198. *Purines, Pyrimidines, and Imidazoles. Part XIX.*[1] *A Synthesis of* N-(5-*Amino*-1-β-D-*ribofuranosylimidazole*-4-*carbonyl*)-L-*aspartic Acid* 5′-*Phosphate.*

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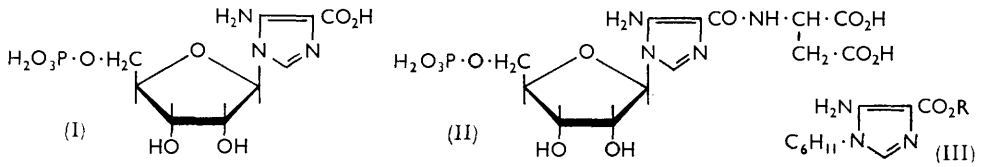
N-(5-Amino-1-β-D-ribofuranosylimidazole-4-carbonyl)-L-aspartic acid 5′-phosphate (succino-AICAR) has been synthesised from methyl 5-amino-1-(2,3-*O*-isopropylidene-β-D-ribofuranosyl)imidazole-4-carboxylate by a series of hydrolysis, phosphorylation, and coupling reactions. The unusual instability of the diazonium salt of succino-AICAR has been examined and the results discussed and compared with results obtained with some synthetic models.

IN the biosynthesis of purine nucleotides *de novo* the 1-nitrogen atom is derived from L-aspartic acid although there is no parallel contribution of the carbon skeleton of the amino-acid to the ring system.[2] The mechanism of the incorporation of nitrogen involves acylation of L-aspartic acid by the aminoimidazolecarboxylic acid ribotide (I), which we have recently synthesised,[1,3] in the presence of adenosine triphosphate, magnesium ions, and an enzyme fraction of avian liver to give the imidazole nucleotide peptide (II) (succino-AICAR).[4] The last compound is split in the presence of an enzyme (adenylosuccinase)* (found in avian, beef, or human liver, *Escherichia coli*, *Neurospora crassa*, *Salmonella typhimurium*, and baker's yeast) to the amide of acid (I) (AICAR) with elimination of fumaric acid,[5] and this reversible step appears to operate by a *trans*-mechanism similar to that involved in the stereospecific aspartase-controlled deamination of L-aspartic acid.[6] Succino-AICAR (II) and the corresponding dephosphorylated nucleotide also accumulate, in varying proportions, in cultures of ten out of thirteen adenine-requiring mutants of *E. coli* and *S. typhimurium* examined.[7,8] This has been attributed to mutational loss of the cleaving enzyme adenylosuccinase, which is seen to be bifunctional since, in addition to catalysing the cleavage of the acid (II) to the amide, it is also concerned in the conversion of adenylosuccinic acid into adenylic acid, and so provides the unusual case of a double block in a common biosynthetic pathway. The accumulation of succino-AICAR is also unusual in that metabolic lesions in systems involving phosphorylated intermediates normally lead to the accumulation of aglycones or dephosphorylated nucleotides.[8]

Owing to the importance of these intermediates, we have synthesised succino-AICAR (II) by an unambiguous method [9] which might also be applied to analogues.

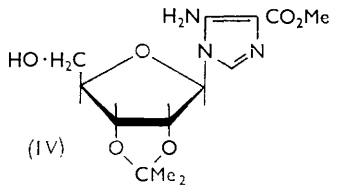


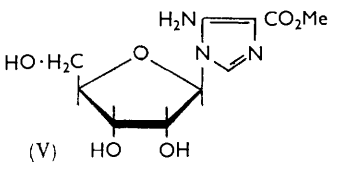
In preliminary experiments on formation of amide bonds of the type present in the acid (II), the ester [1] (III; R = Et) and the ribosyl derivative [1] (V) reacted only slowly

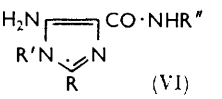
* The new recommended name of this enzyme is " Adenylosuccinate AMP-lyase No. 4.3.2.2."

[1] Part XVIII, Shaw and Wilson, *J.*, 1962, 2937.
[2] Buchanan and Hartman, *Adv. Enzymol.*, 1959, **21**, 199; Hartman and Buchanan, *Ann. Rev. Biochem.*, 1959, **28**, 365.
[3] Shaw and Wilson, *Proc. Chem. Soc.*, 1961, 381.
[4] Lukens and Buchanan, *J. Biol. Chem.*, 1959, **234**, 1791.
[5] Miller, Lukens, and Buchanan, *J. Biol. Chem.*, 1959, **234**, 1806.
[6] Miller and Buchanan, *J. Biol. Chem.*, 1962, **237**, 491.
[7] Gots and Gollub, *Proc. Nat. Acad. Sci. U.S.A.*, 1957, **43**, 826.
[8] Gollub and Gots, *J. Bacteriol.*, 1959, **78**, 320.
[9] Cf. Shaw and Wilson, *Proc. Chem. Soc.*, 1962, 115.

with ammonia at high temperatures. However, reaction of the sodium salt (III; R = Na) with chloroacetonitrile in dimethylformamide gave an excellent yield of the cyanomethyl ester (III; R = CH_2·CN). " Active " esters of this type have been used in many peptide syntheses [10] but this particular compound proved little more reactive than the ethyl ester and only at the boiling point of cyclohexylamine did it give the cyclohexylamide (VI; R = H, R′ = R″ = C_6H_{11}). A similar high-temperature reaction of the cyanomethyl ester with dimethyl L-aspartate gave no useful results. However, reaction of the sodium salt (III; R = Na) with ethyl chloroformate in dimethylformamide and subsequent condensation of the product, presumably a mixed anhydride, with benzylamine gave the benzylamide (VI; R = H, R′ = C_6H_{11}, R″ = Ph·CH_2). The last compound was similarly obtained when a solution of the acid (III; R = H) in pyridine was treated with dicyclohexylcarbodi-imide and benzylamine at room temperature, and this method was eventually chosen as the basis for part of the synthesis of the amide-acid (II).







The isopropylidene ester (IV) was hydrolysed with aqueous-alcoholic sodium hydroxide under conditions which did not cause decomposition of model compounds and has been used [1] for the preparation of the acid (I). The sodium salt obtained was converted into a pyridine salt by passage through an exchange resin in the pyridine form, and a solution of the pyridine salt in dry pyridine was treated at room temperature successively with dicyclohexylcarbodi-imide and dimethyl L-aspartate; the resulting peptide was treated, without isolation or further purification, with 2-cyanoethyl phosphate [11] and a further quantity of the carbodi-imide. The product was heated with acetic acid to eliminate the isopropylidene group, and then kept at 100° with 0·5N-lithium hydroxide to remove the cyanoethyl and ester groups, and to hydrolyse any unchanged cyanoethyl phosphate. This gave the acid (II) which was purified by chromatography on Amberlite CG-400 resin (Br^- form) in a manner analogous to that used for the isolation of the naturally occurring material; [4] this gave the barium salt in an overall yield (estimated spectroscopically) of about 15% from the ester (IV).

The identity of the synthetic material was confirmed (i) by vigorous hydrolysis to glycine and aspartic acid,(ii) by comparison of ultraviolet absorption spectra at various pH values with values given for the natural material and for some synthetic analogues (see Table 1) where excellent agreement is obtained, (iii) by instability of the diazonium salt in the Bratton–Marshall test [12] for arylamines, (iv) by the absorption spectrum of the dye produced in this reaction, and (v) by paper-chromatographic comparison with an authentic specimen (the two materials formed identical running spots which became intensely pink, especially on storage; a similar pink colour has been mentioned [8] for both the ribotide and the corresponding riboside). In addition, our synthetic material in a phosphate buffer in the presence of an adenylosuccinase preparation from *Salmonella* was converted, in a yield very similar to that recorded for the natural material,[7] into the amide of acid (I) which was identified spectroscopically. This similar behaviour suggests that the synthetic aspartate has undergone little or no racemisation. At the same time we have been informed by Dr. Joseph S. Gots, University of Pennsylvania, that a sample of our synthetic ribotide worked well with more highly purified enzyme isolates.

[10] Schwyzer, Iselin, and Feurer, *Helv. Chim. Acta*, 1955, **38**, 69, 83; Schwyzer, Iselin, Feurer, and Kagi, *ibid.*, p. 80; Iselin, Feurer, and Schwyzer, *ibid.*, p. 1508; Albertson, *Org. Reactions*, 1962, **12**, 157.
[11] Tener, *J. Amer. Chem. Soc.*, 1961, **83**, 159.
[12] Bratton and Marshall, *J. Biol. Chem.*, 1939, **128**, 537.

TABLE 1.

Absorption spectra (λ in mμ) of synthetic and natural succino-AICAR and some related model compounds.*

Compound	pH	$\lambda_{max.}$ (1)	$\varepsilon_{max.}$ †	$\lambda_{max.}$ (2)	$\lambda_{min.}$	Ratios of optical densities 250/260	260/280	Bratton–Marshall, $\lambda_{max.}$	Ratio of optical densities 540/600
(II) (note 1)	1	269—270	11,300	244	254—255	0·99	1·12	555—560	1·32
	7	269	—	—	—	0·89	1·22		
	7·8 ‡	269	13,800	—	—	0·7	1·13		
II) (note 2)	2	269·5	—	—	—	0·9	1·05	560	1·18
	7	268	—	—	—	0·8	1·25		
(II) (note 3)	1	269—270	11,000	243	253	0·91	1·06	560	1·31
	8	269—270	13,300	—	—	0·75	1·2		
(VI; R = R′ = R″ = Me)	1	264—265	10,600	245 §	—	—	—		
	8	267—268	11,300	—	—	—	—		
VI; R = Me, R′ = C_6H_{11}, R″ = H	1	267	10,000	245 §	—	—	—		
	8	270—271	10,350	—	—	—	—		

* Measurements were made with a Unicam S.P. 500 spectrophotometer. † Values for the ribotides are based on the results of phosphorus analysis. ‡ "Tris" chloride buffer. § Shoulder.

(1) Synthetic material described in this paper. The substance gave a fading purplish colour in the modified Pauly imidazole test (Koessler and Hanke, *J. Biol. Chem.*, 1919, **39**, 497). A similar colour is reported for the natural material.[7] (2) Natural material isolated from a mutant strain B 97 of *Escherichia Coli*.[7] (3) Natural material prepared enzymically.[4]

The β-configuration originally assigned to succino-AICAR is now confirmed since we have shown [1] that the ester (IV) has a β-configuration at the glycosidic link by conversion of its immediate precursor (V) into inosine.

A characteristic property of succino-AICAR, not shared by related aminoimidazoles, is the instability of its diazonium salt;[4,7] so much so that when the Bratton–Marshall assay is carried out under the usual conditions at room temperature and with a coupling delay of about 5 min. no colour is obtained. To obtain a reasonable intensity of colour, diazotisation and coupling must be carried out rapidly and at low temperature. The instability of the diazonium salt is even more surprising when compared with the diazonium salt of the amide (VI; R = R′ = R″ = H) which is reported to have been kept in the solid state at room temperature for $2\frac{1}{2}$ years without decomposition though it cyclises readily in aqueous ammonia to the aza-analogue (IX; R = R′ = R″ = H) of hypoxanthine.[13] The stability of the latter salt has been ascribed [13] to the formation, by incorporation of the various canonical forms into the π-electron system of the ring, of an internal diazonium salt (VII) in which the ring system serves as the anionic component. Such a highly stabilised form is not of course possible with a 1-substituted imidazole where there is no ionisable ring-hydrogen atom. It seemed most likely, however, that diazotisation of succino-AICAR would lead eventually to the purine aza-derivative (VIII) and in order to test this we have followed spectral changes occurring during decay of the diazonium salt and compared the final stable spectra with absorption spectra of the purine aza-derivatives (IX; R = R′ = R″ = Me; and R = Me, R′ = C_6H_{11}, R″ = H) at different pH values. These derivatives were prepared by reaction of the amides (VI; R = R′ = R″ = Me; and R = Me, R′ = C_6H_{11}, R″ = H) with nitrous acid and ammonia, and their structures were confirmed by analysis and by absence of coupling ability and of infrared bands in the 2300—2000 cm.$^{-1}$ region characteristic of the diazonium ion. The spectral results are recorded in Table 2. After about 30 min. the absorption spectrum of diazotised succino-AICAR in acid and alkaline solution is largely unchanged and in this sense very similar to the aza-derivative (IX; R = R′ = R″ = Me) which it closely resembles, whereas the aza-derivative (IX; R = Me, R′ = C_6H_{11}, R″ = H), which shows similar absorption in acid and neutral solution, has two-peaked absorption in alkaline solution presumably

[13] Shealy, Struck, Holum, and Montgomery, *J. Org. Chem.*, 1961, **26**, 2396.

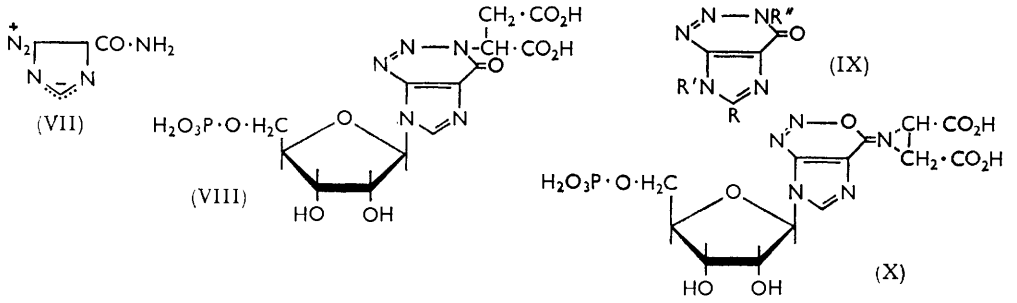
TABLE 2.

Absorption spectra (λ in mμ) of diazotised succino-AICAR and some imidazotriazines.*

Compound	pH	$\lambda_{infl.}$	$\lambda_{min.}$ (1)	$\lambda_{max.}$	$\lambda_{min.}$ (2)	$\varepsilon_{max.}$ †
(IX; R = Me, R′ = C_6H_{11}, R″ = H)...	2	245	—	292	261	5000
	7	246	—	293	261	—
	12	252 ‡	238	295	265	—
(IX; R = R′ = R″ = Me)	2	245	—	298	260	5300
	7	245	—	297	260	—
	12	247	—	298	261	—
Diazotised (II) §	2	247	—	291	255	5600
	12	249	—	295	258	—

* Measurements on an Optica CF 4 double-beam recording spectrophotometer. † $\varepsilon_{max.}$ of the related aminoimidazolecarboxyamides in acid solution are respectively 10,000, 10,600, and 11,300. ‡ An absorption peak. § In a silica cuvette (1 cm. light-path) a small amount of succino-AICAR was dissolved in N-hydrochloric acid (1 drop) and water (3 drops), then 0·1% aqueous sodium nitrite (1 drop) was added and the solution was kept at room temperature for 8 min.; 0·5% aqueous ammonium sulphamate (1 drop) and water (3 ml.) were then added. The spectrum of the final solution was measured after 20 min. and remeasured after adjustment of the pH with sodium hydroxide solution. All solutions were freshly prepared and a reagent control was run in a matched cuvette. The volume of the final solution was calculated from its weight.

due to enolisation of the CO·NH group. In addition, the intensities of absorption of the diazotised ribotide solution and of the two model aza-derivatives are almost exactly half that of the corresponding aminoimidazolecarboxyamide from which they are derived.



These results confirm the view that the final product of decay of the diazotised ribotide is the purine aza-derivative (VIII). However, whereas in the experimental conditions the diazonium salt disappears from solution in 2—3 min. (a half-life of 36 sec. has been recorded [7] for the diazonium salt), the intensity of absorption of the solution continues to decrease for some 20—30 min. before finally becoming constant at a value which, in comparison with those for the analogues, suggests quantitative transformation into the purine aza-derivative (VIII). The results are consistent with the formation of an unstable intermediate from the diazotised ribotide which decays to (VIII). Now, generally, the enhanced reactivity of the diazotised succino-AICAR may be ascribed to the electrophilic succinic acid grouping which favours polarisation of the amide bond to anionic forms such as ·CO·N⁻· and ·C(O⁻):N·, and since the latter is likely to be the preferred anion we suggest that the unstable intermediate may be the compound (X) in which the extended conjugation could account for the more intense absorption of the intermediate than of the aza-derivative (VIII).

The decay of the diazonium salts of the related derivatives (VI; R = H, R′ = R″ = Me; and R = R′ = Me, R″ = H) in acid solution has also been examined, for periods of about 11 min., by observing the change in optical density of the purple chromophore obtained in the Bratton–Marshall test. The first of these compounds showed decay of ~41% and the second of ~14%. In these cases the greater instability of the diazonium derivative of the *N*-methylcarboxyamide may be ascribed to the increased basicity of the amide group.

Experimental

Cyanomethyl 5-Amino-1-cyclohexylimidazole-4-carboxylate.—A solution of sodium 5-amino-1-cyclohexylimidazole-4-carboxylate [1] (0·6 g.) and chloroacetonitrile (0·2 ml.) in dimethylformamide (4 ml.) was heated on a water-bath for 10 min. Initially a solid was precipitated but this soon dissolved. The cooled solution, with water, gave a solid precipitate of the *cyanomethyl ester* (0·4 g.) which crystallised from ethanol as plates, m. p. 183° (Found: C, 58·3; H, 6·25; N, 22·8. $C_{12}H_{16}N_4O_2$ requires C, 58·1; H, 6·5; N, 22·6%).

Cyclohexylamide of 5-Amino-1-cyclohexylimidazole-4-carboxylic Acid.—The foregoing cyanomethyl ester (0·1 g.) and cyclohexylamine (1 ml.) were boiled together under reflux for 15 min. and then evaporated with water to a crystalline residue. The *amide* (0·05 g.) recrystallised from aqueous ethanol as plates, m. p. 210—211° (decomp.) (Found: C, 65·95; H, 8·7; N, 19·7. $C_{16}H_{26}N_4O$ requires C, 66·2; H, 9·05; N, 19·3%). After diazotisation it coupled with β-naphthol or α-naphthylethylenediamine to form red dyes. Attempts to prepare the amide under milder conditions in a reasonable time were unsuccessful.

Benzylamide of 5-Amino-1-cyclohexylimidazole-4-carboxylic Acid.—The above-mentioned sodium salt (0·45 g.) and ethyl chloroformate (0·5 ml.) were kept in dimethylformamide (5 ml.) at 0° for 30 min.; benzylamine (0·5 ml.) was then added to the mixture which was set aside at room temperature for 10 min., diluted with water, and extracted with ether, and the ether extract was extracted with dilute hydrochloric acid. The acid solution was made alkaline with sodium hydroxide solution, yielding a sticky solid. This was collected, and when triturated with ether left the crystalline *amide*. This separated from aqueous ethanol as plates (0·1 g.), m. p. 184—185° (Found: C, 68·4; H, 7·65; N, 18·7. $C_{17}H_{22}N_4O$ requires C, 68·45; H, 7·45; N, 18·8%), gave a red colour with diazotised sulphanilic acid, and after diazotisation coupled with β-naphthol to form a red dye. (*b*) A solution of 5-amino-1-cyclohexylimidazole-4-carboxylic acid [1] (0·45 g.) in pyridine (10 ml.) was treated with benzylamine (0·38 ml.) and dicyclohexylcarbodi-imide (0·7 g.) in pyridine (5 ml.) and set aside for 2 days, then evaporated *in vacuo* with water, and the residue was extracted with ether. The ether solution was worked up as above, to give the amide (0·25 g.), m. p. 185°.

N-(*5-Amino-1-β-D-ribofuranosylimidazole-4-carbonyl*)-L-*aspartic Acid 5′-Phosphate.*—A solution of methyl 5-amino-(2,3-*O*-isopropylidene-β-D-ribofuranosyl)imidazole-4-carboxylate [1] (0·286 g.) in ethanol (6 ml.) and N-sodium hydroxide (6 ml.) was boiled under reflux for 2·5 hr., then concentrated *in vacuo* to half-volume. Pyridine (4 ml.) was added and the mixture was placed on a column of Zeo-Karb 225 (pyridine form) resin which was eluted with 50% aqueous pyridine in 20-ml. fractions. Diazotisable amine (detected by the Bratton–Marshall test) appeared in fractions 2—4 which were combined and concentrated *in vacuo* to 5 ml., then six times concentrated to half volume after addition of more pyridine (6 × 5 ml.). To this solution was added dimethyl L-aspartate [14] (0·295 g.) in dry pyridine (5 ml.) and then, after cooling to 0°, dicyclohexylcarbodi-imide (0·38 g.). The mixture was set aside at room temperature with exclusion of moisture for 60 hr., then dry pyridine (10 ml.) containing 2-cyanoethyl phosphate [11] (4 mmoles; prepared from a standard solution by repeated evaporation *in vacuo* with dry pyridine) and a further quantity of dicyclohexylcarbodi-imide (3·5 g.) were added. The mixture was set aside at room temperature for 2 days, then treated with water (4 ml.) and, after 1 hr., evaporated *in vacuo* to a gum which was evaporated again with water (10 ml.). The residue was heated at 100° with 10% acetic acid (40 ml.) during 90 min. and the solution then evaporated *in vacuo*; the last traces of acetic acid were removed from the residue by further repeated evaporation with water. The residue was suspended in 0·5N-lithium hydroxide (40 ml.) and after 1 hr. at 100° the mixture was cooled and filtered from the solid precipitate. Several volumes of cold water were used to wash the collected solid which was then discarded. The filtrate and washings were combined, adjusted to pH 7 with dilute hydrobromic acid, and concentrated to about 60 ml. *in vacuo*. This solution was treated with barium bromide (1 g.) followed by absolute ethanol (180 ml.) and kept overnight at 0°; there resulted a buff precipitate which was collected by centrifugation and washed successively with 90% ethanol (10 ml.), absolute ethanol (2 × 10 ml.), and dry ether (10 ml.), then dried in air. The solid (0·938 g.) contained about 12% of succino-AICAR (estimated spectroscopically) and was substantially free from extraneous ultraviolet-absorbing material but contained a trace of ninhydrin-reacting impurity. A solution of this solid (0·927 g.) in 0·008N-hydrobromic acid (*ca.* 150 ml.) was

[14] Fischer and Koenigs, *Ber.*, 1907, **40**, 2058.

filtered from some insoluble residue and placed on a column (1·8 × 18 cm.) of Amberlite CG-400 type II (200—400 mesh with 5—40 micron fines removed) resin in the Br^- form and was eluted with 0·008N-hydrobromic acid. Fractions from the column were examined for ultraviolet-absorbing material, and for arylamine by the modified Bratton–Marshall test.[7] Succino-AICAR appeared after the first 1·5 l. of eluate and was eluted in the next 970 ml. The fractions containing succino-AICAR were combined, adjusted to pH 5·6 with saturated barium hydroxide solution, concentrated *in vacuo* to 150 ml., adjusted to pH 8·5 with barium hydroxide, and further concentrated *in vacuo* to 14 ml.; some solid was deposited during this process. The pH of the solution was readjusted to 8·5, and the precipitate removed at the centrifuge, washed with water (3 ml.), and discarded. Absolute ethanol (54 ml.) was added to the combined supernatant fluid and washings to give a precipitate which was collected by centrifugation after 2 hr. at 0°, washed with absolute ethanol (2 × 15 ml.), and air-dried. This purified barium salt of succino-AICAR (56 mg.) was free from ultraviolet-absorbing and ninhydrin-reacting impurities, had the reported spectra (see Table 1) of succino-AICAR, gave a purple colour in the modified Bratton–Marshall test with the absorption max. recorded for the ribotide, and behaved on paper chromatograms like the natural material. A portion of the barium salt (43 mg.) was further purified by precipitation of a clarified aqueous solution with ethanol. The barium salt of succino-AICAR (32·2 mg.) was thus obtained as a colourless halogen-free powder which apparently retained barium hydroxide and water [Found: N, 5·45; P, 3·1%; N : P, 3·9. Calc. for $C_{13}H_{15}Ba_2N_4O_{12}P,Ba(OH)_2,5H_2O$: N, 5·7; P, 3·15%; N : P, 4·0]. It was identified by its spectra (see Table 1) and by paper-chromatographic comparison with an authentic specimen [identical-running pink spots in butanol–acetic acid–water (2 : 1 : 1)]. A portion of the barium salt (0·85 mg.) in a few drops of 5N-hydrochloric acid was heated in a sealed tube at 120° for 5¼ hr. Paper-chromatography of the solution in butanol–acetic acid–water (12 : 3 : 5) revealed aspartic acid and glycine.

Enzymic Conversion of Succino-AICAR into AICAR.[8]—In each of two reaction tubes were placed an adenylosuccinase solution (0·4 ml.), a solution of succino-AICAR (0·1 ml. containing $0·118 \times 10^{-6}$ mole of the ribotide), and a 0·05M-phosphate buffer (pH 7·2; 0·5 ml.). The control tube was treated with 7·5% trichloroacetic acid (1 ml.) immediately after mixing. The assay tube was incubated at 37° for 15 min., then treated with 7·5% trichloroacetic acid (1 ml.). Protein was removed at the centrifuge and 0·4 ml. of the supernatant liquid was assayed by the modified Bratton–Marshall method with a coupling delay of 8 min. (only AICAR then gives a colour). The solution gave an absorption peak at 545—550 mμ analogous to that shown by AICAR,[15] and calculations indicated 45% conversion compared with 44—48% conversion obtained with the natural material.

7-Cyclohexyl-4,7-dihydro-6-methyl-4-oxo-3H-imidazo(4,5-d)-v-triazine (IX; R = Me, R′ = cyclohexyl, R″ = H).—A solution of 5-amino-1-cyclohexyl-2-methylimidazole-4-carboxyamide [16] (0·72 g.) in N-hydrochloric acid (7 ml.) and water (50 ml.) was added dropwise during 20 min. to aqueous sodium nitrite (0·21 g. in 3 ml.) at 0° and set aside at 0°. Crystals (0·253 g.) were obtained. This *product* recrystallised from water as needles, m. p. 180° (decomp.) (Found: C, 56·7; H, 6·45; N, 29·9. $C_{11}H_{15}N_5O$ requires C, 56·6; H, 6·5; N, 30·05%). A further quantity (0·269 g.) was obtained by making the mother-liquors alkaline with ammonia and evaporation *in vacuo* to a low volume.

4,7-Dihydro-3,6,7-trimethyl-4-oxo-3H-imidazo(4.5-d)-v-triazine (IX; R = R′ = R″ = Me).—A solution of the methylamide [16] (74 mg.) of 5-amino-1,2-dimethylimidazole-4-carboxylic acid in N-hydrochloric acid (1 ml.) at 0° was added in portions during 10 min. to ice-cooled aqueous sodium nitrite (40 mg. in 1 ml.) and set aside at 0° overnight. Then a few drops of aqueous ammonia (*d* 0·88) were added and the solution was again set aside. A little solid was precipitated. This was collected, the filtrate evaporated to dryness *in vacuo*, and the residue extracted several times with hot benzene. Evaporation of the extract gave a gum which crystallised. The *product* (15 mg.) separated from ether–methanol as needles, m. p. 197° (Found: C, 47·1; H, 5·4; N, 39·5. $C_7H_9N_5O$ requires C, 46·95; H, 5·05; N, 39·1%).

Diazotisation Experiments.—A 10-ml. portion of a stock solution of the preceding methylamide hydrochloride in water was placed in each of two 100-ml. volumetric flasks A and B. To each flask were added 2N-hydrochloric acid (10 ml.) and 0·1% sodium nitrite solution (10 ml.).

[15] Levenberg and Buchanan, *J. Biol. Chem.*, 1957, **224**, 1005.

[16] Shaw, Warrener, Butler, and Ralph, *J.*, 1959, 1648; Shaw and Warrener, *Proc. Chem. Soc.*, 1958, 93.

Flask A was kept at 0° during 1 min. and flask B at room temperature during 10 min. The excess of nitrous acid in each flask was then destroyed by 0·5% ammonium sulphamate solution (10 ml.) and the colour was developed by addition of 0·1% α-naphthylethylenediamine hydrochloride solution 1 min. later. The solutions were made up to the graduation mark with water and the optical densities of the visible absorption maxima (*ca.* 520 mμ) were measured in conjunction with an appropriate reagent control. The experiment was repeated with a solution of 5-amino-1,2-dimethylimidazole-4-carboxyamide.[16] The percentage decrease in optical density between the two flasks in the first experiment was 41% and with the latter compound only 14%. In these conditions the diazonium salt of succino-AICAR is totally destroyed.

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