out by means of infrared absorption.[46] From the results of duplicate determinations and a check analysis of a known mixture of similar composition the analysis with indicated absolute limits of accuracy was reported as: bromobenzene, 17.9 ± 1.0; *o*-bromotoluene, 16.9 ± 0.5; *m*-bromotoluene, 10.1 ± 0.5; *p*-bromotoluene, 55.1 ± 0.5. This represents an over-all yield of 41% (based on mercuric acetate). From the competitive rate expression of Ingold,[23] averaging the toluene/benzene ratio for 41% conversion with that estimated for 100% conversion and allowing a 10% error in the results

$$k_{C_6H_5Me}/k_{C_6H_5} = 30 \pm 3$$

$$k_{para}/k_{meta} = 5.5 \pm 0.6$$

LAFAYETTE, IND.

NOTES

Ion-exchange Chromatography of the Products of the Non-enzymatic Sugar Amino Acid Reaction, and the Occurrence of the Reaction in Liver Extracts

BY A. ABRAMS AND H. BORSOOK

RECEIVED JULY 27, 1953

The non-enzymatic condensations of reducing sugars with amino acids, first investigated by Maillard,[1] and often referred to as the browning reaction, have recently been reviewed.[2,3] Despite the many years that these reactions have been under investigation, the nature of the products has remained uncertain mainly because of the inadequacies of the available analytical methods. Recently the methods of paper chromatography have been applied to the problem[4-6] with considerable success but the use of ion-exchange chromatography does not seem to have been reported.

Using ion-exchange chromatography and carboxyl-C^{14}-L-leucine we have been able to resolve some of the products formed when the amino acid reacts with D-glucose and with D-ribose. A non-enzymatic reaction with glucose also was observed to take place when leucine was added to liver homogenates, the reaction having been brought about under conditions typical for browning reactions in natural systems. The sugar amino acid reaction in concentrated liver extracts was reported some years ago by Agren.[7,8]

Only one radioactive product was detected when the glucose and leucine reacted, and no visible browning took place. Paper chromatographic evidence made it probable that it was the simple glycosyl amino acid (or Schiff base) which is generally considered to be the initial product formed in the browning reaction although direct evidence is lacking.[3]

The reaction between ribose and leucine carried out under approximately the same conditions as for glucose resulted in the formation of a number of radioactive products and considerable brown color. It should be noted that only those products which retain the carboxyl group of the leucine have been detected. These products must represent those formed in the initial stages of the reaction since the later stages involve the loss of the carboxyl group as CO_2.[9]

These findings need to be taken into account in interpreting metabolic findings with amino acids, especially radioactive amino acids. It has already been pointed out that amino acids and glucose in the same mixture may cause a considerable error in the determination of glucosamine with Ehrlich's reagent.[10,11] It is of interest to note also that the stimulation of the growth of certain bacteria by heated culture media, has been ascribed to the products of the Maillard reaction.[12]

Materials and Methods.—Ion-exchange chromatography was carried out on Dowex-50 cation resin (250–500 mesh) in conjunction with an automatic fraction collector, eluting with various strengths of hydrochloric acid according to the directions of Moore and Stein,[13] except that the column was kept at 4° by the use of a jacketed column, through which cold water was allowed to circulate.[14]

The radioactivity of each fraction was determined by means of a Geiger–Müller end window counter on 0.2-ml. aliquots dried on Tygon painted aluminum cups[14] or copper cups.

The leucine was C^{14}-carboxyl labeled and had a specific activity of 26500 c.p.m./mg.[15]

Experimental and Results

The Reaction in Liver Extracts.—A typical experiment leading to the formation of a glucose–leucine compound follows. A guinea pig liver, 10 g. wet weight, was homogenized in 40 ml. of a salt mixture[16] at *p*H 7.4 to which 5.24 mg. of radioactive leucine had been added. The homogenate was incubated for 3 hours at 37°, and the proteins were removed by heating at 100° at *p*H 5, and the soluble extract was brought to dryness by evacuation in a rotary dryer at a temperature of about 60–80°. The residue was taken up in 5 ml. of 1.5 *N* hydrochloric acid and subjected to ion-exchange analysis as described under Methods. The results are shown in Fig. 1. It can be seen that a major radioactive component was resolved as a fairly

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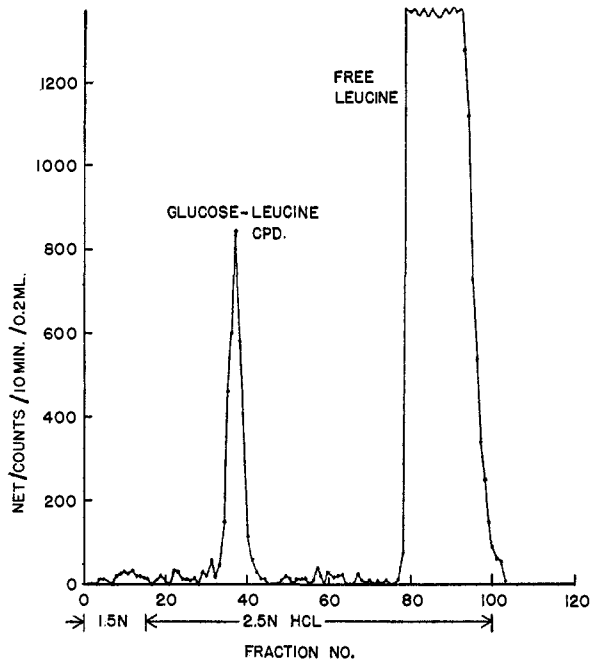

Fig. 1.—Ion-exchange analysis of radioactive products obtained after addition of carboxyl-C^{14}-L-leucine to liver homogenate and treatment as described in text. Volume of each fraction is 5.0 ml.

symmetrical peak, equivalent to 6% of the radioleucine added. A similar result was obtained by adding the labeled leucine to the non-protein extract, showing that the reaction was non-enzymatic. Numerous experiments indicated that the extent of the reaction was related to the conditions used for concentrating and drying the extracts, but we have not attempted to define these conditions rigorously. The browning reaction of concentrated glucose–amino acid mixtures and glucose and proteins in the "dry" state have been shown to be markedly affected by a variety of factors, such as temperature, relative humidity, *p*H and time.[3,17]

The newly formed compound was further purified by rechromatographing on a starch column[18] on which it was eluted close to the "front," and then again on the Dowex resin. Some loss was incurred due to partial hydrolysis in the strong hydrochloric acid (2.5 *N*) at room temperature after elution from the cold column. The hydrochloric acid was removed by evacuation over sodium hydroxide and the substance was chromatographed on Whatman #1 paper with a butanol–acetic acid–water mixture (4:1:1). The compound was located (R_f 0.44) by its radioactivity, and reacted with ninhydrin[19a] to give a dull brown color gradually turning purple. It also reacted with the benzidine[20] and aniline phthalate[21] reagents which are used to detect sugars. In each case the radioactivity coincided with the colored spot produced by the reagents. In addition to the new compound, the paper chromatogram also showed the presence of free leucine (radioactive) and free glucose (non-radioactive). These were evidently formed by partial hydrolysis as mentioned earlier. In another experiment the newly formed radioactive compound produced radioactive leucine after complete acid hydrolysis, as shown by carrier isolation and recrystallization to constant specific activity. It would appear then that the new substance was a compound of glucose and leucine. It was observed that this glucose–leucine compound, while initially colorless, became very dark brown on standing in the dry state over a period of weeks under ordinary atmospheric conditions. This behavior would be expected for an intermediate in the browning reaction.

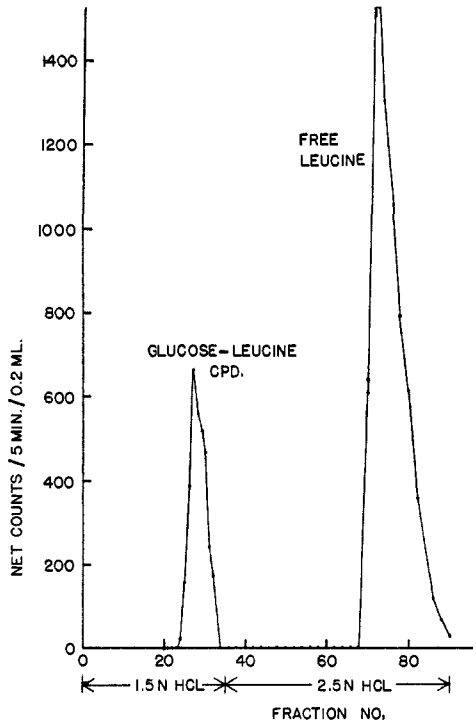

Fig. 2.—Ion-exchange analysis of radioactive products from a heated carboxyl-C^{14}-L-leucine–glucose mixture. Volume of each fraction is 5.8 ml.

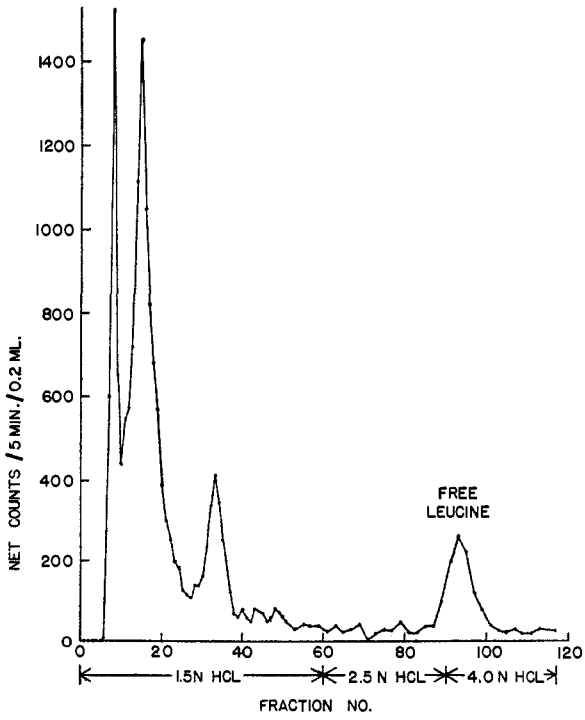

Fig. 3.—Ion-exchange analysis of radioactive products from a heated carboxyl-C^{14}-L-leucine–ribose mixture. Volume of each fraction is 5.0 ml.

The Reaction between Pure Glucose and Radioactive Leucine.—Pure glucose and carboxyl-C^{14}-L-leucine were heated in a "semi-dry" condition so as to simulate the drying conditions used in the preparation of the liver extracts as

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(19) (a) The paper was dipped in 1% ninhydrin in pyridine[b] and the color was allowed to develop under a heating lamp; (b) L. B. Rockland and J. C. Underwood, personal communication.

(20) R. H. Horrocks, *Nature*, **164**, 444 (1949).

(21) S. M. Partridge, *ibid.*, **164**, 443 (1949).

described in the previous section. Radioactive leucine (5.0 mg.) and D-glucose (100 mg.) were dissolved in a few drops of 0.1 *M* acetate buffer, *p*H 5, in a small tube, which was then heated at approximately 80° for one hour in a water-bath. The semi-dry mixture, *still completely colorless*, was dissolved in 5.0 ml. 1.5 *N* hydrochloric acid and chromatographed on a cold Dowex-50 ion-exchange resin column. The result is shown in Fig. 2. The pattern is very similar to that obtained with liver extracts (Fig. 1). The radioactivity in the new compound amounted to 14% of radioactivity added as leucine, and only about 50% of the total radioactivity was recovered. Paper chromatographic analysis gave results similar to those already described for the compound isolated from liver extracts.

The Reaction between D-Ribose and Radioactive Leucine.—A mixture of 100 mg. of D-ribose and 5.0 mg. carboxyl-C^{14}-L-leucine were heated at *p*H 5 in a "semi-dry" condition as described for the glucose–leucine mixture. A great deal of browning took place, in contrast to the glucose–leucine reaction mixture. The chromatographic analysis is shown in Fig. 3. It should be noted that only a small amount, if any, free leucine remained. The brown colored products emerged at the "front" and then gradually diminished. A number of well defined radioactive peaks appeared but these products have not been analyzed further. The greater reactivity of ribose as compared to glucose is in accord with the observation of other investigators.[22]

Acknowledgment.—The financial support of Eli Lilly and Company is gratefully acknowledged.

(22) V. M. Lewis and C. H. Lea, *Biochim. Biophys. Acta*, **4**, 532 (1950).

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Absorption Spectra of Fuming Sulfuric Acid Chromogens Obtained from the Estrogens and Other Steroid Compounds[1]

BY LEONARD R. AXELROD

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Concentrated sulfuric acid has been observed to form chromogens with steroids which give absorption spectra different for each compound.[2] These absorption spectra have been utilized to aid in the qualitative identification of steroid metabolites.[3,4] Fuming sulfuric acid has now been found to form chromogens with the estrogens and other steroids which give specific absorption spectra for each compound different from those with concentrated sulfuric acid. The procedure is as follows: Three ml. of reagent grade fuming sulfuric acid (assay: 15–16% free SO_3) is added to 30–50 micrograms of steroid in a glass-stoppered test-tube. After one-half hour in the dark at room temperature, the optical density of the solution from 220–600 mμ is read in a Beckman D. W. spectrophotometer. Quartz cells with ground glass stoppers obtained from Pyrocell Co., New York, were utilized to protect the apparatus from the acid. Fuming sulfuric acid was used as a blank.

Table I summarizes the results obtained with 22 of the steroids studied. The shapes and peaks of the absorption spectra were found to be specific for each compound.

(1) This investigation was supported by a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

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TABLE I

Compounds[5]	Absorption maxima
Estriol	430
Estradiol-17β	300, 430
Estradiol-17α	300, 420
7-Ketoestrone	242, 310, 425
Equilenin	310, 380, 445
Equilin	305, 380, 435
Δ^6-Dehydroestrone	300, 365, 435
Estrone	295, 380
Methoxydoisynolic acid	265, 320, 390
Diethylstilbestrol	425
17α-Ethinylestradiol	No maxima
17-Hydroxycorticosterone	240, 500
17-Hydroxy-11-dehydrocorticosterone	295, 440
17-Hydroxy-11-desoxycorticosterone	240, 275, 505
Corticosterone	240, 275, 410, 485
11-Desoxycorticosterone	240, 280, 490
Dehydroepiandrosterone	300, 405
Epiandrosterone	235, 300, 395
Testosterone	300
Androsterone	295, 390
Progesterone	300, 440
Pregnane-3α,20α-diol	285

It was furthermore found that the absorption spectra of most compounds change with time so that a new spectrum evolves if the chromogen solution is allowed to stand at room temperature for longer periods of time. For example, 17-hydroxy-11-dehydrocorticosterone after 24 hours exhibits maxima at 250, 280 and 495 mμ. This phenomenon has proven most useful for obtaining the qualitative identification of a single sample of steroid compounds over a period of 24 hours.

(5) Generously donated by Drs. E. Alpert, T. F. Gallagher, E. B. Hershberg, H. B. MacPhillamy, W. H. Pearlman, L. A. Sweat and O. Wintersteiner.

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A Triazolopyrimidine Analog of 6-Mercaptopurine[1,2]

BY CARL TABB BAHNER, BILL STUMP AND MARY EMMA BROWN

RECEIVED JULY 22, 1953

The compound 6-mercaptopurine has been shown to inhibit growth of certain bacteria[3] and tumors.[4–6] Roblin, Lampen, English, Cole and Vaughn[7] prepared several triazolopyrimidines which were found to inhibit bacterial growth and one of them, 8-azaguanine, was found to inhibit certain tumors.

(1) This research was supported in part by a grant from the Damon Runyon Memorial Fund for Cancer Research and in part by a research grant from the National Institutes of Health, U. S. Public Health Service.

(2) Presented in part at the Southeastern Regional Meeting of the American Chemical Society, Auburn, Alabama, October 24, 1952.

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