FRUCTOSE-AMINO ACIDS IN LIVER: STIMULI OF AMINO ACID INCORPORATION IN VITRO*

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We have reported that the incorporation *in vitro* of amino acids into proteins of rabbit reticulocytes was stimulated by a boiling water extract of commercially dried liver or by the filtrate of boiled plasma. Certain amino acids, *i.e.* histidine, leucine, phenylalanine, and valine, had a stimulating effect. The liver and plasma extracts certainly contained these amino acids, but even optimal concentrations could account for less than half the stimulating action of the extracts; indeed the amino acids were synergistic with the extracts. Practically all the familiar metabolites, vitamins, and cofactors were tested; none could account for the stimulating effects of the liver extract or of boiled plasma filtrate (1).

Most of the stimulating activity of the liver extract we have used can be accounted for by the above amino acids acting in conjunction with iron and a class of substances not hitherto identified in animal or plant tissues. These substances are 1-deoxy-1-(*N*-amino acid)-2-ketohexoses. They can be viewed as formed from the corresponding *N*-glucosides of the amino acids by Amadori rearrangement (2–5).

It is not established whether the sugar residue, after the rearrangement, forms a furanose or pyranose ring. Compounds of the type of III have been designated, according to Fischer (6), as *N*-substituted isoglucosamines. When R is an amino acid residue, III gives a positive, weak test with the Elson-Morgan color reagent for glucosamine (7). The keto group at C-2 is responsible for the characteristic ability of these compounds to reduce in 0.1 N NaOH oxidation-reduction dyes (*e.g.*, methylene blue or 2,6-dichlorophenolindophenol) or ferricyanide rapidly at room temperature.

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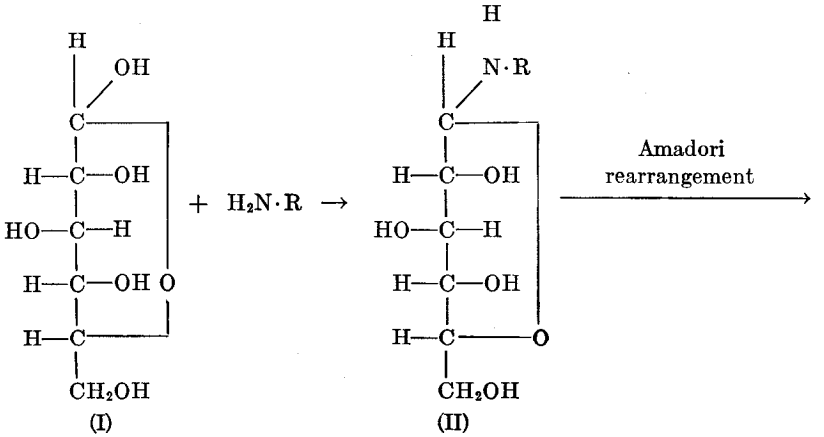
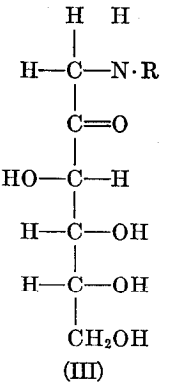
It can be seen that the same Amadori rearranged product is obtained when the parent sugar is either glucose or mannose; the sugar radical after the rearrangement has the configuration of a fructose compound; analogously galactose gives a tagatose compound. The completely descriptive names are cumbersome; for convenience they will be designated here as





fructose- (or tagatose-)alanine (or glycine, etc.) and abbreviated F-Ala, F-Gly, T-Ala, T-Gly, etc., according to the configuration of the sugar residue after the Amadori rearrangement and the constituent amino acid. The Amadori rearrangement occurs slowly in the solid state at 25° and rapidly in hot alcoholic solutions.

We report here the method which we have used to isolate fructose-amino acids from liver, some of the characteristic chemical and physical properties of these substances, and their stimulation of amino acid incorporation *in vitro* into the proteins of rabbit reticulocytes.

Procedure

The production and washing of the reticulocytes, the preparation of the C^{14}-amino acids, the saline solutions, and the reaction mixture which contained the stimulating amino acid mixture were as described previously (1). The incubation time was 4 hours.

We have modified somewhat the procedure of washing the proteins to remove free labeled amino acid. At the end of the incubation the contents of each beaker were added to 80 ml. of water and the reticulocyte protein precipitated with 20 ml. of 35 per cent trichloroacetic acid. When the protein had settled well, usually after 3 hours, the clear supernatant solution was decanted and drained off, and the protein dissolved in 2.5 ml. of 1 N NaOH and then reprecipitated with 100 ml. of 7 per cent trichloroacetic acid. On the next day the precipitate was washed, by centrifugation, five times with 7 per cent trichloroacetic acid, twice with a mixture of equal volumes of acetone and ether, twice with acetone, and finally twice with ether. The proteins were dried for 1 hour at 85°, and their radioactivity was measured as described previously (8).

Reduction Test—A simple and rapid test to follow isolation of the ketose-amino acids in liver or of the synthetic compounds from a reaction mixture was devised, based on reduction at room temperature of ferricyanide and conversion of the ferrocyanide to Prussian blue.

Usually we have used an aliquot of 0.1 ml. To this was added 1 ml. of 0.1 per cent potassium ferricyanide in water, followed by enough 1 N NaOH to make the solution 0.1 N, not counting the alkali required, which was determined beforehand, to bring the pH to the turning point with phenolphthalein. The reaction was allowed to proceed at room temperature for exactly 5 minutes, after which time was added 1 ml. of a ferric sulfate solution prepared according to Folin and Malmros (9). After another 5 minutes, 5 ml. of water were added. The blue color was then measured at 690 mμ.

The aliquot must be so adjusted that the ferricyanide is not completely reduced. For quantitative comparisons the time from the addition of alkali to the addition of ferric sulfate is critical, the longer the time the more reduction. We have chosen a reaction time of 5 minutes because in this time there is a linear relation between the amount of blue color finally obtained and the amount of ketose-amino acid up to 0.2 mg. One can measure by the above procedure 0.01 mg. and detect 0.002 mg.

Ascorbic acid, creatinine, cysteine, glutathione, and tyrosine react positively to this test and need to be taken into account if one uses the above procedure to assay crude extracts of biological material. Glucose gives about 2 per cent of the reduction of an equivalent weight of ketose-amino acids.

Reduction Test for Ketose-Amino Acids on Paper Chromatograms—0.1 per cent potassium ferricyanide was made up in 80 per cent ethanol; not all the ferricyanide went into solution. Just before use one-tenth its volume of 10 per cent NaOH in 80 per cent ethanol was added to it, which brought all the ferricyanide into solution. The alkaline ferricyanide solution was sprayed on the paper lightly. After 5 minutes the ferric reagent (as above) was sprayed on lightly. A positive test was a dark blue spot against a colorless or light blue background. The color was stable. It should be noted that there was no heating. 10 γ were detected easily.

Ninhydrin Test—Paper chromatograms were dipped in 1 per cent ninhydrin in pyridine and drained, and the color was then developed quickly under a heating lamp or more slowly for ½ to 1 hour at room temperature.

Determination of Total Sugar—Total sugar was determined by three methods, with carbazole in Dische's procedure (10, 11), Dreywood's anthrone reagent (12), and hot reduction of ferricyanide in alkaline solution by the procedure of Park and Johnson (13), except that instead of their ferric solution containing Duponol, to keep the Prussian blue suspended, we have used Folin and Malmros' (9) solution containing gum ghatti. The first two of these methods depend on formation of a colored product by condensation with either anthrone or carbazole of a furfural derivative formed from the sugar by concentrated sulfuric acid; the results obtained by either of these methods and by hot reduction of ferricyanide in alkali are markedly different and indicative of whether the sugar is a simple glucoside or conjugated as in the fructose-amino acids.

Glucosamine Color Reaction—After glucosamine is heated in 0.25 N Na_2CO_3 with acetylacetone, it gives with *p*-dimethylbenzaldehyde a purple compound. Fructose-amino acids react positively to this test, but the color per equivalent is much lighter than that yielded by glucosamine. The procedure of Elson and Morgan (7) was followed exactly, except that the color was read in a spectrophotometer at 550 mμ, which is the region of maximal absorption.

Determination of Acid and Basic Groups—The acid and basic groups were determined by electrometric titration in a CO_2-free atmosphere with a glass electrode, the acid groups in 90 per cent acetone, the basic groups in water.

Isolation of Fructose-Amino Acids from Liver—500 gm. of dried hog liver powder[1] were suspended in 2 liters of 7 per cent trichloroacetic acid, stirred slowly at room temperature for 2 hours, and then filtered with the aid of Super-Cel through a No. 50 Whatman paper with suction. The filter cake was suspended in 500 ml. of 7 per cent trichloroacetic acid, stirred thoroughly, and again filtered. Both filtrates were combined, and then an equal volume of 10 per cent $Pb\cdot(C_2H_3O_2)\cdot 3H_2O$ in aqueous acetic acid

[1] Hog liver, dried at 75°, obtained from the Viobin Laboratories, Monticello, Illinois.

(pH 4.0) was added. After standing for 3 hours, the suspension was filtered and the precipitate was washed with water until the filtrate was chloride-free. The washed precipitate (after removal of the lead) had no stimulating activity in our test system. The combined filtrate and washings were brought to pH 9.5 with 5 N NaOH and allowed to stand for about 3 hours, when the precipitate had settled, leaving about half the total volume of the suspension as a clear supernatant solution. Partly by decantation and finally by centrifugation the precipitate was collected in 100 ml. centrifuge tubes and washed by repeated suspension in water and recentrifugation. The supernatant solutions were discarded. A stage is reached in the washing of the precipitate when, even after prolonged centrifugation, the supernatant solution remains turbid. At this stage the latter was very nearly chloride-free. The precipitate washed to this point was suspended in about 200 ml. of water and brought into solution with glacial acetic acid, final pH about 4.5. The lead was removed with H_2S, and, after removing the excess H_2S by aeration, the filtrate from the lead sulfide was lyophilized. Usually the residue weighed about 12 gm.; it was pale yellow and somewhat hygroscopic.

The next stage in the purification was chromatography on Dowex 50. The resin was cleaned by the procedure of Moore and Stein (14) and used in the H^+ form. A column of resin 22 × 450 mm. was washed with redistilled water until the eluate gave a negative test with $AgNO_3$ and the pH was above 5. Then 6 gm. of the material recovered from the lead precipitation, dissolved in 10 ml. of water, were washed into the column with water, and 20 hold-up volumes of water were passed through at a rate of 1 hold-up volume per hour. Then 20 hold-up volumes of each of the following strengths of trichloroacetic acid were passed through in succession: 0.2, 0.5, 0.75, and 1.0 M. The trichloroacetic acid eluates were collected in 0.5 hold-up volume fractions.

In exploratory experiments it had been found that stimulating activity in our test system paralleled the intensity of reduction of ferricyanide (method described above). Accordingly, this test was made the basis of the chromatographic separation. 0.1 to 0.3 ml. aliquots of each fraction were taken for the test, according to the intensity of the reduction obtained. The volumes of the reagents used were the same for 0.1 to 0.3 ml. aliquots except for the additional alkali needed for neutralization. Fig. 1 is a typical chromatogram.

The fractions constituting each band in Fig. 1 were pooled and lyophilized after removal of the trichloroacetic acid with ether. The material in every one of these bands stimulated the incorporation of leucine *in vitro* into rabbit reticulocyte proteins; the eluates between the bands had no stimulating action; there was some stimulating material in the water eluate.

So far only bands I, II, and III have been worked up further. They

were rechromatographed twice on the same resin in columns of the same dimensions, and the bands were located as before by the reduction test. 20 hold-up volumes of water were passed through and discarded. Bands I and II were eluted with 0.05 M and band III with 0.1 M trichloroacetic acid after 20 hold-up volumes of the 0.05 M solution had passed through. After each chromatography the trichloroacetic acid was extracted with ether, and the solution was then lyophilized. After the second chromatography the dried residue was dissolved in absolute methanol, from which it was precipitated by the addition of 10 volumes of dried ether. The solution in methanol and precipitation by ether were repeated twice more.

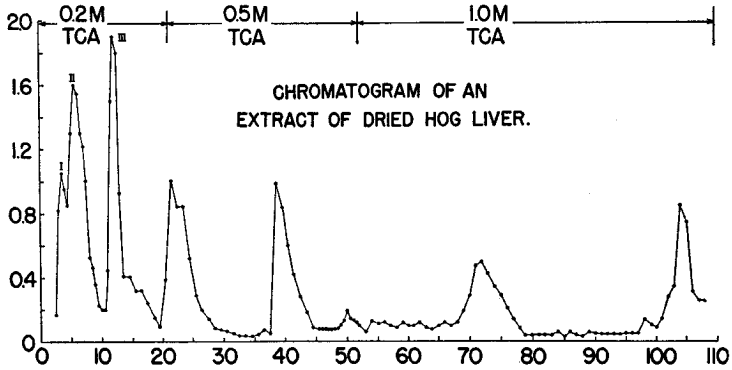


FIG. 1. Chromatogram of an extract of dried hog liver. Resin, Dowex 50 H^+; column dimensions, 22 × 450 mm.; eluent, varying strengths of trichloroacetic acid; material chromatographed, trichloroacetic extract of dried hog liver and material carried down with lead hydroxide on bringing lead acetate solution to pH 9.5. Ordinates, reduction values in terms of optical density of ferriferrocyanide formed; abscissas, hold-up volumes.

This procedure yielded, in the case of band II, a single component, fructose-L-glutamic acid. Both bands I and III were mixtures of fructose-alanine and fructose-glycine; the proportion and mode of linkage of the sugar were different in the two bands, but in each case the components of the mixture had very similar chromatographic properties. 500 gm. of dried liver usually gave 75, 250, and 200 mg., respectively, of compounds corresponding to bands I, II and III.

The composition of bands II and III was proved by comparison with corresponding synthetic compounds (for the nature of the material in band I see below). The details of the synthesis will be given in a later communication.

Sugar-protein compounds are formed slowly during storage of dried foods, and there is evidence (15) that they are of a type that can and does undergo

Amadori rearrangement to compounds of the above type III. The material in the bands of Fig. 1 may have been formed during and after the drying of the liver or in the isolation procedure. To test this point a hog liver was obtained directly from the animal, immediately frozen with solid CO_2, minced at 0°, and then precipitated with 7 per cent trichloroacetic acid at 0°. From this point on the procedure was identical with that described above. The chromatography of the fresh differed from that of the dried hog liver in that 0.35 M trichloroacetic acid was interposed before and 0.75 M

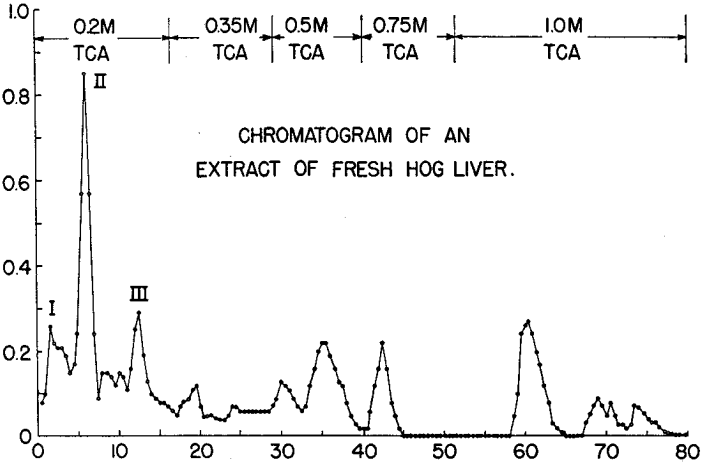


FIG. 2. Chromatogram of an extract of fresh hog liver. Resin, Dowex 50 H^+; column dimensions, 22 × 450 mm.; eluent, varying strengths of trichloroacetic acid; material chromatographed, cold trichloroacetic acid extract of fresh hog liver and material carried down with lead hydroxide on bringing lead acetate solution to pH 9.5. Ordinates, reduction values in terms of optical density of ferriferrocyanide formed; abscissas, hold-up volumes.

after the 0.5 M acid. The chromatogram, Fig. 2, is seen to be qualitatively similar to that given by the dried hog liver, Fig. 1. Bands II and III from fresh and from dried liver proved to be the same material. There was too little of band I from fresh liver to characterize it as extensively as the corresponding band from dried liver.

Comparison of Band II Material with Synthetic Fructose-L-Glutamic Acid—Table I shows that fructose-L-glutamic acid and the material in band II (Figs. 1 and 2) from both dried and fresh hog liver were the same.

The hydrolysis was carried out in 1.0 N HCl at 100° for 2 hours. With all three preparations the yield of amino acid was much less than half the theoretical. The solutions became dark brown, almost black. Both the

low yield of amino acid and the intense browning on heating with strong acid are characteristic of fructose-amino acids.

The difference in optical rotation of the two preparations recorded in Table I is within the experimental error of the micromethod used.

It is characteristic of all the fructose- and tagatose-amino acids we have prepared that they yield low values for sugar by methods based on conversion to furfural derivatives, as in the anthrone (12) and carbazole methods (10, 11), and nearly theoretical values by methods based on their reducing

TABLE I

Comparison of Chemical and Physical Properties of Synthetic Fructose-L-Glutamic Acid with Band II Material from Dried and Fresh Hog Liver

	Synthetic	Dried liver	Fresh liver
Amino acid released on acid hydrolysis	Glutamic	Glutamic	Glutamic
Total N, theoretical, %	4.53	4.53	
" " observed, %	4.67	4.40	
$[\alpha]_D^{25}$ (H_2O), degrees	−40 (*c* 3.27) −38 (*c* 6.32)	−34 (*c* 6.55)	
No. of titratable groups, acid	2	2	
" " " " basic	1	1	
pK′ of basic group	8.1–8.6	8.1–8.6	
Glucose, theoretical, equiv. per mole	1.00	1.00	1.00
Determined by anthrone	0.39	0.42	0.37
" " carbazole	0.72	0.55	0.85
" " hot reduction of ferricyanide	0.91	0.82	0.87
Reduction, % of that by equivalent amount of synthetic fructose-glycine	106	104	109
Glucosamine color, % of that given by equivalent weight of glucosamine	3.8	5.0	4.9
Paper chromatography			
Butanol-acetic acid-water, R_F	0.10, 0.22	0.10, 0.22	0.11, 0.22
Propanol-ammonia, R_F	0.095, 0.24	0.082, 0.22	0.086, 0.22

property, as in the hot reduction of ferricyanide. This is shown in Table I.

In Table I and throughout this communication the reduction values obtained at room temperature were rates measured under standarized conditions, as described above, and compared with the reduction under the same conditions by an equivalent amount of synthetic fructose-glycine.

With the glucosamine color reagent, fructose-glutamic acid gave, under the conditions employed, about 5 per cent of the color produced by an equivalent weight of glucosamine. A positive but low value by this test is characteristic of fructose-amino acids. Gottschalk (16) reported a value for fructose-DL-phenylalanine as 10 per cent that of glucosamine, which is the same as that of fructose-alanine and fructose-glycine (see Table II).

In the filter paper chromatography a mixture of butanol-acetic acid-water in the proportion of 4:1:1 and of propanol-ammonia (1 per cent), 3:1, was used. The chromatograms were developed with both the ninhydrin and reduction reagents. The synthetic fructose-L-glutamic acid and those isolated from dried and from fresh liver all gave two reducing spots with cold reagents, but the material of only one of these spots, the compound with

TABLE II

Comparison of Chemical and Physical Properties of Synthetic Fructose-L-Alanine and Fructose-Glycine with Band III Material from Dried and Fresh Hog Liver

	Synthetic		Band III	
	F-Ala	F-Gly	Dried liver	Fresh liver
Amino acid released on acid hydrolysis	Alanine	Glycine	Alanine, glycine	Alanine, glycine
Total N, theoretical, %	5.58	5.92	5.58–5.92	5.58–5.92
" " observed, %	5.78	6.05	5.53	5.75
$[\alpha]_D^{25}$ (H_2O), degrees	−52 (*c* 7.06)	−65 (*c* 3.61)	−59 (*c* 7.1)	
No. of titratable groups, acid	1	1	1	
" " " basic	1	1	1	
pK′ of basic group	8.2	8.1	8.2	
Glucose, theoretical, equiv. per mole	1.00	1.00	1.00	1.00
Determined by anthrone	0.19	0.22	0.23	0.20
" " carbazole	0.04	0.06	0.06	
" " hot reduction of ferricyanide	0.99	1.05	0.93	0.99
Reduction, % of that by equivalent amount of synthetic fructose-glycine	122	100	140	116
Glucosamine color, % of that given by equivalent weight of glucosamine	10	9	9	10
Paper chromatography				
Butanol-acetic acid-water, R_F	0.14	0.087	0.087, 0.14	0.087, 0.14
Propanol-ammonia, R_F	0.21	0.16	0.15, 0.20	0.15, 0.20

the lower R_F, was ninhydrin-positive. On paper electrophoresis at pH 7 only one spot was obtained in each case. It may be that the relation of the material giving the two reducing spots with cold reagents is analogous to that of glutamic and pyrrolidonecarboxylic acids. If that is the case here, the compound with the higher R_F, which does not react positively with ninhydrin, would be the ring structure involving the hydrogen on the conjugated nitrogen.

Comparison of Band III Material with Synthetic Fructose-L-Alanine and Fructose-Glycine—Table II shows that the material in band III from both

dried and fresh hog liver was a mixture of fructose-L-alanine and fructose-glycine.

Comparison of Band I Material with Synthetic Fructose-L-Alanine and Fructose-Glycine (Table III)—Bands II and III behaved in every respect as the corresponding synthetic compounds or a mixture of them. Band I

TABLE III

Chemical and Physical Properties of Material Obtained from Band I Isolated from Dried Hog Liver

All values are calculated on the basis of 2 hexose and 1 amino acid residues per equivalent.

		Amino acid released on acid hydrolysis
		Alanine, glycine, traces of aspartic acid
Total nitrogen, %	Calculated	3.38–3.50
	Observed	3.67
No. of titratable groups	Acid	1
	Basic	1
Glucose, equiv. per mole		
Determined by anthrone	Calculated	1.2
	Observed	1.2
" " carbazole	Calculated	1.05
	Observed	0.9
" " hot reduction of ferricyanide	Calculated	1.00
	Observed	1.05
Reduction, % of that by equivalent amount of synthetic fructose-glycine	Calculated	112
	Observed	128
Glucosamine color, % of that given by equivalent weight of glucosamine	Calculated	10
	Observed	9.5
Paper chromatography		
Butanol-acetic acid-water, R_F		0.04
Propanol-ammonia, R_F		0.09

was different; it was eluted before fructose-glutamic acid; yet on acid hydrolysis the amino acids liberated were alanine and glycine, with a trace of aspartic acid. The filter paper chromatograms, unlike those of band III, gave only one spot with the ninhydrin or cold reduction reagents. An interpretation consistent with all the analytical data is that band I is a mixture of two compounds, each of which consists of two hexoses and one amino acid, either alanine or glycine. 1 of the 2 hexose residues is in the fructose-amino acid form, which would give low values with the anthrone and carba-

zole reagents and a nearly theoretical value in the hot reduction of ferricyanide. The other hexose residue is so linked that its power to reduce ferricyanide (in the hot or cold) is lost, but yields nearly the same value that free glucose does with the anthrone and carbazole reagents; this behavior is in accord with a normal glycosidic linkage. The values for reduction at room temperature and glucosamine color also are in accord with a structure consisting of two hexoses, one in a fructose-amino acid linkage

TABLE IV

Comparison of Alanine and Glycine Conjugates with Glucose, Mannose, or Galactose, with Band III Material from Dried or Fresh Hog Liver

Compound	F-Ala		F-Gly		T-Ala	T-Gly	Band III from	
Original sugar	Glucose	Mannose	Glucose	Mannose	Galactose	Galactose	Dried liver	Fresh liver
$[\alpha]_D^{25}$, degrees	−52	−55	−65	−66	−10.3	−13.5	−59	
c in H_2O	7.06	6.71	3.61	3.66	6.3	6.68	7.1	
Glucose, theoretical	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Determined by anthrone	0.19	0.19	0.23	0.22	0.12	0.17	0.23	0.20
Determined by carbazole	0.06	0.04	0.06	0.06	0.06	0.10	0.06	
Determined by hot reduction of ferricyanide	1.05	0.99	0.99	1.01	0.80	0.78	0.99	
Reduction, % of that by equivalent amount of synthetic fructose-glycine	124	124	100	104	224	208	140	116
Glucosamine color, % of that given by equivalent weight of glucosamine	10	10	10	9	10	10	9	10

and the other glycosidic. There was too little movement on the paper chromatograms to decide whether the material is a mixture of two such compounds, in one of which the amino acid is alanine and in the other glycine, or whether it is one compound consisting of four hexoses and two amino acids.

The hexose residue was identified by an indirect method, because treatment such as heating with acid, although it yields the constituent amino acid in low amount, does not give the sugar but furfural derivatives. The evidence in Table IV indicates that the hexose residue in the material of band III, obtained from dried or fresh liver, corresponds to fructose. In

the synthesis, a fructose-amino acid is obtained whether one uses glucose or mannose. In the condensation with the amino acid at the C-1 position of the sugar and the subsequent rearrangement the difference between glucose and mannose, which is at the C-2 position, is abolished. On the other hand the analogous compounds derived from galactose are tagatose-amino acids. The decisive comparison in determining the identity of the sugar radical is based on optical rotation. Table IV shows that F-Ala is the same, whether made from glucose or mannose, and that this is the case also with F-Gly, and band III corresponds to a mixture of F-Ala and F-Gly, and that these compounds are quite different from the analogous compounds made from galactose. The anthrone and carbazole sugar values were the same for all. Galactose was 70 per cent of the value given by glucose in the hot reduction of ferricyanide; correspondingly F-Ala, F-Gly (from either

TABLE V

Partial Reconstitution of Stimulation of Leucine Incorporation by Known Constituents of Liver Extract

Results expressed as per cent of blank control.

Added to control reaction mixture	None	Liver extract	F-Ala	F-Glu	F-Gly	Band I	Band II	Band III
None	100	170	100	100	100	100	100	100
Amino acids	160	350	160	160	160	160	160	160
" " + 0.5 γ Fe per ml.	210	350	290	260	285	290	290	290
" " + 5.0 " " " "	290	350	300	300	300	300	300	300

glucose or mannose), and band III gave nearly theoretical values by hot reduction of ferricyanide; T-Ala and T-Gly were lower. On the other hand reduction values for T-Ala and T-Gly were higher at room temperature than those for F-Ala, or F-Gly, which were the same whether the original sugar was glucose or mannose.

Stimulation of Amino Acid Incorporation—Table V presents the salient characteristics of the stimulation of the incorporation of labeled leucine into rabbit reticulocyte proteins by liver extract and by fructose-amino acids, whether synthetic or isolated from liver. Similar results were obtained with the incorporation of labeled glycine, histidine, and lysine. The liver extract referred to was prepared as described previously (1); 4 ml. of reaction mixture contained an amount equivalent to 0.5 gm. of fresh liver, which was optimal in the test system used. The results reported for the individual sugar-amino acids and for the material isolated from liver were those obtained with 5×10^{-4} M, which in every case was close to the lowest concentration giving maximal stimulation. The same results were obtained for bands I, II, and III from chromatograms of dried and fresh hog liver.

As Table V shows, the addition of the amino acid mixture plus iron nearly triples the rate of incorporation. Iron is ineffective unless the amino acids are added also; the amino acids stimulate alone, though less than the combination. The effect of the liver extract is doubled by the amino acids, but adding iron has no further augmenting effect, because the liver extract is rich in available iron. After removal of all heavy metals by 8-hydroxyquinoline, the liver extract had no stimulating effect; this effect was completely restored by the addition to the reaction mixture of 5 γ per ml. of ferrous iron. This concentration is the minimum for maximal stimulation, whether alone or with other stimulating factors. Ferric iron is only slightly less effective than ferrous iron.

Fructose-amino acids had no stimulating effect unless both the amino acid mixture and iron were added also. The concentration of added iron needs to be relatively low, *e.g.* 0.5 γ per ml. With 5.0 γ of iron the stimulation by iron plus amino acids is so high that added fructose-amino acids have only a slight effect. It appears that one of the functions of the fructose-amino acids is to make low concentrations of iron more effective.

DISCUSSION

The fructose-amino acids, whose isolation from liver and identification are described above, are the first to be found free in biological material. We are of the opinion that liver contains other fructose- (hexose-)amino acids in addition to these, indicated in the bands beyond III in Figs. 1 and 2. A number of synthetic fructose-amino acids were eluted from Dowex 50 columns in the same relative positions and required for elution the same strengths of trichloroacetic acid.

One point can be made at the present time regarding the mechanism of the stimulation of amino acid incorporation by fructose-amino acids. It is that a fructose-amino acid does not act as a carrier of its amino acid into the protein. There was no incorporation of leucine when fructose-C^{14}-L-leucine was used instead of C^{14}-L-leucine. The details of these experiments will be reported in a later communication, where the interrelation of the several stimulating factors we have found will be dealt with.

Even the greatest stimulation attainable by amino acids and iron, with or without the fructose-amino acid, falls short of the maximum obtained with amino acids plus liver extract. Because the maximal stimulation obtained with one fructose-amino acid compound was not augmented by the addition of ten other fructose-amino acids, our present, tentative interpretation is that there is another stimulating factor in liver.

The stimulating factor in plasma also requires the amino acids and iron; it differs from the fructose-amino acids we have so far isolated and synthesized in that its greatest effect is seen with a high iron concentration that obscures the stimulation by the fructose-amino acids. We are now engaged

in the isolation of this factor and the, as yet, unidentified factor in liver, which may be the same as the stimulating factor in plasma.

SUMMARY

1. A method is described of isolating fructose-amino acids (1-deoxy-1-amino-2-ketohexoses) from liver.

2. A test is described by which 10 γ of such compounds can be measured.

3. Aqueous extracts of dried and fresh liver contain a number of fructose-amino acids. Fructose-L-glutamic acid and a mixture of fructose-L-alanine and fructose-glycine were isolated and their identity proved by comparison with the synthetic compounds.

4. Some of the physical, chemical, and biological properties of the fructose-amino acids isolated are described.

5. Fructose-amino acids acting with certain amino acids and iron account for most of the stimulation exerted by the original liver extract on the incorporation of amino acids *in vitro* into the proteins of rabbit reticulocytes.

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