Acknowledgments

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Cobamides and Ribonucleotide Reduction. IV. Stereochemistry of Hydrogen Transfer to the Deoxyribonucleotide*

T. J. Batterham, R. K. Ghambeer,† R. L. Blakley, and Carol Brownson

ABSTRACT: Determination of coupling constants from the proton magnetic resonance (pmr) spectrum of deoxyadenosine in deuterated dimethyl sulfoxide has permitted calculation of the approximate dihedral angles for vicinal protons in the deoxyribose moiety. The agreement between these and the angles calculated for the C-2′ *endo* conformation of the deoxyribose moiety indicate that deoxyadenosine in dimethyl sulfoxide solution has considerable conformational purity and that the preferred conformation is the C-2′ *endo* form. The chemical shifts of the $2'_\alpha$ and $2'_\beta$ protons are sufficiently different for these protons to give separate octets, the downfield octet owing to the $2'_\beta$ proton. Deuterated deoxyadenosine was derived from the deoxyadenosine triphosphate formed by the action of ribonucleotide reductase of *Lactobacillus leichmannii* on adenosine triphosphate (ATP) in deuterium oxide. In the pmr spectrum of the deuterated deoxyadenosine the H-$2'_\alpha$ octet was absent and the H-1′, H-$2'_\beta$, and H-3′ peaks showed simplification consistent with deuteration predominantly, if not exclusively, in the $2'_\alpha$ position. It is concluded that in the reductase reaction the 2′-hydroxyl group of ATP is replaced by a hydrogen with retention of the configuration at C-2′.

In studies on the deoxyadenosylcobalamin-dependent reduction of ribonucleoside triphosphates to the corresponding deoxyribonucleoside triphosphates by ribonucleotide reductase of *Lactobacillus leichmannii*, proton magnetic resonance (pmr) has been used to show that when ATP[1] is enzymically reduced in deuterium oxide, a deuterium atom is incorporated into the deoxyribose moiety of the dATP product at the 2′ position (Blakley *et al.*, 1966). We have now obtained an unequivocal analysis of peak patterns from the deoxyribose protons of unlabeled deoxyadenosine

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[1] Abbreviations used: ATP, adenosine triphosphate; dGTP, deoxyguanosine triphosphate; dATP, deoxyadenosine triphosphate; CoA, coenzyme A.

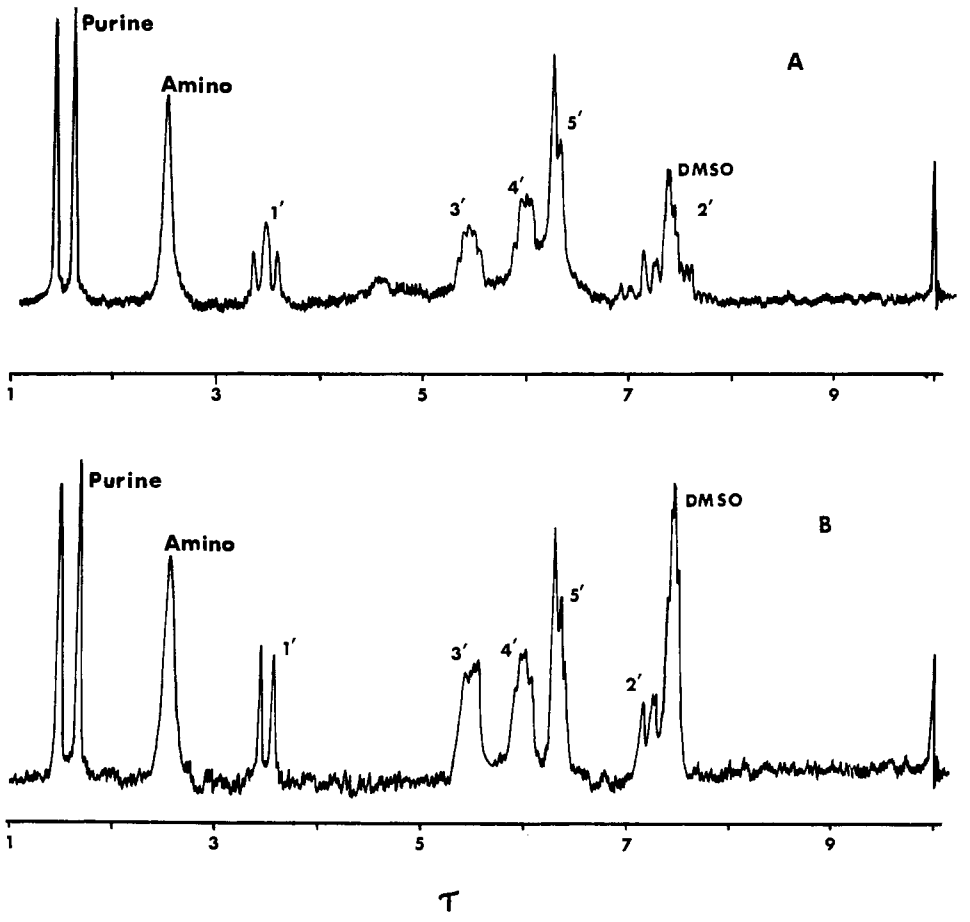


FIGURE 1: Pmr spectrum of deoxyadenosine (A) and deuterated deoxyadenosine (B) in deuterated dimethyl sulfoxide.

and have used this to determine the configuration of the deuterated enzymic product.

Materials and Methods

ATP, dGTP, and deoxyribonucleosides were obtained from P-L Biochemicals, Inc., and Sigma. DL-Lipoic acid was obtained from Sigma; it was reduced (Gunsalus and Razzell, 1957) without distillation of the product. Bacterial alkaline phosphatase (chromatographically purified) was a product of Worthington Biochemicals. Deoxyadenosylcobalamin was a gift from Dr. H. P. C. Hogenkamp. Deuterium oxide (99.7%) was obtained from the Australian Institute of Nuclear Science and Engineering, and deuterated dimethyl sulfoxide from Fluka.

Enzymic Reduction of ATP. The reaction mixture containing 10 mM ATP, 30 mM dihydrolipoate, 1 mM EDTA, 0.5 mM dGTP, 40 μM 5'-deoxyadenosylcobalamin, and 0.3 M sodium 3,3-dimethylglutarate, pH 7.3, in a total volume of 80 ml was freeze dried. Equilibration of exchangeable protons with 20 ml of D_2O (99.7%) was allowed to take place for 1 hr at room temperature under nitrogen after which the solvent was removed by freeze drying. The process was repeated. The enzyme (42.3 mg) was freeze dried separately with about 75% retention of activity. The freeze-dried reactants and enzyme were dissolved in a total of 80 ml of D_2O (99.7%) and incubated under nitrogen at 37° for 6 hr in the dark. Deoxyadenosine from enzymically synthesized dATP was isolated as previously described (Blakley *et al.*, 1966).

Pmr Measurements. The pmr spectra of solutions in deuterated dimethyl sulfoxide were determined at 60 mcycles/sec and 33.5° on a Perkin-Elmer R10 spectrometer. Chemical shifts, measured from tetramethylsilane as internal standard, are quoted in τ units.

Results

The pmr spectra of nucleosides in deuterated dimethyl sulfoxide have been studied by a number of workers (Gatlin and Davis, 1962; Miles, 1963; Miles *et al.*, 1963; Kokko *et al.*, 1961). Unfortunately peaks from the 2' protons of deoxyadenosine occur in the same region of the spectrum as those from the residual protons of the solvent and in undeuterated dimethyl sulfoxide are completely hidden by the solvent peak, a congruence which previously led to an erroneous

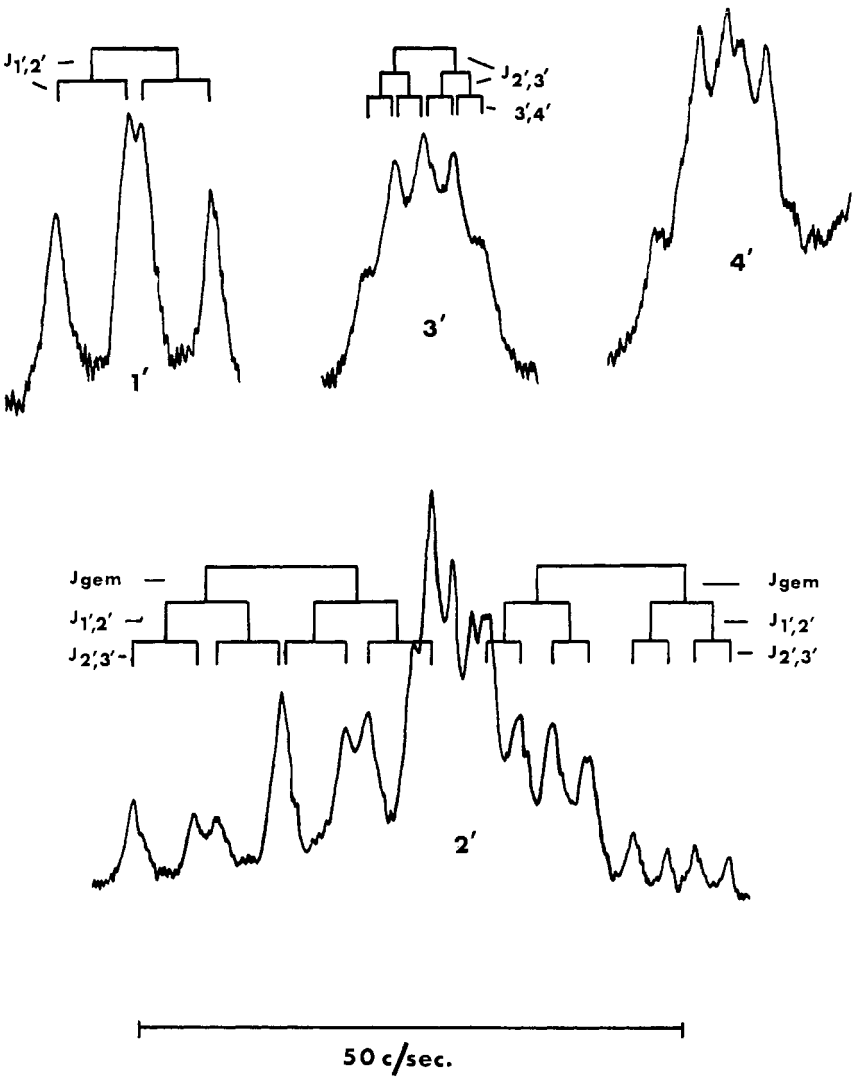


FIGURE 2: Expansions of multiplets from H-1′, H-2′, H-3′, and H-4′ of deoxyadenosine in deuterated dimethyl sulfoxide. J_{gem} is coupling between the 2′-methylene protons, that is, $J_{2'\alpha,2'\beta}$.

assignment (Gatlin and Davis, 1962). Using the deuterated solvent we have obtained a clear spectrum of deoxyadenosine (Figure 1) in which peaks are readily assigned to specific protons by comparison with spectra in other solvents (Blakley *et al.*, 1966). Multiplets from H-1′, H-3′, and the 2′-methylene group were expanded to give well-resolved patterns (Figure 2) which have been analyzed using the iterative computer program, LAOCOON II (private communication from A. A. Bothner-By). Parameters obtained are listed in Table I.

The coupling constant $J_{i,j}$ for a pair of vicinal protons H_i, H_j is a function of the dihedral angle $\theta_{i,j}$ between the two CH bonds. Karplus (1963) has made valence bond calculations of $J_{i,j}$ which yield results approximated by the equation $J_{i,j} = A + B \cos \theta_{i,j} + C \cos 2\theta_{i,j}$. Parallel calculations by Conroy (1960) in which a molecular orbital method was used have given results summarized by curve 1 in Figure 3. While these relationships are not sufficiently accurate to permit the calculation of absolute values of dihedral angles from coupling constants, they give values which are sufficiently accurate to permit a choice between various possible conformations, in this case between possible conformations of the deoxyribose ring.

If θ_1 is the dihedral angle between H-1′ of deoxyadenosine and H-2′$_\beta$ the more distant of the two 2′ protons, and if it is assumed that the projected angle between the geminal CH bonds is 120°, then θ_2 the dihedral angle between H-1′ and H-2′$_\alpha$, the nearer 2′ proton, will be $\theta_1 - 120°$. Corresponding values of θ_1 and θ_2 are marked along the frame of Figure 3 and curves 1 and 2 indicate the values of J which correspond to pairs of values for θ_1 and θ_2. Curve AV indicates the average of such pairs of values of J corresponding to pairs of θ_1, θ_2 values. As shown in Table I, the observed coupling constants between H-1′ and the 2′ protons are 6.0 and 8.8, respectively, which, from Figure 3, corresponds to dihedral angles of 147

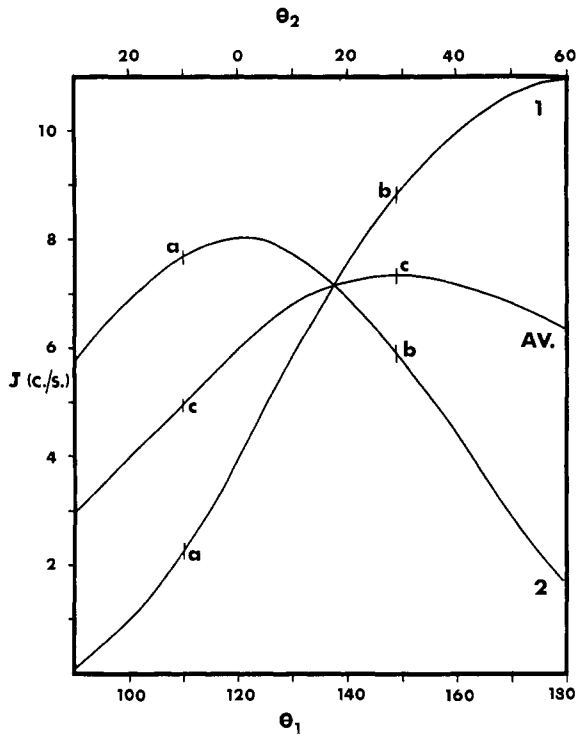


FIGURE 3: Theoretical (Karplus type) curves showing the relationship between coupling constants (J) and dihedral angles (θ_1 and θ_2) for a single proton coupled to a vicinal methylene group. Curve 1 shows the relationship between θ_1 and J, and curve 2 shows the relationship between θ_2 and J, where θ_2 is always the smaller angle. The AV curve is the average of curves 1 and 2. (a) Values for $J_{2'\alpha,3'}$ and $J_{2'\beta,3'}$ for deoxyadenosine; (b) values for $J_{1',2'\alpha}$ and $J_{1',2'\beta}$ for deoxyadenosine; and (c) average $J_{1',2'}$ values observed for thymidine.

TABLE I: Proton Magnetic Resonance Parameters for Deoxyadenosine in Deuterated Dimethyl Sulfoxide.

Chemical Shifts (τ)		Coupling Constants (cycles/sec)	
H-1′	3.48	$J_{1',2'\alpha}$	6.0
H-2′$_\alpha$	7.52	$J_{1',2'\beta}$	8.8
H-2′$_\beta$	7.31	$J_{2'\alpha,2'\beta}$	13.9
H-3′	5.46	$J_{2'\alpha,3'}$	2.3
H-4′	6.02	$J_{2'\beta,3'}$	7.2
H-5′	6.30	$J_{3',4'}$	2.5

TABLE II: Comparison of Dihedral Angles in the Sugar Moiety of Deoxyadenosine Obtained from Coupling Constants with Those Calculated for the "C-2′ *endo*" Conformation.

Coupling Constants (cycles/sec)		Dihedral Angles (deg)	
		From J	C-2′ *endo*
$J_{1',2'\alpha}$	6.0	30	30
$J_{1',2'\beta}$	8.8	147	150
$J_{2'\alpha,2'\beta}$	13.9		
$J_{2'\alpha,3'}$	2.3	110	110
$J_{2'\beta,3'}$	7.2	13	10
$J_{3',4'}$	2.5	112	110

and 30° (θ_1 and θ_2). Similarly the observed coupling constants between H-3′ and the 2′ protons, 2.3 and 7.2, respectively, correspond to dihedral angles of 110 and 13°, respectively.

Jardetzky (1960) has considered the likely conformations of the ribose ring in ribonucleosides and has calculated the H-1′, H-2′ dihedral angle present in each of these conformations. Similar conformations must be considered for deoxyribonucleosides, and the large value of the H-1′, H-2′$_\beta$ dihedral angle obtained above is consistent with the C-2′ *endo* or C-3′ *endo* puckered conformations (where *endo* means that the atom is located on the same side of the plane defined by the ring atoms C-1′, O, and C-4′ as C-5′). However, the values calculated above for the H-3′, H-2′$_\alpha$ and H-3′, H-2′$_\beta$ dihedral angles fit the C-2′ *endo* conformation but not the C-3′ *endo* conformation. Table II compares the dihedral angles calculated from the coupling constants with those calculated for the C-2′ *endo* conformation. The coupling constants, therefore, indicate that deoxyadenosine in dimethyl sulfoxide solution has considerable conformational purity (a not unexpected finding, in view of the size of the substituent groups) and that the preferred conformation is the C-2′ *endo* form, a conformation giving minimal nonbonded interaction between substituents as may be seen from the three projections of it in Figure 4. This conformation differs from that of crystalline deoxyadenosine for which X-ray data have established the C-3′ *endo* conformation (Sundaralingam, 1965).

The coupling constants in Table II allow assignment of the downfield octet in the 2′ multiplet to H-2′$_\beta$. This conclusion is in agreement with the observation, made with Dreiding models, that H-2′$_\beta$ can be more effectively deshielded by the β-oriented purine moiety than can H-2′$_\alpha$.

Although Jardetzky (1960) has stated that purine ribonucleosides and deoxyribonucleosides have a different conformation from the corresponding pyrimidine nucleosides, the proposed conformation of deoxyadenosine (in deuterated dimethyl sulfoxide) is similar to that proposed by Lemieux (1961) for thymidine (in deuterium oxide) on the basis of observed coupling constants. We have obtained similar coupling constants for thymidine in deuterated dimethyl sulfoxide (Table III). In the case of thymidine the chemical shifts of the α and β 2′ protons are so similar that the pmr spectrum in many solvents is simplified and gives only

TABLE III: Comparison of Coupling Constants (in cycles per second) for Thymidine with Average Values for Deoxyadenosine.

Coupling Constant	Thymidine In D_2O[a]	Thymidine In DMSO[b]	Deoxyadenosine in DMSO[b]
$J_{1',2'}$ (av)	7.0	7.0	7.1
$J_{2',3'}$ (av)	5.8	4.8	4.75
$J_{3,4}$	3.2	2.8	2.5

[a] Lemieux (1961). [b] Deuterated dimethyl sulfoxide.

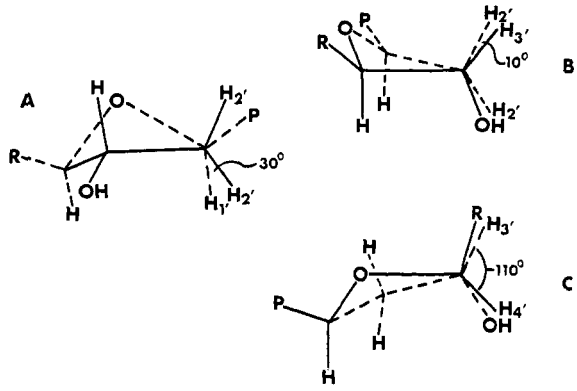


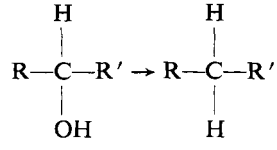
FIGURE 4: Perspective drawings of the proposed conformation for deoxyadenosine in deuterated dimethyl sulfoxide. (A) Along the 1',2' bond; (B) along the 2',3' bond; and (C) along the 3',4' bond.

average values for $J_{1',2'_\alpha}$ and $J_{1',2'_\beta}$ and for $J_{2'_\alpha,3'}$ and $J_{2'_\beta,3'}$. However, as shown in Figure 3 and Table III, these average values for thymidine agree well with the averages of the separate values obtained for deoxyadenosine. In spectra of the latter compound the chemical shifts of the 2' protons are separated by 13 cycles/sec, a separation large enough to ensure that deceptively simple spectra are not observed and that a complete analysis of the system can be obtained.

Configuration at 2' in Enzymically Synthesized Deoxyadenosine. In the enzymically synthesized deuterated deoxyadenosine the H-$2'_\alpha$ octet is absent (Figure 1) and the expected simplification of peaks from H-1', H-$2'_\beta$, and H-3' establishes that deuteration has occurred predominantly, if not exclusively, in the H-$2'_\alpha$ position.

Discussion

The demonstration that deuterium from the solvent is incorporated into the C-$2'_\alpha$ position of dATP by ribonucleotide reductase permits a more complete comparison of the reaction catalyzed by this enzyme with other cobamide-dependent enzymic reactions. The reduction at C-2' of ATP catalyzed by ribonucleotide reductase has an apparent resemblance to the reduction catalyzed by dioldehydrase at C-2 of propanediol (Weissbach and Dickerman, 1965). Thus both these reactions may be represented as follows



In neither enzyme system is there any intramolecular rearrangement of the carbon chain of the respective substrates. This is in marked contrast to the rearrangement of the skeletal structure of the respective substrates catalyzed by glutamate mutase and methylmalonyl-CoA mutase (Weissbach and Dickerman, 1965). In the dioldehydrase reaction, the newly introduced hydrogen at C-2 comes ultimately from C-1 of the same molecule or of a different molecule of the substrate (Abeles and Zagalak, 1966); in the ribonucleotide reductase reaction, on the other hand, the ultimate hydrogen donor *in vitro* is dihydrolipoate, although the physiological donor is probably thioredoxin (Orr and Vitols, 1966). However, an important difference between the reactions catalyzed by propanediol dehydrase and ribonucleotide reductase, respectively, has now been shown in regard to their stereochemistry. The replacement of the hydroxy group by hydrogen at C-2 on the propanediol proceeds with inversion of the configuration at C-2 (Zagalak *et al.*, 1966), whereas the corresponding substitution of the hydroxy group at C-2' of ATP by a hydrogen proceeds with retention of configuration.

It seems possible that in the ribonucleotide reductase reaction, substitution of the hydroxy group in ATP by hydrogen is a nucleophilic substitution reaction perhaps involving the transfer of a hydride ion. In this case the reaction is probably of the SN1 type since SN2 reactions are associated with inversion of configuration (Ingold, 1953). If an SN1 mechanism is assumed, it may be supposed that the hydroxy group at C-2' of the ATP is released as an anion or, after protonation, as a water molecule, leaving behind the ATP carbonium ion. The latter would then receive a hydride ion by a nucleophilic attack and give rise to the product. Although SN1 reactions at an optically active center normally result in racemization, none appear to occur in the ribonucleotide reductase reaction. However, the absence of racemization could be accounted for by a steric effect of the enzyme to which the reacting nucleotide is bound. That is, the enzyme may prevent the approach of a hydride ion to the postulated ATP carbonium ion except from one direction.

It may be noted that cobamide-dependent enzymes do not appear to show any uniformity in regard to the stereochemistry of the reactions they catalyze. Thus in the glutamate mutase (Sprecher *et al.*, 1966a) and

propanediol dehydrase reactions (Zagalak *et al.*, 1966) inversion of substrate configuration occurs, whereas in the methylmalonyl-CoA mutase (Sprecher *et al.*, 1966b) and ribonucleotide reductase of *L. leichmannii* reaction the configuration of substrate is retained.

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The Isolation and Identification of L-Lanthionine and L-Cystathionine from Insect Haemolymph*

D. Rajagopal Rao,† A. H. Ennor, and B. Thorpe

ABSTRACT: Lanthionine and cystathionine have been isolated in crystalline form from the deproteinized haemolymph of *Bombyx mori* (silkworm) and *Antheraea pernyi* (Japanese oak moth) by ion-exchange chromatography. Both compounds have been characterized as the L-enanthiomorphs by enzymatic and physical methods. A preliminary survey of the distribution of lanthionine and cystathionine throughout a number of phyla has been carried out and the results of this, together with some preliminary experiments on the *in vivo* incorporation of isotopically labeled materials, are reported.

The presence of free L-lanthionine in insect issues is invariably associated with a complete absence or, at best, barely detectable traces of cysteine, cystine, and methionine.

During the course of investigations into the metabolism of certain amino acids in insects, the presence of two unusual amino compounds was detected by paper chromatography in the haemolymph of *Bombyx mori* (silkworm) and *Antheraea pernyi* (Japanese oak moth). The compounds have been identified as L-lanthionine and L-cystathionine and the present report deals with their detection, isolation, and characterization. In addition, the results of some experiments on the amino acid composition of haemolymph from *A. pernyi* together with preliminary experiments on the distribution of lanthionine and cystathionine are reported together with some results of isotopic incorporation studies.

Experimental Section

Materials. All reagents were of analytical grade and solvents used for chromatography were purified by fractional distillation. *meso*-Lanthionine and D- and L-cystathionine were gifts from Dr. D. McHale of Walton Oaks Experimental Station, Vitamins Ltd., Tadworth, Surrey, U.K., and Dr. N. Horowitz of

* From the Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T., Australia. *Received September 29, 1966.* A preliminary report of some of these findings has been published (Rajagopal Rao *et al.*, 1966).

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