rather than to cause the absence of the wild-type enzyme. It is of interest that in both of these cases hybrid enzymes are formed in heterozygotes. Dimerization which has been proposed as the basis for hybrid vigor[1] may be a common feature of maize enzymes.

Summary.—Two alleles of the E_3 gene in maize specify electrophoretically separable esterases. As is the case with the pH 7.5 esterases formed by the E_1 gene, a hybrid esterase with an intermediate migration rate is found in the E_3 heterozygotes. The enzyme specified by one of the E_3 alleles has a migration rate identical to that formed by one of the E_1 alleles.

* Research supported by National Science Foundation grant GB 302.

[1] Schwartz, D., these PROCEEDINGS, **46**, 1210 (1960).

PLASMA AMINO ACID PATTERNS IN ALCOHOLISM: THE EFFECTS OF ETHANOL LOADING

BY FRANK L. SIEGEL,* MARY K. ROACH, AND LEON R. POMEROY

CLAYTON FOUNDATION BIOCHEMICAL INSTITUTE, UNIVERSITY OF TEXAS, AUSTIN

Communicated by Roger J. Williams, January 31, 1964

Reports from several groups provide evidence which indicates that alcoholics have metabolic patterns which distinguish them from nonalcoholic subjects. Previous findings from this laboratory[1] include imbalances in the formed element composition of the blood of alcoholics, and of elevated blood levels of glucose, sodium, potassium, and calcium in this group. It was also reported that alcoholics have significantly elevated urinary excretion of hippuric acid, sodium, potassium, and chloride, and lowered excretion of creatinine. Application of these findings is complicated by ethnic group differences.[2] More recently, we have presented a preliminary report[3] indicating imbalances of plasma amino acids in alcoholics, and group-distinguishing alterations of the plasma amino acid pattern following acute ethanol ingestion by alcoholics and controls. Other laboratories have found that alcoholics, as a group, exhibit a defect of tryptophan metabolism,[4] show reduced activity of the sympathetic nervous system,[5] tend to be vitamin B6- and B12-deficient,[6,7] and to have low plasma levels of zinc[8] and magnesium.[9]

Such metabolic imbalances may be regarded as the summation of two factors: the various metabolic and nutritional effects of chronic alcohol consumption, and constitutional factors related to the etiology of alcoholism. Only when partition of these factors has been achieved can the importance of the genetic aspects be assessed. The present report on patterns of free amino acids in plasma describes the use of amino acid data obtained before and after ethanol loading to generate an hypothesis of a constitutional defect in alcoholism. The relative importance of environmental stressers in the development of alcoholism is not denied, but it is suggested that constitutional factors may convey unusual susceptibility to such stress in alcoholism-prone individuals. Reed[10] and McClearn[11] have documented evidence for genetic factors governing alcohol consumption in laboratory animals, and Williams[12] has indicated the potential value of approaching the problems of

disease susceptibility from a biochemical focus which considers the importance of individual differences within the normal range.

In the metabolic imbalances which have been cited as distinguishing alcoholics, as a group, from nonalcoholics, considerable intergroup overlap exists between the values of each parameter. Such overlap precludes the direct use of single measurements for diagnosis and prognosis. By comparing biochemical patterns of individuals, in addition to measurements of single variables, the value of biochemical analyses should be enhanced considerably.

Accordingly, fasting patterns of plasma amino acids of alcoholics and controls were investigated in an effort to evaluate the following four facets of the problem: (1) differences between mean levels of single amino acids of alcoholics and controls; (2) differences between the interrelations of individual plasma amino acid levels within each of the two groups; (3) the effects of ethanol loading on the patterns of free amino acids in the plasma of fasting alcoholics and controls; and (4) differences in over-all amino acid patterns of individual alcoholics and controls, and the application of such differences to the construction of a model system of chemically aided diagnosis.

Materials and Methods.—Subjects: The control group consisted of 25 healthy males from local church groups and the university staff. Most members of this group consumed alcohol in moderation, and none was an inordinately heavy drinker. The group of alcoholics consisted of 25 males, 12 of whom were selected from a local chapter of Alcoholics Anonymous. The remaining 13 were drawn from the alcoholic ward of the local state hospital. They were ambulatory patients with a diagnosis of chronic alcoholism and free from complicating pathology. The hospitalized group of alcoholics received some multivitamin supplementation.

Experimental: Fasting blood samples were taken by venipuncture before the subjects had breakfast. Twenty-five milliliters of blood were collected and processed for amino acid analysis by the method of Stein and Moore.[13] The protein-free filtrates were concentrated by lyophilizing rather than by evaporating *in vacuo*. Analyses were made by means of amino acid analyzers,[14] using a 30–60° temperature program rather than the conventional 30–50° temperature change, in order to facilitate the estimation of glucosamine and to improve the resolution of tyrosine and phenylalanine. Ethanol loading effects on the free amino acids in plasma were determined for ten fasting members of the control group, each of whom consumed measured doses of ethanol one hour before blood was collected. From five of these subjects, two additional blood samples were collected at 2-hr intervals. All subjects were intoxicated as judged by behavioral criteria, and all had blood alcohol values greater than 90 mg%. Blood alcohol levels were determined enzymatically by NAD reduction.[15] Blood was collected from each of eight intoxicated alcoholics upon their admission to the state hospital facility. These subjects were also fasting.

Results.—Group differences between fasting levels of single amino acids: Table 1 lists group free amino acid means, ranked by decreasing significance of the intergroup differences. The value for total micromoles of amino acids was obtained by summation of the values of all variables except urea. Because of the individual differences in total free amino acids, the data were re-evaluated as the micromolar contribution of each amino acid to a subject's free amino acid total. In this case

TABLE 1

FREE AMINO ACID PLASMA LEVELS IN FASTING ALCOHOLICS AND CONTROLS*

Amino acids, etc.	Control group mean (N = 25)	Alcoholic group mean (N = 25)	P
Tryptophan†	(2.95)	(1.89)	0.001
Methionine	2.35	1.78	0.001
Glutamic Acid	3.14	4.43	0.01
Leucine	12.77	10.85	0.01
Taurine	4.27	5.36	0.01
Valine	21.77	18.79	0.01
Isoleucine	6.63	5.78	0.02
Citrulline	4.12	3.50	0.05
Urea	402.52	340.56	NS
Ethanolamine	0.57	0.83	NS
Proline	18.23	15.90	NS
Ornithine	5.00	5.68	NS
Histidine	6.54	6.04	NS
Alanine	31.01	28.03	NS
Lysine	14.03	13.08	NS
Phenylalanine	5.32	5.04	NS
Serine	8.30	8.70	NS
Tyrosine	5.12	5.30	NS
Glycine	20.67	19.92	NS
Threonine	11.15	11.47	NS
Cystine/2	8.51	8.38	NS
Glutamine‡	57.68	58.33	NS
α-Aminobutyric acid	1.92	1.89	NS
Glucosamine	0.57	0.57	NS
Arginine	7.13	7.13	NS
Total μmoles	259.8	248.7	NS

* Values are expressed as μmoles/100 ml of plasma.
† The tryptophan values should be considered to be minimal due to losses of this compound during analysis.
‡ Includes about 10% asparagine.

there is a close correspondence of the resulting means with the data of Table 1. However, the group difference of citrulline is no longer significant, while the higher ornithine level in the alcoholic group becomes significant.

Pearson product moment intercorrelation coefficients of plasma amino acid levels within each group: Intercorrelation matrices expressing the correlations between individual amino acid levels for each of the two groups were generated through use of conventional techniques, on a Control Data 1604 computer. Tables of correlation coefficients were thus obtained for both the raw data and for the amino acid levels expressed as the per cent contribution to the free amino acid pool made by each amino acid. Here again, expressing the data in the latter way minimizes the effect of individual differences in total free amino acids and is a more accurate reflection of the mutual relations among amino acids. The obtained values of these correlation coefficients range up to 0.85 for the valine-leucine correlation of alcoholics. A coefficient of 1.0 represents perfect correlation. For the alcoholic group, the level of glutamine had a significant negative correlation ($P < 0.02$) with the levels of valine, leucine, isoleucine, and alanine, and a positive correlation with glycine levels. None of these relationships was significant in the control group.

The effects of ethanol loading on plasma amino acids: As Table 2 indicates, ethanol depresses the total amino acid value for the control group, while no significant alteration takes place in the alcoholic group. Table 3 lists those amino acids in each group, the levels of which are significantly depressed by ethanol loading, while Table 4 lists those amino acids which significantly increase. The magnitude of the ethanol effects is illustrated by the depression of serine levels to mean values of 5.86

TABLE 2

THE EFFECT OF ETHANOL LOADING ON TOTAL PLASMA FREE AMINO ACIDS OF ALCOHOLICS AND CONTROLS*

Group	Basal level	After ethanol loading	P
Control	259.8	220.7	0.01
Alcoholic	248.7	270.2	NS

* Values are expressed as μmoles/100 ml of plasma.

and 5.12 μmoles/100 ml of plasma in controls and alcoholics, respectively. By way of comparison, a recent literature review compiled by Soupart[16] lists a range of 7.16–19.4 μmoles/100 ml of plasma for serine levels in fasting adults; these data were compiled from studies in six laboratories. Individual values for intoxicated subjects ranged down to 3.11 in the present study, while the highest value for serine

TABLE 3

PLASMA AMINO ACID LEVELS DEPRESSED BY ETHANOL*

Amino acids, etc.	Control group mean (N = 10)	P	Alcoholic group mean (N = 8)	P
Serine	5.86†	0.001	5.12†	0.001
Threonine	7.91†	0.001	NS (12.48)	NS
Methionine	1.63	0.001	E (2.85)	E
Leucine	9.51†	0.001	E (16.62)	E
Alanine	21.99†	0.001	NS (28.96)	NS
Lysine	11.04	0.01	NS (12.24)	NS
α-Aminobutyric acid	1.29	0.01	NS (1.89)	NS
Citrulline	3.14	0.01	NS (4.23)	NS
Glycine	16.07	0.01	15.58†	0.02
Valine	17.84†	0.01	E (23.25)	E
Isoleucine	5.25	0.01	E (9.23)	E
Proline	13.67	0.01	E (26.99)	E
Tryptophan	1.87	0.02	NS (2.11)	NS
Glucosamine	NS (0.551)	NS	0.435	0.05

* Levels after ethanol ingestion; values are expressed as μmoles/100 ml of plasma.
† Indicates that this parameter is depressed significantly also when data are expressed as per cent molar contribution to the total free amino acid pool in plasma. Parentheses around a value indicate that there is no significant depression (NS) or else that there is significant elevation (E).

from intoxicated subjects from either group was only 7.72. The ranges of basal values of both groups are in close agreement with the literature values. Serine provides an example of an amino acid, the level of which is affected by alcohol in the same manner in both groups. For many other amino acids, the effect of ethanol in the alcoholic group is opposite to that in the control group, and this difference serves to distinguish the groups from one another. All of the alcoholic and control

TABLE 4

PLASMA AMINO ACID LEVELS ELEVATED BY ETHANOL

Amino acids, etc.	Control group mean (N = 10)	P	Alcoholic group mean (N = 8)	P
Ethanolamine	0.984	0.05	NS (0.707)	NS
Proline	NS (13.67)	NS	26.99†	0.001
Methionine	D (1.63)	D	2.85†	0.01
Isoleucine	D (5.25)	D	9.23†	0.01
Leucine	D (9.51)	D	16.62†	0.01
Glutamine	NS† (61.50)	NS	NS (60.02)	NS

* Levels after ethanol ingestion; values are expressed as μmoles/100 ml of plasma.
† Indicates that this parameter is elevated significantly also when data are expressed as per cent molar contribution to the plasma amino acid total. Parentheses around a value indicate that there is no significant elevation (NS) or else that there is significant depression (D).

subjects who participated in the ethanol loading study could be correctly assigned to their respective nonoverlapping groups by their values for the ratio of proline levels before and after ethanol loading. A similar group-distinguishing effect was shown by the glutamine contribution to the amino acid pool; this value rose significantly in controls, while no significant change was found in the alcoholic group. No simple relations were found between the levels of plasma amino acids and blood alcohol levels.

Studies of urinary excretion of amino acids by these subjects have ruled out explanation of the data presented herein in terms of alterations of renal function.

Discussion.—The following six experimental findings taken together suggest a chemical defect which could contribute to the etiology of alcoholism:

(1) Alcoholics displayed elevated basal levels of glutamic acid, and depressed basal levels of methionine, leucine, valine, and isoleucine.

(2) No difference was found between basal levels of proline or glutamine in alcoholics and controls.

(3) Significant negative correlation was found between the glutamine content of the free amino acid pool in the plasma of alcoholics and the content of leucine, isoleucine, valine, and alanine. These relations in the control group are not significant.

(4) Elevation of the glutamine contribution to the free amino acid pool in plasma took place in controls following ethanol loading. There is no change in this parameter in the alcoholic group.

(5) Elevation of proline, methionine, isoleucine, and leucine levels within the plasma amino acid pool of the alcoholic group following ethanol loading was found. No such increases took place in the control group.

(6) Decreased content of serine, glycine, and glucosamine in the alcoholic amino acid pool followed ethanol loading. Serine and glycine, but not glucosamine, also decrease in the control group.

Ravel[17] had previously shown that glutamine protects *S. faecalis* R from ethanol poisoning, and Rogers[18] has reported that the level of alcohol voluntarily consumed by laboratory animals is decreased by the administration of glutamine. Preliminary clinical trial results[19–21] indicate that glutamine may also reduce the craving for alcohol of chronic alcoholics. It seems interesting, in view of these protective effects against alcohol that glutamine affords, that ethanol administration is followed by a glutamine increase only in the control group. An involvement of glutamine in alcoholism is further indicated when our results are considered in the light of findings of Meister on glutamine synthesis. Reports from his laboratory[22] indicate that the keto acids derived from alanine, valine, leucine, phenylalanine, tyrosine, and methionine can participate in transamination reactions with glutamine. The reverse reactions would result in the synthesis of glutamine from any of the above amino acids and α-ketoglutaramate, the keto acid derived from glutamine. Although Snell[23] has shown that glutamine can be synthesized nonenzymatically from α-ketoglutaramate, via vitamin B6-catalyzed transamination, this reaction is difficult to demonstrate *in vivo*, because ω-amidase activity is normally high enough to cause rapid destruction of α-ketoglutaramate. Meister[24] also has shown that ω-amidase deamidation of α-ketoglutaramate is markedly decreased when appreciable transamination occurs with pyruvate, phenylpyruvate, or α-ketovalerate.

In addition, it may be noted that brain levels of ω-amidase are only a third of those in liver, and it is in brain where a metabolic defect in alcoholics would be expected to exert its greatest influence.

Of several possible explanations of these findings, we have adopted the following as a working hypothesis to aid in the planning of definitive experiments: that a block in the glutamine synthetase system exists in alcoholics, and that they derive a significant portion of their endogenous glutamine by another metabolic pathway. A possible precursor of glutamine in alcoholics is α-ketoglutaramate, the keto acid analogue of glutamine. Transamination of this compound with the amino acids shown above to be statistically related yields glutamine and would be expected to increase the vitamin B6 requirements of alcoholics. One alternate possibility, an ω-amidase block, seems ruled out by the accumulation of high levels of proline in

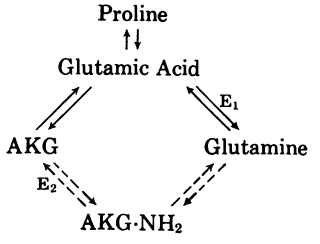

FIG. 1.—Conventional (→) and postulated (---->) routes of glutamine biogenesis. The abbreviations AKG and AKG·NH_2 represent α-ketoglutarate and α-ketoglutaramate, while E_1 and E_2 represent the enzymes glutamine synthetase and ω-amidase.

the plasma of ethanol-loaded alcoholics, who show no corollary increases of plasma glutamic acid and glutamine. The metabolic pathways involved are illustrated in Figure 1.

This hypothesis would account for the elevated basal levels of glutamic acid in terms of a block in its conversion to glutamine, while the lowered levels of methionine, leucine, and valine are consonant with their utilization as transamination precursors of glutamine. Since the keto acids formed from the transamination of isoleucine and leucine are ketogenic, their increase in alcoholics following ethanol loading should result in increased lipogenesis in this group. Elko[25] has reported that intoxicating doses of alcohol increase liver fat without increasing fat mobilization from depots or free fatty acids in blood. The increase in proline with no increase in glutamic acid, observed in the alcoholic group, may be considered in terms of the enzyme systems responsible for their interconversion. A mitochondrial enzyme, proline oxidase, converts proline to pyrolline carboxylic acid, the immediate precursor of glutamic acid. This system should be less active as a result of ethanol-induced respiratory depression by NADH.[26] The microsomal enzyme system responsible for the conversion of pyrolline carboxylic acid to proline has an NADH requirement and would not suffer from the effects of respiratory depression. If the glutamine synthetase pathway is blocked, the predicted net result of ethanol loading on the glutamic acid-proline enzyme systems would be an increase in proline at the expense of glutamic acid or precursors of glutamic acid. The depression of respiration may also be responsible for the ethanol-induced decrease in serine and

glycine levels. Inhibition of glycolysis would act to impair the *de novo* synthesis of serine.

An investigation of over-all free amino acid patterns in the plasma of individual alcoholics and controls by means of multiple discriminant analysis techniques will be discussed in a further publication of this series.[27]

We wish to acknowledge our deep appreciation to Professor Roger J. Williams for his inspiration, support, and encouragement. We are grateful for the cooperation of the superintendent and staff of the Austin State Hospital and for the expert technical assistance of J. M. Patterson and W. B. DeVille. Our thanks are also due to the staff of the Computation Center of the University of Texas for their cooperation and the use of their facilities.

* Requests for reprints should be directed to Dr. Siegel, whose present address is: Joseph P. Kennedy, Jr., Laboratory, Department of Pediatrics, University of Wisconsin Medical School, Madison 6, Wisconsin.

[1] Williams, R. J., R. B. Pelton, H. M. Hakkinen, and L. L. Rogers, these PROCEEDINGS, **44**, 216 (1958); and previous reports from this laboratory.

[2] Williams, R. J., these PROCEEDINGS, **47**, 221 (1961).

[3] Siegel, F. L., M. K. Roach, and W. B. DeVille, *Fed. Proc.*, **22**, 680 (1963).

[4] Olson, R. E., D. Gursey, and J. W. Vester, *New Engl. J. Med.*, **263**, 1169 (1960).

[5] Kissen, B., V. Schenker, and A. Schenker, *Quart. J. Studies Alc.*, **20**, 480 (1959).

[6] Pluvinage, R., and R. Lelns, *Bull. Mem. Soc. Med. Hop. Paris*, **113**, 1030 (1951).

[7] Goodhart, R. S., *Am. J. Clin. Nutr.*, **5**, 612 (1957).

[8] Smith, W. O., and J. F. Hammarsten, *Am. J. Med. Sci.*, **237**, 413 (1959).

[9] Vallee, B. L., W. E. C. Wacker, A. F. Bartholomay, and F. L. Hoch, *Ann. Internal Med.*, **50**, 1077 (1959).

[10] Reed, J. G., *Texas Univ. Publ., Biochem. Inst. Stud.*, **4**, 139 (1951).

[11] McClearn, E. G., and D. A. Rodgers, *Quart. J. Studies Alc.*, **20**, 691 (1959).

[12] Williams, R. J., in *Biochemical Individuality* (New York: John Wiley, 1959), pp. 183–195.

[13] Stein, W. H., and S. Moore, *J. Biol. Chem.*, **211**, 915 (1954).

[14] Spackman, D. H., W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

[15] Bucher, T., and H. Redetzki, *Klin. Wochschr.*, **29**, 615 (1951).

[16] Soupart, P. in *Amino Acid Pools* (New York: Elsevier, 1962), p. 233.

[17] Ravel, L. M., B. Felsing, E. M. Lansford, Jr., R. H. Trubey, and W. Shive, *J. Biol. Chem.*, **214**, 497 (1955).

[18] Rogers, L. L., R. B. Pelton, and R. J. Williams, *J. Biol. Chem.*, **214**, 503 (1955).

[19] Fincle, L. P., *Bedford Res.*, (Vet. Adm. Hosp.), **7**, 2 (1961).

[20] Rogers, L. L., and R. B. Pelton, *Quart. J. Studies Alc.*, **18**, 581 (1957).

[21] Trunnell, J. B., and J. Wheeler, presented at Southwest Regional Meeting, American Chemical Society, 1955.

[22] Meister, A., *J. Biol. Chem.*, **210**, 17 (1954).

[23] Metzler, D., and E. E. Snell, *J. Am. Chem. Soc.*, **74**, 979 (1952).

[24] Meister, A., *J. Biol. Chem.*, **200**, 571 (1953).

[25] Elko, E. E., E. R. Wooleo, and N. R. De Luzio, *Am. J. Physiol.*, **201**, 923 (1961).

[26] Peisach, J., and H. J. Strecker, *J. Biol. Chem.*, **237**, 2255 (1962).

[27] Siegel, F. L., L. R. Pomeroy, and M. K. Roach, these PROCEEDINGS, in press.