mixture melted at 112–114°. A mixture melting point with the derivative from a known sample of 1,5-diphenyl-1-pentene, m.p. 113.5–115°, came at 113–114.5°.

The *trans*-1-amino-2,6-diphenylpiperidine can be isomerized to the *cis* isomer in 15% yield with lithium aluminum hydride in refluxing ether for 24 hours. Similarly, reduction of the *trans*-1-nitroso-2,6-diphenylpiperidine with lithium aluminum hydride in ether for 12 hours gave 18% of the *cis* amino compound.

It would appear that this novel oxidation is a useful one for specific types of ring closures in cyclic systems and represents a new type of elimination process. Other examples and modifications of this new reaction and its mechanism will be described in more detail at a later date.

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RECEIVED FEBRUARY 6, 1957

THE SUB-FRACTIONATION OF HUMAN GAMMA-GLOBULIN IN A CONTINUOUSLY DEVELOPING *p*H GRADIENT[1]

Sir:

Human gamma globulins migrate homogeneously in an electrophoretic field. However, other physico-chemical fractionation techniques including ultracentrifugation, convection-electrophoresis and low temperature-ethanol fractionation have established the heterogeneity of this material. Thus Cann and Kirkwood[2] utilized differences of isoelectric points of gamma globulins and afforded separation by means of electrophoresis-convection.

Kolin's technique[3] which utilizes a combination of *p*H and conductivity gradients has been used for the separation and identification of human hemoglobins.[4] The technique described by Kolin, however, proved unsatisfactory for the separation of gamma globulins.

In the present study, a rapid method for the preparative fractionation of human gamma globulins has been developed. A very even gradient with narrow limits of *p*H but with considerable spread was produced by the action of an electric potential on a weak buffer. Empirically, the following system afforded good separation: negative electrode, low density buffer (*p*H 3.5), high density buffer (*p*H 4.5), low density buffer (*p*H 4.0), positive electrode.

The high density buffer (*p*H 4.5) was introduced in the bottom of the U-tube of a Kolin isoelectrophoresis cell. The low density buffer of *p*H 3.5 was layered on one side in contact with the negative electrode, while the second low density buffer of *p*H 4.0 was layered on the other side in contact with the positive electrode. Then 0.15 ml. of the globulin solution[5] was introduced via a tuberculin syringe through an inlet in the bottom of the U-tube into the high density buffer, coming to rest between the high density buffer (*p*H 4.5) and low density buffer (*p*H 4.0). A potential of 200 volts between the electrodes was applied for 10 minutes creating an expanded *p*H gradient. The protein solution showing only faint signs of heterogeneity was removed by micro-pipet. Gamma-globulin solution (0.15 ml.) was introduced and again exposed to the same potential gradient of 200 volts. The preformed *p*H gradient in the system continued to develop further and the top fraction (I) separated very clearly within a few minutes, being removed after nine minutes by micro-pipet. Fraction I contained 76 μg. of protein. Seven minutes later a second band (II) separated clearly and this contained 47 μg. of protein. After seven more minutes fractions III and IV became clearly defined; these contained 25 and 121 μg. of protein respectively. Average nitrogen values were derived from over 40 experiments on the basis of pooled samples of 2 to 3 runs each.

Figure 1 illustrates the extent of separation obtained with the system employed. The center

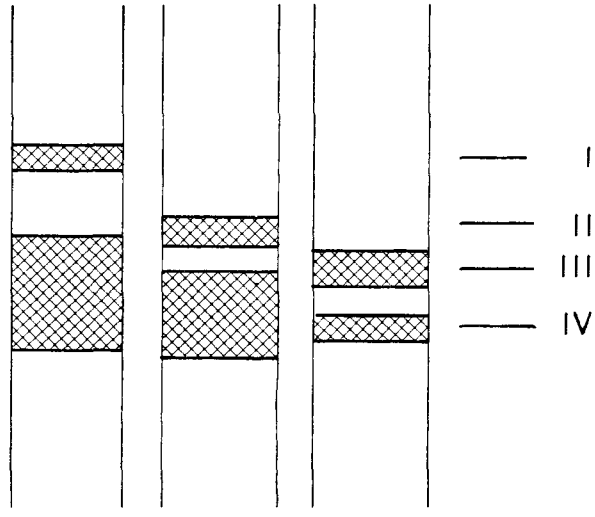

Fig. 1.

part of the isoelectrophoresis cell is photographed. The globulin fractions are made visible by dark-field illumination. The picture on the left shows the separation of fraction I. In the middle picture separation of fraction II can be seen, and in the picture on the right separation of fractions III and IV is depicted.

Detailed data on the individual fractions will be reported separately.

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RECEIVED DECEMBER 7, 1956

FURTHER INTERMEDIATES IN THE BIOSYNTHESIS OF INOSINIC ACID *de novo*[1]

Sir:

A previous report[2] has described the isolation and characterization of a new ribotide which is an

(1) This investigation was supported by a grant from the USPHS, National Heart Institute, H 2271.

(2) J. R. Cann and J. G. Kirkwood, *Cold Spring Harbor Symposia, Quant. Biol.*, **14**, 9 (1950).

(3) A. Kolin, *J. Chem. Phys.*, **23**, 407 (1955).

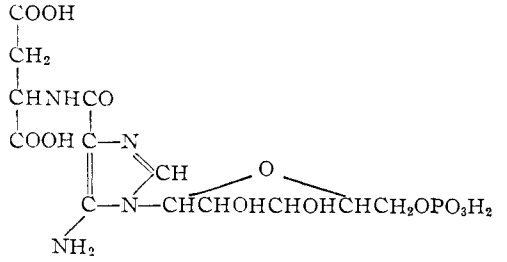
(4) A. H. Tuttle, *J. Lab. Clin. Med.*, **47**, 811 (1956).

(5) Squibb Poliomyelitis Immune Globulin, Human, Lot 305-1.

(1) This work has been supported by grants-in-aid from the National Cancer Institute, National Institutes of Health, United States Public Health Service, and the National Science Foundation.

(2) L. N. Lukens and J. M. Buchanan, *Federation Proc.*, **15**, 305 (1956).

intermediate in the *de novo* synthesis of inosinic acid by enzymes of avian liver. On the basis of the evidence presented, the structure of this ribotide was formulated as 5-amino-4-imidazole-N-succinocarboxamide ribotide.



5-Amino-4-imidazole-N-succinocarboxamide ribotide

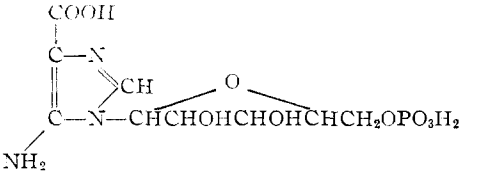
Its formation from AIR[3] and conversion to AICAR are described by reactions 1 and 2

$$\text{AIR} + CO_2 + \text{ATP} + \text{Aspartic Acid} \xrightarrow[\longleftarrow]{\text{enzyme fraction I}} \text{SAICAR} + \text{ADP} + P_i \quad (1)$$

$$\text{SAICAR} \xrightleftharpoons[\longleftarrow]{\text{enzyme II}} \text{AICAR} + \text{Fumaric Acid}^4 \quad (2)$$

Enzyme fraction I, which catalyzes reaction 1 reversibly, has been partially purified from extracts of pigeon or chicken liver. The purification and properties of enzyme II are described in the following communication.[4] Either enzyme fraction is devoid of activity in the reaction catalyzed by the other. Although it is likely that enzyme fraction I contains at least two enzymes, no attempts have yet been made to separate them. The assays for both enzyme fraction I and enzyme II employ the Bratton–Marshall[5,6] test for arylamines in which both AIR and AICAR produce colored compounds while SAICAR does not.[7] As shown in equation 1, one mole of ATP is broken down to ADP and inorganic phosphate for every mole of SAICAR which is synthesized. ATP is much more effective at equimolar levels than the guanosine, inosine, cytidine or uridine triphosphates.

It has now been found that a new ribotide is formed when AIR is incubated with enzyme fraction I and bicarbonate. This ribotide, on the basis of the evidence described below, is believed to be 5-amino-4-imidazolecarboxylic acid ribotide.



5-Amino-4-imidazolecarboxylic acid ribotide

Its formation and conversion to SAICAR are described by equations 3 and 4.

$$\text{AIR} + CO_2 \rightleftharpoons \text{C-AIR} \quad (3)$$

$$\text{C-AIR} + \text{ATP} + \text{Aspartic Acid} \rightleftharpoons \text{SAICAR} + \text{ADP} + P_i \quad (4)$$

The over-all reaction given by equation (1) is the sum of reactions (3) and (4).

Reaction 3 was first detected as a result of the observation that, when AIR, bicarbonate and enzyme fraction I were incubated together, the reaction mixture gave a red color in the Bratton–Marshall test rather than the orange color produced by pure AIR.[6] Subsequent isolation of C-AIR showed that it reacts in the Bratton–Marshall test to give a purple product with an absorption maximum at 519 mμ. Since, in contrast to AIR, C-AIR has a specific absorption band in the ultraviolet region, reaction 3 may be followed quantitatively by observing the increase in optical density at 265 mμ. A complete dependence of this reaction on added bicarbonate can be demonstrated. The best yields of C-AIR, around 50% based on AIR, are realized when the concentration of bicarbonate is relatively high (0.3 *M*). The presence in C-AIR of the carbon atom from the added bicarbonate was confirmed by isolating radioactive C-AIR after incubation of unlabeled AIR and C^{14}-bicarbonate in the presence of enzyme. When this radioactive C-AIR was incubated with enzyme fraction I in the absence of bicarbonate, it was quantitatively converted to unlabeled AIR.

In Table I evidence is presented for the occur-

TABLE I

CONVERSION OF C-AIR TO AICAR IN ABSENCE OF CARBON DIOXIDE

All vessels contained: Mg acetate, 15 μmoles; K acetate 20 μmoles; potassium L-aspartate, 1 μmole; disodium salt of ATP, 0.15 μmole; Tris chloride buffer, *p*H 8.4, 40 μmoles; enzyme fraction I, 0.4 mg.; enzyme II, 12 mg. Where indicated the following compounds were included: C-AIR 0.15 μmole; AIR, 0.07 μmole and $KHCO_3$, 20 μmoles. The total volume was 0.55 ml. The vessels were incubated for 5 minutes at 38° and the reaction terminated by the addition of 0.1 ml. of 30% trichloroacetic acid. After removal of the precipitated protein by centrifugation, a 0.40-ml. aliquot was removed from each vessel to a separate tube, to which was added 0.10 ml. of 5 *N* H_2SO_4. The tubes were capped with glass marbles, placed in a boiling water-bath for 15 minutes, then cooled to room temperature and the Bratton–Marshall reagents were added as described elsewhere.[6]

Additions	AICAR formed, mμmoles
C-AIR	18.0
C-AIR + $KHCO_3$	18.5
AIR	3.4
AIR + $KHCO_3$	14.0

(3) These abbreviations, trivial names and the systematic names of four intermediates of inosinic acid biosynthesis are used: AIR, 5-aminoimidazole ribotide, 5-amino-1-β-D-(5'-phosphoribosyl)-imidazole; C-AIR, 5-amino-4-imidazolecarboxylic acid ribotide, 5-amino-1-β-D-(5'-phosphoribosyl)-4-imidazolecarboxylic acid; SAICAR, 5-amino-4-imidazole-N-succinocarboxamide ribotide; N-(5-amino-1-β-D-(5'-phosphoribosyl)-4-imidazolecarbonyl)-L-aspartic acid; AICAR, 5-amino-4-imidazolecarboxamide ribotide, 5-amino-1-β-D-(5'-phosphoribosyl)-4-imidazolecarboxamide. The compounds are named as having a β configuration since they are precursors of inosinic acid, which is known to be of the β form. Abbreviations for other compounds are: ATP, adenosine triphosphate; ADP, adenosine diphosphate; P_i, inorganic phosphate, and Tris, tris-(hydroxymethyl)-aminomethane.

(4) R. W. Miller, L. N. Lukens and J. M. Buchanan, THIS JOURNAL, **79**, 1513 (1957).

(5) A. C. Bratton and E. K. Marshall, Jr., *J. Biol. Chem.*, **128**, 537 (1939).

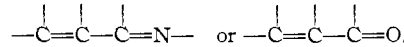
(6) B. Levenberg and J. M. Buchanan, *ibid.*, **224**, 1005 (1957).

(7) If the Bratton–Marshall procedure is modified by keeping the sample in ice during and for ten minutes after the addition of the reagents, SAICAR produces a purple chromophore which absorbs maximally at 550 mμ. The diazonium salt of SAICAR is very unstable and decomposes before it can couple under the usual test conditions. We are indebted to Dr. Joseph Gots for this explanation of the previously puzzling failure of SAICAR to give a color in this test.

rence of reaction 4. As expected, the conversion of C-AIR, isolated as described below, to SAICAR (and AICAR) does not require bicarbonate, whereas bicarbonate is required for the conversion of AIR to SAICAR. Excess enzyme II was added so that any SAICAR formed was converted to AICAR according to reaction (2). The AICAR formed was measured by the Bratton–Marshall procedure after quantitatively destroying AIR and C-AIR by heating in 1 N H_2SO_4 for 15 minutes at 100°.

C-AIR is rapidly decarboxylated by heat or acid to yield AIR. Its isolation was therefore performed at 3° and exposure to acid was kept at a minimum. The deproteinized reaction mixture at *p*H 9 was placed on a Dowex 1 acetate column from which the residual AIR was rapidly eluted under pressure with 0.04 M ammonium acetate buffer, *p*H 5.1. A solution of 0.01 M Tris bromide *p*H 9 was then passed through the column with pressure until the eluate had reached a *p*H of 8 to 9. C-AIR was obtained by a gradient elution in which a solution containing 0.2 M potassium acetate and 0.01 M Tris bromide, *p*H 9, dropped into a mixing vessel containing 400 ml. of 0.01 M Tris bromide, *p*H 9. The ribotide, after concentration, was precipitated from an 85% ethanol solution as the barium salt.

The structure of C-AIR was first suggested by the finding that only two substrates, AIR and bicarbonate, were required for its formation. The conversion of C-AIR to SAICAR by reaction 4, and the known incorporation of bicarbonate into position 6 of inosinic acid, made highly unlikely any position of attachment of the bicarbonate to the imidazole nucleus other than that shown. In addition, the possession of a specific ultraviolet absorption band by C-AIR with λ_{max} at 249 mμ at *p*H 8.5, provides physical evidence for the proposed structure of C-AIR, since it has been shown that the ultraviolet absorption of purines and related compounds is due to chromophoric systems of the type



Further evidence of the structure of C-AIR comes from the report of Rabinowitz[9] that the aglycone, 5-amino-4-imidazolecarboxylic acid, decomposes similarly in acid solution to form 5-aminoimidazole.

(8) L. F. Cavalieri, A. Bendich, J. F. Tinker and G. B. Brown, THIS JOURNAL, **70**, 3875 (1948).

(9) J. C. Rabinowitz, *J. Biol. Chem.*, **218**, 175 (1956).

(10) Predoctoral Fellow of the National Science Foundation.

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RECEIVED FEBRUARY 16, 1957

THE ENZYMATIC CLEAVAGE OF 5-AMINO-4-IMIDAZOLE-N-SUCCINOCARBOXAMIDE RIBOTIDE[1]

Sir:

The enzymatic conversion of 5-amino-4-imidazole-N-succinocarboxamide ribotide (SAICAR) to 5-amino-4-imidazolecarboxamide ribotide (AICAR) and to a mixture of fumaric and malic acids previously has been reported as a step in the *de novo* synthesis of inosinic acid.[2] It has now been found through purification of this enzyme that fumaric acid is the initial product of the cleavage of SAICAR and that this enzyme is in all probability identical with adenylosuccinase described by Carter and Cohen.[3]

The enzyme was obtained free from fumarase from extracts of pigeon or chicken liver by a procedure including ethanol precipitation, negative gel absorption on Alumina Cγ, and differential heat inactivation. Other sources of the enzyme are baker's yeast, *Escherichia coli*, *Salmonella typhimurium* and *Neurospora crassa*. The equilibrium constant of the splitting reaction determined with an enzyme purified 35-fold from baker's yeast[3] is approximately 7×10^{-3} mole per liter. The Michaelis–Menten constant for SAICAR is 1.9×10^{-4} mole per liter.

Because of the similarity of this reaction to the cleavage of adenylosuccinic acid[4] (AMP-S) to adenylic acid and fumaric acid and to the cleavage of argininosuccinic acid[4] to arginine and fumaric acid, an investigation of the specificity of the SAICAR splitting enzyme was undertaken. The splitting of SAICAR was inhibited 50% in the presence of an equimolar concentration of AMP-S but was not inhibited by a 6-fold excess of argininosuccinic acid. While preparations of the SAICAR splitting enzyme split AMP-S, no evidence could be obtained for the cleavage of argininosuccinic acid. During purification from yeast, the ratio of SAICAR splitting activity to adenylosuccinase activity remained constant within 5%. Both AMP-S and SAICAR were found to protect the SAICAR splitting enzyme from denaturation during heating steps in the purification procedure.

Additional evidence concerning the identity of adenylosuccinase and the SAICAR splitting enzyme was obtained from three adenine-requiring (F group) mutants of *Neurospora*. Mycelial extracts of these mutants have been shown to lack adenylosuccinase.[5] We have now shown that these mutants lack the ability to split SAICAR, while the wild strain (74A) is active in splitting both of the succino compounds. Partridge and Giles[6] have recently demonstrated that two revertants of an "F" mutant regain the same per cent. of the lost enzymatic activity whether assayed for adenylosuccinase or the SAICAR splitting enzyme. These facts may indicate that the loss of both activities is the result of a single genetic event. The indication that a single mutation exerts the same effect on two enzymatic activities makes it probable that only one enzyme is involved.

(1) This work is supported by grants-in-aid from the National Cancer Institute, National Institutes of Health, United States Public Health Service and the National Science Foundation. For the proper systematic names of intermediates of purine biosynthesis see the accompanying communication, L. N. Lukens and J. M. Buchanan, THIS JOURNAL, **79**, 1512 (1957).

(2) L. N. Lukens and J. M. Buchanan, *Federation Proc.*, **15**, 305 (1956).

(3) C. E. Carter and L. H. Cohen, *J. Biol. Chem.*, **222**, 17 (1956). We wish to thank Dr. Carter for a sample of adenylosuccinate.

(4) S. Ratner and B. Petrack, *ibid.*, **200**, 175 (1953). We wish to thank Dr. Ratner for a sample of argininosuccinate.

(5) C. W. H. Partridge and N. H. Giles, *Arch. Biochem. and Biophys.*, in press.

(6) N. H. Giles and C. W. H. Partridge, personal communication. We wish to thank these investigators for samples of their mutant strains of *Neurospora*.