocyanic acid on malic semi-aldehyde. The attempts to utilise Strecker's method for the synthesis of β -hydroxyglutamic acid were made with 10 % alcoholic solutions of the aldehyde, adding anhydrous hydrocyanic acid (1 mol.) together with a few drops of alcoholic ammonia and then adding alcoholic ammonia (2 mols.) on the second day. The conditions were substantially those used by Fischer in his synthesis of serine. On the third day, fuming hydrochloric acid was added for the purpose of hydrolysing any amino-cyanohydrin that might be formed. In other experiments, the ammonia was added first and the hydrocyanic acid later, but the

results were no better. After saturating with hydrochloric acid and boiling for an hour, the mixture was evaporated to dryness and the residue extracted with alcohol, leaving most of the ammonium chloride behind. The alcohol was then removed by evaporation and the solution made alkaline with sodium carbonate and boiled to remove ammonia. The solution was then just acidified with acetic acid and precipitated with mercuric acetate. The small precipitate was decomposed with hydrogen sulphide and any β -hydroxyglutamic acid was isolated as silver salt after removing traces of chlorides. 10 g. of the aldehyde yielded about 0.25 g. of silver salt equivalent to 0.11 g. of acid and on analysis it was found to be decidedly impure. It gave, however, the qualitative reactions for β -hydroxyglutamic acid previously described, so that it is not improbable that a minute amount of the amino-acid had been formed. But it was clear that at any rate in its present form the synthesis was not a practicable one.

Experiments with derivatives of acetonedicarboxylic ester.

The direct reduction of α -isonitroso-acetonedicarboxylic ester gave unsatisfactory results, as already stated. The nitroso-compound was prepared in large quantities according to Pechmann's [1891] directions, and practically every reducing agent that had any prospect of success was tried. The reduction in either acid or neutral media gave products that on analysis furnished results similar to those required for β -hydroxyglutamic acid or its anhydride, but the product was mostly not an amino-acid as judged by the nitrous acid reaction, nor did it give an amino-acid as the main product on warming with mineral acids. For reasons already given, it was decided to attempt to lessen the reactivity of the carbonyl group of the acetonedicarboxylic ester and its nitroso derivative by condensation with orthoformic ester, since as shown in the following section, the ethoxyglutaconates thus formed may be reduced by sodium amalgam to β -hydroxyglutaric acids.

Reduction of β -ethoxyglutaconic ester. Diethyl β -ethoxyglutaconate was prepared by the condensation of acetonedicarboxylic ester and orthoformic ester with acetyl chloride as described by Claisen (cp. D.R.P. 80739) and the product was fractionated under reduced pressure. The ester (10 g.) was mixed with alcohol (75 cc.) and water (75 cc.) and then reduced with 4 % sodium amalgam (100 g.) which was added rapidly. The reaction, which was slow, was carried on at first in an ice bath with constant stirring, but later at room temperature. After standing overnight, phenolphthalein was added and the solution exactly neutralised with nitric acid. The filtered solution was then precipitated with excess of silver nitrate solution and the silver salt washed with water. A portion of the silver salt on ignition was found to contain 59.0 % of silver compared with 59.6 for silver β -hydroxyglutarate and 55.4 % for silver β -ethoxyglutarate. That β -hydroxyglutaric acid was the main or sole product of the reaction was proved by decomposing the remaining silver salt suspended in water with hydrogen sulphide, and evaporating the filtrate. The residue

crystallised completely and, after spreading on a porous plate and washing with a little ether, melted at 93–95°. The yield was 55 % of the calculated. The β -hydroxyglutaric acid was further identified by its *bis*-phenylhydrazide prepared by warming the acid with three parts of phenylhydrazine for 15 minutes at 120°. The substance crystallised from glacial acetic acid in fine white needles melting at 234–235° as described by Pechmann. The free acid was also analysed:

Analysis. 0.1265 g.; 0.1878 CO₂; 0.0623 H₂O.

			C	H
Calculated for C ₅ H ₈ O ₅	...		40.5	5.41
Found	...	...	40.5	5.47

α -Nitroso- β -ethoxyglutaconic ester (?). On adding ethyl orthoformate (1 mol.) to α -nitroso-acetonedicarboxylic ester (1 mol.) no obvious reaction takes place, but on adding an acid catalyst, such as acetyl chloride or a little alcoholic hydrochloric acid, a vigorous reaction occurs with liberation of heat. On warming to 60° under diminished pressure, ethyl formate and, when acetyl chloride has been used, ethyl acetate are found in the distillate. The residual product is apparently a mixture of the α -nitroso- β -ethoxyglutaconic ester with unchanged α -nitroso-acetonedicarboxylic ester, as judged by the results of elementary analysis. The former compound requires 50.9 % carbon, 6.57 % hydrogen, the latter requires 46.7 % carbon and 5.63 % hydrogen. The observed results varied from 46.0–50.1 % carbon and 5.65–6.61 % hydrogen. It would therefore appear as if the ethoxy-compound were readily reconvertible into the ketonic ester.

The ease with which the ethyl group of ethoxyglutaconic ester could be eliminated on reduction has been shown in the foregoing. A further example is furnished by the action of excess of amyl nitrite (2 mols.) on β -ethoxyglutaconic ester (1 mol.) in the presence of 2 % of alcoholic hydrochloric acid. A crystalline product was readily separated which proved to be hydroxyisoxazolidicarboxylic ethyl ester. It crystallised from alcohol in needles melting at 104° as described by Pechmann.

Analysis. 0.1835 g.; 0.3162 CO₂; 0.0820 H₂O.

			C	H
Calculated for C ₉ H ₁₁ O ₆ N	...		47.0	4.80
Found	...	...	47.2	4.96

The reaction may be represented as follows:

The preceding reactions clearly indicated the difficulty that was likely to be encountered in obtaining pure α -nitroso- β -ethoxyglutaconic ester starting

either from ethoxyglutaconic ester or α -nitroso-acetonedicarboxylic ester. Accordingly the products of the interaction of α -nitroso-acetonedicarboxylic ester (1 mol.) and orthoformic ester (1 mol.) after standing with a trace of alcoholic hydrochloric acid for 24 hours at room temperature were reduced in 50 % alcoholic solution without further purification, using a large excess of sodium amalgam at 0°. The reaction was kept feebly acid with sulphuric acid at first and later was allowed to become alkaline. A current of carbon dioxide was passed during the reaction which lasted 12–18 hours. As stated in the introduction, the isolation of the products of the reaction is difficult and has not been satisfactorily solved. The separation of the products by precipitating with mercuric acetate and then isolating the silver salt of the acid contained in the mercury precipitate gave a product which resembled hydroxyglutamic acid in its properties but was accompanied by other substances closely similar in elementary composition. It may be mentioned that a small amount of apparently pure β -hydroxyglutamic acid and its copper salt have been isolated with great difficulty, but much further work is needed to clear up the course of the reaction and the sharp separation of the products. The study of the reaction will be continued. The purest specimen of acid hitherto obtained by the foregoing method contained 36.4 % carbon, 5.63 % hydrogen and 8.4 % amino-nitrogen. The copper salt, which was greenish-blue resembling that obtained by alternative methods, contained 29.4 % of copper and 6.15 % nitrogen. These results are in fair agreement with the calculated figures. But this method of synthesis in its present form is much less satisfactory than that from hydantoin-propionic acid previously described.

II. THE *p*-NITROPHENYLOSAZONES OF MALIC SEMI-ALDEHYDE AND TARTRONIC SEMI-ALDEHYDE.

One of the main pieces of evidence which led the writer to assign to the amino-acid separated from caseinogen [1918] the structure of β -hydroxyglutamic acid was the fact that on oxidation it gave an aldehyde (II) which reacted with *p*-nitrophenylhydrazine to give a well-defined osazone (III), thus indicating that the amino and hydroxyl groups were attached to contiguous carbon atoms.

It appeared desirable, in order to confirm this deduction, to prepare the osazone in some entirely independent fashion, and this has now been done and the apparent identity of the products established. The preparation of its

lower homologue is also described, a substance which has since been obtained by the oxidation of hydroxyaspartic acid. The synthesis of the latter amino-acid from chloromalic acid will be described in a separate communication.

Malic semi-aldehyde p-nitrophenylosazone. γ -Diethoxyacetoacetic ester [Dakin and Dudley, 1914] (3 g.) was shaken with 15 cc. of normal sodium hydroxide at room temperature and the mixture allowed to stand overnight. The solution was then neutralised with dilute sulphuric acid and unchanged ester removed by extracting twice with ether. The aqueous solution was then made decidedly acid to Congo-red with more sulphuric acid and nitrophenylhydrazine (3 g.) dissolved in 15 cc. of glacial acetic acid was added. An immediate precipitate was formed and the reaction was completed by heating for 20 minutes on the water-bath. The precipitate was filtered off, washed well with warm methyl alcohol and recrystallised from boiling nitrobenzene. The yield was 1.0 g. The reactions may be represented as follows:

Analysis. 0.1856 g.; 0.3470 CO₂; 0.0650 H₂O.

	C	H
Calculated for C ₁₆ H ₁₄ O ₆ N ₂ ...	51.1	3.72
Found	51.0	3.89

The *osazone* is sparingly soluble in most organic solvents but it dissolves very readily in boiling nitrobenzene, crystallising out on cooling in the form of deep brown-red thick prismatic needles. It dissolves readily in pyridine even in the cold forming a salt, but is practically insoluble in water, alcohol, ether or chloroform. Its melting point varies considerably with the rate of heating and, if this is slow, may be as low as 275°, while with rapid heating it is 289–291° (uncorr.) or 297–299° (corr.). Its melting point was unchanged on mixing it with the *osazone* prepared from natural β -hydroxyglutamic acid as described previously [Dakin, 1918]. On adding alcoholic sodium hydroxide to a trace of the substance, a clear blue coloration is obtained, characteristic of two adjacent nitrophenylhydrazine groups.

Tartronic semi-aldehyde p-nitrophenylosazone. A solution of tartronic semi-aldehyde (II) was obtained according to Fenton's [1905] directions. Dihydroxymaleic acid (I) (1.48 g.) was added to mercuric chloride (5.5 g.) in water (15 cc.) and the mixture kept at 65–70° for an hour. The solution was then filtered from calomel and the residual mercury removed with hydrogen sulphide. Excess of hydrogen sulphide was removed by exposure to reduced pressure and then nitrophenylhydrazine (3.5 g.) dissolved in hot glacial acetic acid (15 cc.) was added. An immediate red precipitate was formed and the mixture was warmed for 20 minutes on a steam bath. The *osazone* (III) was

then filtered off and well washed with warm methyl alcohol. The yield was 2.2 g. The reactions may be represented as follows:

The *osazone* is sparingly soluble in alcohol, ether or chloroform, but readily dissolves in pyridine with formation of a salt which does not readily separate out. The osazone can be recrystallised from boiling nitrobenzene but it is much less soluble than the preceding osazone and the crystalline form is quite different. It separates from nitrobenzene solution in nodular rosettes of fine needles with a few separate long fine needles. The melting point of the recrystallised substance is about 302° uncorr. (310° corr.) with effervescence. Slight darkening occurs above 270°. A trace of the substance dissolved in alcoholic sodium hydroxide with an intense clear blue colour.

Analysis. 0.1780 g.; 0.3192 CO₂; 0.0570 H₂O.

	C	H
Calculated for C ₁₈ H ₁₂ O ₆ N ₆ ...	48.9	3.23
Found	48.9	3.56

An identical osazone has been obtained from the oxidation products of synthetic hydroxyaspartic acid, under the same conditions as those used for the oxidation of β -hydroxyglutamic acid.

III. OPTICAL ACTIVITY OF NATURAL β -HYDROXYGLUTAMIC ACID.

In the first communication on β -hydroxyglutamic acid [Dakin, 1918] from caseinogen it was stated that "in aqueous solution the substance shows a low dextro-rotation which is increased on addition of hydrochloric acid." The difficulties in obtaining trustworthy observations on the optical activity of the acid are mainly two-fold. In the first place, the difficulty of satisfactorily recrystallising the acid and the fact that its purification as then described rested almost as much on methods for the removal of other substances as for the direct separation of the acid in some characteristic form. The second difficulty was not appreciated at the beginning of the work and was the cause of considerable confusion until it was established that *d*- β -hydroxyglutamic acid passed over into a laevorotatory hydroxypyrrolidonecarboxylic acid on evaporating its aqueous solution or drying at 100°.

Accordingly, experiments have been made on the rotation of specimens of β -hydroxyglutamic acid, two of which were prepared from the repeatedly crystallised strychnine salt to be described in the next section, while one was obtained from the products of tryptic digestion, and all of them were carefully handled at low temperature to avoid formation of the laevorotatory anhydride. The observations are probably fairly accurate, though the possibility of the need of revision is by no means excluded.

It was found with all specimens that the free acid had a very slight dextro-rotation which was much increased in the presence of hydrochloric acid. Further, a dextro solution of the free acid on evaporation and drying at 105–110° gave a strongly laevorotatory solution which could be changed back to dextro on treating with hydrochloric acid—the first change in rotation on drying being due to the formation of hydroxypyrrolidonecarboxylic acid and the second to its reconversion into hydroxyglutamic acid. No apparent racemisation accompanied these changes. Alkaline solutions of the potassium or sodium salts of β -hydroxyglutamic acid were found to be practically inactive, but on adding Walden's uranium solution, a fairly strong laevo-rotation was observed. This behaviour shows a close analogy to malic acid and other hydroxy-acids.

Specimen A was obtained as described in a previous paper [Dakin, 1918] and was redissolved in 50 % alcohol, precipitated with mercuric acetate in feebly acid solution and the hydroxyglutamic acid obtained by decomposing the washed precipitate with hydrogen sulphide and evaporating at low temperature (25–30°). It crystallised readily and on combustion gave 37.0 % carbon and 5.57 % hydrogen. (Calculated 36.8 % carbon and 5.52 % hydrogen.)

Specimen B was obtained by the same methods as previously employed, but the caseinogen, instead of being hydrolysed with sulphuric acid, was thoroughly digested with trypsin. The yield was disappointing, only 4.5 g. being obtained from 350 g. caseinogen.

Specimens C and D were prepared similarly to A, and then converted into the strychnine salt (see next section) which was repeatedly crystallised and then decomposed with sodium carbonate. The strychnine was filtered off, and traces remaining in solution removed by shaking with amyl alcohol. The acid was then removed by precipitation with mercuric acetate in feebly acid solution, followed by decomposition with hydrogen sulphide. The polarimetric observations are recorded in the following table:

Conditions	Specimen	Concentration	Length of tube, dm.	Observed rotation	$[\alpha]_D^{20}$
Free acid in aqueous solution	A	5.43	2.2	+0.05°	+ 0.4°
" " " " " " " " " " " "	B	3.0	1.0	+0.02	+ 0.7
" " " " " " " " " " " "	C	4.0	2.0	+0.08	+ 1.0
" " " " " " " " " " " "	D	4.0	2.0	+0.10	+ 1.2
Acid in 20 % hydrochloric acid	A	2.0	2.0	+0.62	+15.5
" " " " " " " " " " " "	B	3.0	1.0	+0.48	+16.0
" " " " " " " " " " " "	C	1.33	2.0	+0.43	+16.2
" " " " " " " " " " " "	D	1.33	2.0	+0.47	+17.6
Acid dissolved in <i>N</i> potassium hydroxide	B	3.0	1.0	0	—
" " " " " " " " " " " "	C	2.0	2.0	0	—
Filtered solution from mixture containing 0.4 <i>N</i> potassium hydroxide and 12 % uranium nitrate	A	1.08	1.0	–0.13	–12.0
Ditto, 0.2 <i>N</i> potassium hydroxide, and 20 % uranium nitrate	C	0.60	2.2	–0.32	–24.3
Acid heated 2 hours 105°	A	1.73	2.0	–0.37	–10.9
" 2 " " " " " " " " " "	B	1.0	1.0	–0.15	–15.0
" 4 " " " " " " " " " "	C	1.20	2.0	–0.27	–11.3
* Solution C after heating with 20 % hydrochloric acid	C*	1.20	1.0	+0.20	+16.6

Taking a mean of the various observations, it may be stated that the apparent specific rotation of the free acid in 4 % solution is about $+0.8^\circ$, while a 2 % solution in 20 % hydrochloric acid has a rotation of $+16.3^\circ$. The potassium salt apparently has a negligible rotation but is strongly laevorotatory on addition of uranium nitrate. The acid becomes strongly laevorotatory on drying at 105° , but the numbers have no great quantitative value since the conversion of the hydroxyglutamic acid into its anhydride is not known to be perfectly complete.

IV. ALKALOID SALTS OF β -HYDROXYGLUTAMIC ACID AND RELATED ACIDS.

One of the main difficulties in working with hydroxyglutamic acid lay in the paucity of simple derivatives, capable of purification by repeated crystallisation. The metallic salts are either very soluble substances that require precipitation by alcohol or insoluble precipitates that are incapable of recrystallisation. It therefore became necessary to search for other salts with more acceptable properties. Of the common alkaloids only the salts of strychnine and brucine proved to be of value since the others mostly underwent hydrolytic dissociation too easily. For purposes of comparison, the corresponding salts of aspartic, glutamic and pyrrolidonecarboxylic acid have been prepared. Many of these salts are finely crystalline products and can be readily recrystallised from appropriate solvents but it is to be regretted that their melting points are not sharp in most cases, though their behaviour on heating is often characteristic.

All the salts contained equimolecular proportions of base and acid and were prepared by dissolving the acid in warm water, then adding the alkaloid till the solution became neutral to litmus, and adding a little methyl alcohol from time to time to aid in the solution of the base. The neutral solution was then evaporated at low temperature to avoid hydrolytic decomposition and recrystallised from an appropriate solvent. The specific rotation of the salts was determined in aqueous solution.

Strychnine d- β -hydroxyglutamate. The salt is very soluble in water and on slow evaporation of its solution separates out in the form of nodular masses which are spread on porous plates and recrystallised. Butyl alcohol containing a little water has been found to be decidedly the best solvent for this purpose. Methyl alcohol dissolves the salt too readily, while the solubility in ethyl alcohol is not much greater at the boiling temperature than at room temperature. 5 g. of the strychnine salt require about 60 cc. of butyl alcohol and will give about 3.5 g. of fine prismatic needles. The composition of the salt does not change on repeated crystallisation nor does its behaviour on heating. As observed in a melting point tube, a change takes place fairly sharply at a temperature varying from 165 to 175° , giving a white opaque waxy mass which does not melt till about 245° , when it gives a clear brown oil without effervescence. On dissolving the salt crystallised from butyl alcohol in water, no strychnine separates, and on evaporation long striated needles of the salt

crystallise out. These contain practically no water of crystallisation (0.3 %) and show the same behaviour on heating as that just described. The strychnine salt of β -hydroxyglutamic acid is preferable to any of the others for purposes of purification and characterisation of the acid.

Specific rotation. $C=1.67$; $l=2$ dm.; $\alpha=-0.88^\circ$; $[\alpha]_D^{20}=-26.3^\circ$.

Analysis. 0.1629 g.; 0.3760 CO₂; 0.0963 H₂O,
0.1145 g.*; 0.2640 CO₂; 0.0630 H₂O.

* Recrystallised five times.

		C	H
Calculated for C ₂₈ H ₃₁ O ₇ N ₃		62.8	6.24
Found		62.9	6.57
„		62.9	6.11

Brucine d- β -hydroxyglutamate. This salt separates out in the form of needles containing water of crystallisation on evaporation of its aqueous solution at low temperatures. It is best purified by spreading on a porous tile, then warming with acetone in which it is insoluble and recrystallising the residue either from a little water or from methyl alcohol. The salt crystallised from water begins to soften at about 90° and melts to a turbid fluid around 110°. The anhydrous salt obtained as prismatic needles by crystallisation from dry methyl alcohol decomposes indefinitely above 200°. Both salts are extremely soluble in water and alcohol but almost insoluble in acetone.

Specific rotation. $C=1.0$; $l=2.0$; $\alpha=-0.50^\circ$; $[\alpha]_D^{20}=-25.0^\circ$.

Analysis. 0.1540 g.; 0.3368 CO₂; 0.0905 H₂O.

		C	H
Calculated for C ₂₈ H ₃₅ O ₉ N ₃		60.3	6.26
Found		59.6	6.53

Strychnine d-glutamate. The salt is prepared as usual but the neutral solution should be concentrated at low temperatures and methyl alcohol added by degrees to replace the water. A syrupy mass results which, on mixing with excess of absolute alcohol, gives stout isolated glistening prisms which dissolve in water without separation of strychnine. The salt is practically insoluble in dry methyl, ethyl or butyl alcohols, but extremely soluble in water. It begins to show signs of decomposition on heating above 200° and melts about 225–230°.

Specific rotation. $C=2.0$; $l=2.0$; $\alpha=-1.02^\circ$; $[\alpha]_D^{20}=-25.5^\circ$.

Analysis. 0.2217 g. dried at 100°; 0.0196 N (Kjeldahl) = 8.84 % N.

Calculated for C₂₈H₃₁O₆N₃ 8.69 „

Brucine d-glutamate. The salt crystallises from water in the form of long stout prismatic needles with a satin lustre. These crystals contain about 15.5 % of water of crystallisation which is lost at 100°. This corresponds to between five (14.2 %) and six molecules (16.4 %) of water. The salt as crystallised from water dissolves readily in hot methyl alcohol and, on cooling, bright rhombic prisms of the anhydrous salt separate out. The water-containing salt dissolves less readily in ethyl or butyl alcohol. After recrystallisation from

methyl alcohol, the anhydrous salt is soluble only with great difficulty in any of the alcohols. The salt containing 5.5 mols. of water softens at about 96° and melts at about 101°, while the anhydrous salt slowly decomposes above 170° but does not melt until about 240°.

Specific rotation. C=2.0 (anhydrous); $l=2.0$; $\alpha = -0.92^\circ$; $[\alpha]_D^{20} = -23.0^\circ$;

Analysis. 0.2 g. dried at 100°; 0.0154 N (Kjeldahl)=7.70 % N.

Calculated for $C_{28}H_{35}O_8N_3$ 7.76 „

Strychnine l-aspartate. The salt is very soluble in water and separates in crusts which have the appearance of stout prisms to the naked eye but are made up of masses of fine interlacing needles. Its solubilities closely parallel those of the corresponding glutamate and it melts fairly sharply to a brown oil with effervescence around 252–255°.

Specific rotation. C=1.164; $l=2.0$; $\alpha = -0.66^\circ$; $[\alpha]_D^{20} = -28.3^\circ$.

Analysis. 0.1500 g.; 0.0134 N (Kjeldahl)=8.90 % N.

Calculated for $C_{25}H_{29}O_6N_3$ 9.0 „

Brucine l-aspartate. As first obtained from aqueous solution, this salt forms rather waxy prisms which become hard and brittle on drying and take on an opaque white appearance. It contains 5 molecules of water of crystallisation which are given off at 90°. (Water: found 14.9 %, calculated 14.6.) The salt is readily soluble in methyl alcohol but does not crystallise out readily on cooling. On adding ethyl acetate, in which it is sparingly soluble, it is deposited as fine needles. The anhydrous salt is moderately soluble in ethyl alcohol and less so in butyl alcohol, but does not crystallise out readily. It is insoluble in acetone. The salt containing water of crystallisation softens at 96° and melts about 100°, while the anhydrous salt decomposes indefinitely above 200°.

Specific rotation. C=1.702 (anhydrous); $l=2.0$; $\alpha = -0.97^\circ$; $[\alpha]_D^{20} = -28.4^\circ$.

Analysis. 0.1500 g.; 0.0121 N (Kjeldahl)=8.05 % N.

Calculated for $C_{27}H_{33}O_8N_3$ 7.97 „

Strychnine l- α -pyrrolidone-carboxylate. The acid used for preparing this and the succeeding salt was obtained by heating glutamic acid under reduced pressure at 165° and then crystallising the products from alcohol. It had a specific rotation in water of -5.7° , and hence was partially racemised since the pure laevo-acid is generally regarded as possessing a rotation of about -7.2° .

The salt crystallises from water in the form of felted fan-shaped masses of fine needles which are readily soluble in ethyl alcohol but sparingly soluble in acetone. It dissolves easily in butyl alcohol and also in chloroform but crystallises better from water than from any of the organic solvents. The salt thus obtained softens on heating at 90° and partially melts. It contains water of crystallisation which is lost at 100°. The anhydrous salt melts indefinitely but incompletely until about 245° is reached.

Specific rotation. C=2.74; $l=2.0$; $\alpha = -1.45^\circ$; $[\alpha]_D^{20} = -26.7^\circ$.

Analysis. 0.2438 g.; 0.0218 N (Kjeldahl)=8.96 % N.

Calculated for $C_{26}H_{29}O_5N_3$ 9.07 „

Brucine 1- α -pyrrolidone-carboxylate. This salt is better suited than the preceding one for purposes of characterisation of the acid. On concentrating the neutral aqueous solution, a syrup was obtained which at once solidified to a mass of stout prisms. The salt is very soluble in methyl alcohol but can be conveniently crystallised from boiling acetone from which it separates in well-formed prisms. On heating the substance, decomposition can be noted as low as 140° and it melts with effervescence to a clear fluid from 180 – 195° .

Specific rotation. $C=5.0$; $l=2.0$; $a=-3.15^{\circ}$; $[\alpha]_D^{20}=-31.5^{\circ}$.

Analysis. 0.4619 g.; 0.0364 N (Kjeldahl) = 7.88% N.
Calculated for $C_{28}H_{33}O_7N_3$ 8.03% „

V. IDENTIFICATION OF β -HYDROXYGLUTAMIC ACID AMONG THE PRODUCTS OF HYDROLYSIS OF GLUTENIN AND GLIADIN.

The experiments in the following section were made with the cooperation of Miss Marjorie Lord Strauss, to whom acknowledgment is due for much valuable assistance.

Hydroxyglutamic acid up to the present has only been isolated from caseinogen, a typical animal protein, after hydrolysis with acids, with trypsin, and possibly to a smaller extent with barium hydroxide. There seems every reason to suppose that search for this amino-acid will disclose its presence in other proteins. The present communication records its presence in two typical vegetable proteins, glutenin and gliadin.

The protein preparations were prepared from wheat flour according to the excellent methods described by Osborne. The methods employed were practically identical with those described in the work on caseinogen [Dakin, 1918]. Portions of 100 g. of each protein were hydrolysed by prolonged boiling with dilute sulphuric acid and the latter subsequently removed exactly with barium hydroxide. The concentrated solution of amino-acids was then thoroughly extracted with butyl alcohol and the aqueous residue containing essentially the strong acids and bases was saturated with hydrochloric acid to separate the bulk of the glutamic acid. The calcium salts of the acids were then separated by alcohol according to Foreman's method. They were dissolved in water, the calcium removed exactly with oxalic acid, and the diluted filtrate boiled with lead hydroxide to remove aspartic acid. Lead was removed from the filtrate with hydrogen sulphide and traces of bases precipitated with phosphotungstic acid in sulphuric acid solution. After removal of the excess of the latter reagents with barium hydroxide, the filtrate was made acid to Congo-red with sulphuric acid and concentrated to about 100 cc. The silver salt of β -hydroxyglutamic acid was then separated in the usual way and decomposed with hydrogen sulphide. The crude acid, as thus separated, was less pure than when prepared from caseinogen. It was purified by dissolving in cold 50% methyl alcohol and precipitating with mercuric acetate in very feebly acid (acetic) solution. The precipitate, on decomposition with hydrogen sulphide and concentration at not over 50° , gave a very soluble syrup which

solidified in the desiccator and had all the properties of β -hydroxyglutamic acid prepared from caseinogen. The yield of acid was small but undoubtedly the losses were considerable. 100 g. of gliadin gave 2.4 g. of the acid, while 1.8 g. were obtained from 100 g. of glutenin. It is worth noting that the yields of the acid from caseinogen have been found to vary enormously. At times, not more than 2-3 % has been obtained in a reasonably pure state, while in other experiments as much as 10 % has been obtained. It is not unlikely that the acid, being a β -hydroxy-acid is not entirely stable to many of the reagents employed. The yields are generally highest when small amounts of protein are worked up at a time.

Acid from gliadin.

Analysis. 0.2112 g.; 0.2861 CO₂; 0.1100 H₂O.

	C	H
Calculated for C ₅ H ₉ O ₅ N...	36.8	5.52
Found	36.9	5.79

On heating the acid previously dried over phosphorus pentoxide to 110-115°, water was split off and the residue, containing 40.9 % of carbon and 5.3 % of hydrogen, was mainly made up of hydroxypyrrolidonecarboxylic acid, for it contained less than 1 % of nitrogen in the (NH₂) form as judged by van Slyke's method with nitrous acid.

The optical properties of the acid resembled those previously recorded. In aqueous solution $[\alpha]_D^{20} = +1.0^\circ$ ($c = 5.87$, $l = 2.0$, $a = +0.11^\circ$). In 20 % hydrochloric acid ($c = 1.96$), $[\alpha]_D^{20} = +14.0^\circ$.

Acid from glutenin. The acid obtained from glutenin resembled the preceding closely. It contained 37.0 % carbon and 5.8 % hydrogen. It showed the characteristic decomposition on heating at 110° but its concentrated aqueous solution showed no detectable rotation in 10 % aqueous solution and in 20 % hydrochloric acid it had $[\alpha]_D^{20} = +6.2^\circ$. It appeared therefore as if partial racemisation had taken place in the process of preparation, for careful investigation failed to indicate the presence of any other acid. The strychnine and brucine salts were also prepared and analysed without disclosing any other cause for the lower rotations. It is not unlikely that racemisation may have been caused by the alkali used in the preparation of the glutenin.

VI. THE FATE OF β -HYDROXYGLUTAMIC ACID IN THE DIABETIC ORGANISM.

The fate of β -hydroxyglutamic acid in the diabetic animal presents certain questions of interest. Lusk has shown that when glutamic acid (III) is fed to a starving dog fully under the influence of phloridzin, about 65 % of the weight of glutamic acid given reappears in the urine as "extra glucose." The writer has shown that substantially the same thing is true for proline (I) and ornithine (IV), both of which, like glutamic acid, contain five carbon atoms. It appears as if three out of the five carbon atoms were concerned in glucose formation, and it is not unreasonable to regard it as likely that their catabolic paths are similar.

Now β -hydroxyglutamic acid has been found to yield glucose in the diabetic animal in precisely the same fashion as the previously mentioned acids. This fact appears to the writer to possess a certain speculative significance based on the probability of similarity in the metabolism of all of these acids. In the first place, if, as appears likely, β -hydroxyglutamic acid may arise in the body from glutamic acid, an example is furnished of " β -oxidation" such as is known to occur in the oxidation of fatty acids, but has not hitherto been observed with the amino-acids. Secondly, the possible conversion of β -hydroxyglutamic acid into malic acid (VII) by oxidation must be regarded as plausible for it involves a type of simple reaction often encountered in animal oxidation, and has, moreover, been effected by the writer *in vitro*. Malic acid is also stated to occur in sheep's sweat in significant quantities. Now Ringer, Frankel and Jonas [1913] have shown that malic acid readily goes over into glucose in the diabetic dog, 13 g. of the acid furnishing 8 g. of glucose. A similar conversion of malic acid into sugar has been observed in acorns by Bloor [1912]. It is therefore not unlikely that malic acid is a product of the intermediary metabolism of hydroxyglutamic acid and, in turn, of the other 5-carbon acids. In picturing the possible further steps in the conversion of malic acid into glucose, one cannot avoid considering favourably the idea that it may part with carbon dioxide with formation of lactic acid (VIII), for this is a reaction which is actually known to occur as the result of enzyme activity in the case of vegetable cells. This change is also observed in the ageing of wines and is apparently affected by *Micrococcus malolacticus* [cp. von der Heide, 1912]. The subsequent conversion of lactic acid into glucose involves no difficulty.

One other point may be referred to in connection with the rôle of β -hydroxyglutamic acid in metabolism. The conversion of proline into glucose obviously requires the opening of the pyrrole nucleus. The reversibility of many biological reactions lends significance to the extraordinarily easy conversion of β -hydroxyglutamic acid into hydroxypyrrolidonecarboxylic acid—and the less easy conversion of glutamic acid into α -pyrrolidonecarboxylic acid (II). Such changes may well be concerned with the synthesis of substances containing the pyrrole nucleus, and, moreover, the reduction of pyrrolidonecarboxylic acids to pyrrolidinecarboxylic acids is known to be feasible *in vitro* from Fischer's experiments.

It should be noted that Ringer, Frankel and Jonas have described experiments which they consider prove that glutamic acid is converted into succinic acid in the body, while they regard malic acid as an intermediate product in the metabolism of aspartic acid. They picture the malic acid giving hydroacrylic acid and malonic acid, but curiously enough ignore the possibility of its giving lactic acid in spite of the fact that malonic acid gives little or no glucose in the diabetic animal. The writer is completely unable to follow the logic of Ringer's structural explanations of glucogenesis and cannot help feeling that too little distinction is made between possible metabolic paths and those actually known to occur.

The relationships in structure of the various five-carbon acids and their possible conversion into glucose *via* malic and lactic acids as previously outlined may be seen from the following scheme:

Experimental. Two experiments were made on the administration of β -hydroxyglutamic acid to a diabetic dog. The conditions of the experiments were those used previously by the writer [1913]. Phloridzin was administered to the starving dog by Coolen's method. 1 g. emulsified in 10 cc. of olive oil was given every day. The urine was collected in 18 hour periods and the bladder was carefully catheterised and washed out with sterile salt solution at the end of each period. The acid was given as the mono-sodium salt in aqueous solution by subcutaneous injection. The glucose was determined gravimetrically and checked by polarimetric readings. The excretion of "acetone bodies" was followed but showed no special change, hence the results are not recorded. The animal used was a terrier dog weighing about 8 kilograms.

Period	Hours	Total N	Glucose	G/N ratio	Substance
I	—	—	—	2.80	
II	18	4.21	13.53	3.22	
III	18	5.32	21.90	4.11	17.4 g. acid = 1.5 g. N
IV	18	5.10	15.74	3.08	
V	18	5.47	19.79	3.62	10 g. acid = 0.86 g. N

The "extra glucose" following the administration of the β -hydroxyglutamic acid may be calculated approximately using Lusk's method. The $\frac{\text{Glucose}}{\text{Nitrogen}}$ ratio in the blank periods averages 3.1. Assuming that the whole of the nitrogen of the amino-acid given is excreted in the course of the following 18 hour period, it is calculated that in the first experiment 17.4 g. of the acid gave rise to 10.06 g. of "extra glucose" while 10 g. of the acid gave 5.5 g. From the mean of the experiments it is concluded that β -hydroxyglutamic acid may furnish about 55–60 % of its weight of glucose in the diabetic dog.

According to the suggestion advanced that β -hydroxyglutamic acid may yield glucose in the diabetic animal *via* lactic acid, it may be calculated that the theoretical yield is 55 %, assuming one molecule of glucose to arise from two of lactic acid and two of hydroxyglutamic acid. The almost precise coincidence of the observed and calculated result must be regarded as partly accidental since the unavoidable experimental errors are considerable, but even allowing for them, the agreement is striking. The "protein sparing" effect of the injected amino-acid is very obvious.

SUMMARY.

Considerable difficulty has been experienced in synthesising inactive β -hydroxyglutamic acid.

Bromination of the acetyl derivative of β -hydroxyglutaric anhydride and subsequent action of ammonia on the product did not yield the desired result.

Small amounts of the amino-acid were obtained on reducing α -isonitroso-acetonedicarboxylic ester but the results were unsatisfactory.

Only traces of the amino-acid were formed by the application of Strecker's reaction to malic semi-aldehyde.

The most satisfactory synthesis so far accomplished had as its starting point glutamic acid. The steps are as follows: glutamic acid $\rightarrow$ α -uraminoglutamic acid $\rightarrow$ hydantoin-propionic acid $\rightarrow$ hydantoin- β -bromopropionic acid $\rightarrow$ hydantoinacrylic acid $\rightarrow$ β -hydroxyglutamic acid.

It is noted that hydroxyaspartic acid has been synthesised from barium chloromaleate.

The hitherto unknown semi-aldehyde of malic acid has been prepared from γ -diethoxyacetoacetic ester. Its *p*-nitrophenylosazone and that of tartronic semi-aldehyde are described.

The optical properties of β -hydroxyglutamic acid have been examined. The specific rotation of the free acid in 4 % aqueous solution is about $+0.8^\circ$, while a 2 % solution in 20 % HCl has a specific rotation of $+16.3^\circ$. The rotation of the potassium salt is practically undetectable, but the addition of uranium nitrate renders the solution strongly laevorotatory. On drying the free acid at 105° it loses water and is converted into hydroxypyrrolidonecarboxylic acid, which has a strong laevo-rotation in aqueous solution.

The strychnine and brucine salts of the following acids are described: *d*- β -hydroxyglutamic, *d*-glutamic, *l*-aspartic, and *l*- α -pyrrolidonecarboxylic.

β -Hydroxyglutamic acid has been isolated from gliadin and glutenin.

When administered to a diabetic (phloridzinised) dog β -hydroxyglutamic acid yields 55–60 % of its weight as glucose, apparently three of its five carbon atoms being concerned in glucose formation. In this it resembles glutamic acid, proline and ornithine, and it seems not unreasonable to regard the catabolic paths of all these acids as similar. The possibility of their conversion into glucose *via* malic and lactic acids is indicated.

REFERENCES.

- Blaise (1903). *Bull. Soc. chim.* [3], **29**, 1013.
Bloor (1912). *J. Amer. Chem. Soc.* **34**, 534.
Bülow and Göller (1911). *Ber.* **44**, 2835.
Dakin (1910). *Amer. Chem. J.* **44**, 48.
—— (1913). *J. Biol. Chem.* **13**, 514.
—— (1918). *Biochem. J.* **12**, 290.
Dakin and Dudley (1914). *J. Chem. Soc.* **105**, 2453.
Erlenmeyer and Bade (1904). *Annalen*, **337**, 218.
Fenton (1905). *J. Chem. Soc.* **87**, 813.
Gabriel (1906). *Annalen*, **348**, 50.
Heide, von der (1912). *Arbb. Kais. Gesundh. Amt.* **42**, 1.
Johnson and Hoffman (1912). *Amer. Chem. J.* **47**, 20.
Pechmann (1891). *Ber.* **24**, 860.
Perkin and Tattersall (1905). *J. Chem. Soc.* **87**, 362.
Ringer, Frankel and Jonas (1913). *J. Biol. Chem.* **14**, 539.
Rosenthaler (1912). *Chem. Zeit.* **36**, 830.
Semenov (1899). *J. Russ. Chem. Soc.* **31**, 386.
Wislicenus (1899). *Ber.* **32**, 2047.