The apparent inability of the animals to control their movements indicated that the cells in certain areas of the brain may have been affected. The levels of methionine and phenylalanine in the rations may have altered the ratio of amino acids in the brain cells[2] to such an extent that permanent changes in the enzyme or metabolic systems of the cells took place. Furthermore, the observation that the symptoms were not reversible by feeding or injecting niacin would substantiate this conclusion, since this compound prevented the onset of the nervous symptoms, when it was present in the ration. A gross examination of the brains of several animals did not reveal any abnormalities.

It is believed that this is the first time a syndrome of this nature has been observed in experiments with rations containing an imbalance of amino acids. There are known human diseases such as cystinuria,[3,4] alkaptonuria,[5] tyrosinosis,[6] and phenylpyruvic oligophrenia,[7,8] which have been related to inborn errors of amino acid metabolism. In these diseases normal metabolic processes involving phenylalanine and tyrosine are either partially or completely inhibited, and the disorders are generally recognized by the persistent excretion of abnormal urinary constituents.[9,10]

When our results are considered in the light of faulty amino acid metabolism, it is feasible that the effects observed were due partially to a limiting ability of the animal to metabolize phenylalanine. It is also possible that the causative agent in this syndrome was the D isomer of this amino acid, since it has been observed that the growth of rats was reduced drastically when this isomer was supplemented at low levels in a 9% casein ration.[1]

Summary. Animals fed a niacin-tryptophan deficient ration supplemented with 0.208% DL-phenylalanine and 0.2% DL-methionine for a period of 12 weeks developed a nervous syndrome. Generous quantities of tryptophan or niacin in the ration protected the animals from this syndrome, but neither compound cured the symptoms once they had progressed beyond a given stage.

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2 Christensen, H. N., Streicher, J. A., Elbinger, R. L., *J. Biol. Chem.*, 1948 **172**, 515.

3 Burke, B. S., Harding, V. V., and Stuart, H. C., *J. Pediat.*, 1943, **23**, 508.

4 Lewis, H. B., Brown, B. H., and White, F. R., *J. Biol. Chem.*, 1935, **109**, 69.

5 Hall, W. K., Rawls, K., and Sydenstricker, V. P., *Fed. Proc.*, 1946, **5**, 136.

6 Medes, G., *Biochem. J.*, 1932, **26**, 917.

7 Dann, M., Marples, E., and Levine, S. Z., *J. Clin. Invest.*, 1943, **22**, 87.

8 Jarvis, G. A., Block, R. J., Bolling, D., Kanze, E., *J. Biol. Chem.*, 1940, **134**, 105.

9 Sealock, R. R., and Silberstein, H. E., *J. Biol. Chem.*, 1940, **135**, 251.

10 Basinski, D. H., and Sealock, R. H., *Fed. Proc.*, 1946, **5**, 121.

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A Modified Diphenylamine Procedure for Fructose or Inulin Determination. (17429)

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The use of inulin clearance as a measure of glomerular filtration rate has prompted the appearance of a number of methods for the determination of inulin or fructose, the majority of which are based either on the Seliwanoff reaction[1] or the reaction with

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diphenylamine described by Ihl.[2] The hexoses when heated in the presence of strong acids form hydroxymethylfurfural and this in turn reacts to form a colored condensation product with either resorcinol or diphenylamine. Although the ketohexoses react much more readily than the aldohexoses, glucose in the concentrations normally found in plasma will yield a significant amount of color. If the plasma glucose remains constant this error is eliminated by the use of a control blank. Since this is not generally the case, the usual procedure has been either to remove the glucose by fermentation or to determine it in each sample and make a correction.

The amount of color produced by fructose or inulin as compared to that produced by glucose varies somewhat with different methods. Factors which influence this ratio are, (1.) acidity of the reacting solution, (2.) concentration of resorcinol or diphenylamine, (3.) duration of heating and (4.) temperature at which the reaction is carried out. While a decrease in any of these factors will decrease the speed of the reaction, the glucose color production is diminished to a greater extent than the fructose. Corcoran and Page,[3] using a diphenylamine procedure at 100°C, found a fructose to glucose color ratio of 40 and say, "Error from this source is abolished by the compensating blank when blood glucose is constant, and the error amounts to only 0.5 mg of levulose per 100 cc with glucose concentrations varying 20 mg per 100 cc." Using their method we have found the ratio to be between 20 and 25; Corcoran has recently informed us that they now find this lower ratio. Findley and White[4] found a ratio of 25; since then it has been found to be more nearly 20. Harrison,[5] who has improved the technic by substituting glacial acetic acid for ethyl alcohol as the solvent for diphenylamine, does not state his ratio, but says, "It is necessary to remove glucose from the blood plasma." Using his method we have found the ratio to be 8. Roe, Epstein and Goldstein,[6] using a resorcinol method, have recently reported a fructose to glucose color ratio of about 2000 at various concentrations of glucose; such a ratio would render any errors due to glucose fluctuations insignificant. We have not been able to confirm their finding. Using their method and working with aqueous glucose solutions containing 0.0625 mg/ml to to 0.5 mg/ml and with fructose solutions containing 0.005 mg/ml to 0.03 mg/ml we have found the color ratio to be approximately 50. Color productions by glucose and by fructose are additive when the two sugars are determined together. We have found also that the color produced by this reaction is quite sensitive to light, either artificial or daylight, and that there can be a difference of as much as 15% between duplicates even when the samples are read within the 30 minutes recommended in their paper, unless special precautions are taken to maintain light exposure at a minimum and uniform for all samples.

In an effort to find a set of conditions which would yield a high fructose to glucose color ratio we have tried many combinations of diphenylamine and HCl with different heating times and temperatures. The following procedure has produced the highest fructose to glucose ratio and yielded the best agreement between duplicates. Although we have not been able to verify Roe, Epstein and Goldstein's exceedingly high color ratio, it still appears to be a satisfactory method since even a ratio of 50 is higher than with previously reported methods; in our hands it has yielded accurate results when colored samples are protected from light. It is still felt, however, that the method presented here has some advantages in that the color ratio is somewhat higher and the color produced is stable. It further avoids the unpleasantness involved in handling their highly concentrated HCl reagent.

Method. Reagent. Dissolve 3 g of diphenylamine† in 100 ml of glacial acetic acid and add

1 Seliwanoff, T., *Ber. chem. Ges.*, 1887, **1**, 181.

2 Ihl, A., *Chem. Ztg.*, 1885, **9**, 451.

3 Corcoran, A. C., and Page, I. H., *J. Biol. Chem.*, 1939, **127**, 601.

4 Findley, T., and White, H. L., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 623.

5 Harrison, H. E., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 111.

6 Roe, J. H., Epstein, J. H., and Goldstein, N. P., *J. Biol. Chem.*, 1949, **178**, 839.

TABLE I.
Optical Densities × 10^3 Produced by Fructose, Inulin, and Glucose Solution.

	Fructose, μg/2 ml					Glucose, mg/2 ml	Fructose
	10	20	30	40	60	1	glucose
1		145 ± .5(6)	222 ± .6(6)	289 ± 1.0(6)	433 ± 1.0(6)		
2		142 ± .5(5)	220 ± 1.0(6)	285 ± .9(6)	436 ± .8(6)	117 ± .7(4)	62
3	89 ± .5(5)	147 ± .5(5)	221 ± .7(5)	291 ± .8(5)	447 ± .5(5)	114 ± .6(4)	65
4	82 ± .3(6)	154 ± .5(5)	236 ± .2(5)	309 ± .9(4)	460 ± .4(4)	124 ± .5(3)	63
5	77 ± .5(3)	148 ± .7(3)	220 ± .7(3)	294 ± 1.0(3)	448 ± .5(3)	118 ± .0(2)	63
6	74 ± .5(3)	152 ± .4(3)	228 ± 2.0(3)	295 ± 1.0(3)	445 ± 1.0(3)	114 ± 1.0(3)	66
7	72 ± .0(3)	148 ± .7(3)	230 ± 2.0(3)	305 ± 1.0(3)	457 ± 2.0(3)	120 ± 1.0(3)	63
			Inulin μg per 2 ml.				
8			229 ± .7(10)				
9		142 ± .7(6)	204 ± .4 (6)	284 ± .7(4)	426 ± 1.0(6)		

The data of a horizontal line were obtained with a single heating. The figure following the ± is S.E. for the samples of a given concentration in the same heating; the figures in parentheses give n, the number of samples. Ten 30 μg fructose samples run simultaneously with the 10 inulin samples of line 8 gave an average optical density × 10^3 of 234 ± 0.8, indicating complete hydrolysis of the inulin. The inulin densities in line 9 are somewhat lower than those usually obtained because some diphenylamine had precipitated after the reagent had stood 2 days in the refrigerator; simultaneous fructose determinations (not shown in table) were also correspondingly low.

60 ml of conc. HCl. This is the same reagent used by Harrison. Prepare fresh each day; if kept in a refrigerator some of the diphenylamine will precipitate and it is difficult to redissolve it. Use of this reagent after partial precipitation of diphenylamine yields a calibration curve which is lower than one obtained with fresh reagent.

Procedure. Pipette 2 ml of a 1:10 plasma filtrate[7] or appropriate urine dilution into a test tube 15 x 125 mm. Add 4 ml of diphenylamine reagent and *mix well* with swirling. Since the reagent has a high viscosity, care must be taken that pipette drainage is adequate. For plasma determinations use as a blank 2 ml of a 1:10 filtrate of plasma obtained just prior to the injection of inulin; 2 ml of water is used for urine blanks. Place in a rack and heat in a water bath at 75°C for 60 minutes. The bath used had a thermoregulator sensitive to ± 0.7°C at 75°C; a mechanical stirrer insured uniform heating of all samples. At the end of the heating period place rack in an ice bath for 1 to 2 minutes. If the tubes are kept too long at the lower temperature there may be some precipitation of diphenylamine. Read the samples in a photoelectric colorimeter with a 640 filter or a spectrophometer at 640 mμ.

When 2 ml samples containing 10 to 60 μg of fructose or inulin in water solution are used, optical density plotted against concentration yields a straight line. This corresponds to a range from 5 to 30 mg per 100 ml of plasma since 2 ml of a 1:10 filtrate are used. Separate calibration curves may not superimpose. Since the curves are straight lines, the inclusion of a standard with each set of determinations makes it possible to correct the values read from the calibration curve.

Table I shows the optical densities obtained with various concentrations of fructose, inulin and glucose. There is good agreement between samples of the same concentration in any one heating batch (represented on a horizontal line). Within the range of concentrations shown in Table I a given amount of fructose yields 64 times as much color as the same amount of glucose; this same ratio holds when fructose color development is compared with that of glucose in concentrations of 0.125 and 0.250 mg/ml (not shown in table). In our experience the plasma glucose level of fasted subjects does not depart more than 10 mg % from the blank value during

† Since this paper went to press we have been using 2.5 g of diphenylamine and have been keeping the tubes covered while they are in the water bath. This has prevented crystallization of diphenylamine.

[7] Somogyi, M., *J. Biol. Chem.*, 1930, **86**, 655.

a clearance experiment: the fluctuation is usually less than this. With a plasma inulin level of 15 mg % a 10 mg % departure from the blank would produce an error of one % in the inulin value determined.

A series of determinations on different solutions containing both glucose and fructose were run in the same heating batch with the corresponding separate glucose and fructose concentrations. The ratios of the observed additive optical densities to the calculated were 0.993, 0.993, 1.003, 1.007, 1.015, 0.994, 0.994, 0.985, 1.010, 1.006, 1.033, 1.006, 1.020 and 0.987.

Summary. A modification of Harrison's diphenylamine method for fructose or inulin determination is described, heating being carried out at 75°C for 60 minutes. With this procedure the ratio of fructose to glucose color production is 64, as compared with a ratio of 8 at 100°C for 30 minutes. Inulin hydrolysis is complete, the standard error of the method is less than 1 percent, the color is light stable and the low color production by glucose permits one to omit fermentation except in the presence of unusual glucose fluctuations.

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False Positive Trichina Precipitin Reactions in Neoplastic Disease. (17430)

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Because of high fever, palpebral edema, painful inflammation of feet and hands, and eosinophilia of 5% in a child with atypical lymphosarcoma, a trichina precipitin test was performed. The reaction was strongly positive in a dilution of 1:1280, the highest titre routinely tested. The clinical signs rapidly subsided during treatment with 4-aminopteroylaspartic acid, a trichina complement-fixation test was negative, and x-ray examination demonstrated lesions of bone of the "moth-eaten" type commonly seen in acute leukemia. Hence, although the precipitin test remained unchanged, there was no longer any clinical reason to consider a diagnosis of trichiniasis and it was felt that the trichina precipitin reaction was a false positive.

This occurrence of an apparently false positive trichina precipitin test in a patient with lymphosarcoma led to a study of sera from other patients with lymphosarcoma, Hodgkin's disease, leukemia, and other neoplastic diseases. Positive reactions were so frequently seen that it seemed desirable to report the phenomenon.

Method. Sera were obtained from hospitalized and clinic patients and personnel of Memorial Hospital. Three categories were studied: (1) those neoplastic diseases which involve principally the reticuloendothelial system (Hodgkin's disease, lymphosarcoma, the leukemias, and multiple myeloma), (2) widespread metastatic disease of other tissue, both carcinomas and sarcomas, (3) apparently healthy controls. Group 2 was included as controls who showed advanced and debilitating disease comparable to that seen in the first group.

Precipitin tests were performed by the standard technic of the Bureau of Laboratories of the New York City Department of Health. The antigen consisted of a saline extract of *Trichinella spiralis* larvae obtained from infected rat muscle by digestion with pepsin and hydrochloric acid. Undiluted serum was pipetted into small bore test tubes and overlaid with antigen in dilutions of 1:160, 1:320, 1:640, and 1:1280 on a basis of weight of dried larvae. A positive test was indicated by the formation, after two hours at room temperature, of an opaque ring. A

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