The Determination of the Optical Configuration of Naturally Occurring Amino Acids using Specific Enzymes and Paper Chromatography

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The problem of determining the optical configuration of naturally occurring amino acids has excited increasing attention of late. It has been known for some time as the result of direct isolation methods that the amino acids in the common vegetable and animal proteins are all of the same L configuration. The existence of D forms, however, has been suspected (Kögl & Erxleben, 1939) and denied (Chibnall, Rees, Williams & Boyland, 1940) in cancer tissues, and has been proved in the case of certain antibiotics of bacterial origin (see reviews in *Biochem. Soc. Symposia*, no. 1, 1948). There is little knowledge, however, of the configuration of the amino acids occurring in the free form in tissue fluids, although in discussions as to the significance of these compounds, the tacit assumption is usually made that they are in fact all of L configuration and are therefore immediately available for protein synthesis. Nevertheless, the fact that there exist in certain tissues, such as sheep's kidney cortex, large quantities of enzyme specifically capable of oxidizing D-amino acids, suggests that these latter compounds may occur at some stage of metabolism. More recently (Auclair & Patton, 1950) good evidence of the presence of D-alanine in insect blood has been obtained.

It is clear that any method of analysis, to be of practical value, must be capable of application to crude biological fluids of largely unknown composition and which may contain only μg . quantities of the amino acid in question. The only feasible tool is therefore an enzyme able specifically to act upon amino acids of one or other configuration, with paper chromatographic analysis of the mixture as a test of reaction or otherwise.

D- and L-amino acid oxidases come to mind as the most readily available enzymes for such a purpose. Lipmann, Behrens, Kabat & Burk (1940) and Lipmann, Hotchkiss & Dubos (1941) have already used the D oxidase in an attempt to determine the configuration of the amino acids in hydrolysates of various proteins. They performed the reactions in Warburg flasks and by following the oxygen uptake and ammonia production drew conclusions as to whether D- or L-amino acids were present and in

what percentage of the total amino nitrogen. The method attained greater specificity when the enzyme solution of D-amino acid oxidase was sprayed directly on to the individual amino acids separated from each other on paper chromatograms. Synge (1949), for instance, was able to show in this way that gramicidin contained D-leucine and D-valine, while Jones (1948) likewise identified D-leucine and L-threonine in aerosporin. Both these workers, however, dealt with mixtures containing only three to five amino acids. A chromatogram with twenty or more amino acids on it would require for the analysis a considerable quantity of enzyme solution and we presume that there would be a tendency for smudging to occur during the reaction on the wet paper which would result in some confusion in distinguishing adjacent amino acids. Auclair & Patton (1950), nevertheless, have used such a method with insect blood. They added the refinement of spraying a duplicate chromatogram with 2,4-dinitrophenylhydrazine to detect the keto acids produced in the reaction.

We have therefore attempted here to simplify the procedure hitherto reported both in the preparation and in the use of the D- and L-amino acid oxidases. We have then applied this method in a preliminary manner to normal human plasma ultrafiltrate, to a hydrolysate of casein, and to seven representative samples of human pathological fluids.

METHODS

Preparation of enzymes

D-Amino acid oxidase was prepared from sheep's kidney cortex by a modification of the procedure of Negelein & Brömel (1939). Our aim was to prepare by as simple a means as possible an active enzyme free, in the amounts to be used, from detectable quantities of amino acids. We therefore omitted stage 2 of Negelein & Brömel's procedure, during which the splitting of the prosthetic group is brought about. In this way we avoided having to prepare the alloxazine-adenine dinucleotide in order to reconstruct the active enzyme. From then on the purification procedure was carried to stage 4, the centrifuged precipitate being dried *in vacuo* over P_2O_5 and then kept in a desiccator over KOH at 4° . When subsequently used, a solution containing 1.5 mg./ml. of enzyme protein ($\text{N} \times 6.25$) was made up in 0.067M sodium pyrophosphate buffer at pH 8.3. The quantity of

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Table 1. Q_{O_2} of L-amino acid oxidase in various snake venoms

Q_{O_2} = μ l. O_2 /mg. dry matter/hr.; sodium phosphate buffer (0.067 M, pH 7.2); snake venom, 0.5 mg.; DL-amino acids 2×10^{-5} M; final volume 2 ml. Q_{O_2} was calculated from the O_2 uptake during the first 30 min.

	DL-Leucine		DL-Methionine		DL-Phenylalanine	
	O_2	Air	O_2	Air	O_2	Air
Cobra	131	82	143	112	172	56
<i>Bothrops atrox</i>	—	—	—	—	245	—
<i>Vipera libetina</i>	—	—	—	—	185	—
Russell viper*	Inactive					

* From the preparation 'Stypven' of Burroughs, Wellcome and Co.

enzyme solution taken for each test showed no detectable amino acids when analysed by paper chromatography. It was free from catalase and no O_2 uptake could be demonstrated without the addition of substrate. The O_2 uptake/mg. protein/hr. at 37° with 0.05 M DL-alanine as substrate varied from 800 to 1300 μ l. according to the freshness of the solution, since even at -10° it lost 30–40% of its activity in a week.

Snake venoms were chosen as sources of L-amino acid oxidases (Zeller & Maritz, 1944). Dried venoms from various species were kindly provided by the late Dr C. H. Kellaway and tested for activity. They were stored at room temperature over KOH. The Q_{O_2} varied according to the species and were lower than those reported by Zeller & Maritz. Table 1 summarizes some of our data. It is well known that the range of oxidase activity is wide and unpredictable. On the basis of these results we used *Bothrops atrox* venom in all our later experiments. The venom solutions (2 mg./ml.) kept at 4° or -10° had a remarkable stability and gave reproducible results over a period of weeks. In the amounts used, the solutions contained no detectable concentrations of amino acids. No O_2 uptake was observed without added substrate.

Since no catalase was present in either of the D- or L-amino acid oxidases used here, the O_2 uptake was represented by 1 mole O_2 for 1 mole of substrate.

Substrates

The substrates tested have been casein hydrolysate, plasma ultrafiltrate, pathological samples of cerebrospinal fluid and urine, and made-up mixtures of amino acids.

The casein was hydrolysed with 6 N-HCl for 24 hr. at 100°, the hydrolysate evaporated repeatedly to dryness *in vacuo* after additions of water and the residue finally diluted to a concentration corresponding to 3.3 mg. of the original casein/ml.

Both the plasma and cerebrospinal fluid were deproteinized by ultrafiltration through collodion bags (Dent & Schilling, 1949) followed by electrolytic desalting in a micro-modification (Dent, 1952) of the apparatus described by Consden, Gordon & Martin (1947). The plasma was finally concentrated to one-fifth of its original volume by evaporation *in vacuo* at 40°. We had confirmed in trial experiments that our L-amino acid oxidase had no action on plasma concentrated without desalting to 0.274 M-NaCl (Zeller, Iselin & Maritz, 1946).

The urine was used as passed.

Incubation

All experiments with pure amino acids and four of the seven with pathological fluids were performed in Warburg

flasks in order to follow the O_2 uptake quantitatively and to determine the time at which oxidation was complete.

In each run six flasks (or more if various concentrations of the substrate had to be tested) were set up. In tests with D-amino acid oxidase the main compartment of the flask contained 0.40 ml. of 0.067 M sodium pyrophosphate buffer (pH 8.3) and either the solution to be analysed or a solution of synthetic amino acid as substrate (0.1 ml. of M DL-alanine). In tests with L-amino acid oxidase the main compartment contained 0.25 ml. of 0.067 M sodium phosphate buffer (pH 7.2) and either the solution to be analysed or a solution of L-amino acid as substrate (0.25 or 0.30 ml. of 0.04 M L-tryptophan). The side arm contained 0.5 ml. of the enzyme solution (namely 0.75 mg. of enzyme protein as D-amino acid oxidase or 1 mg. of dry snake venom as L-amino acid oxidase). In control blanks the enzyme was omitted. The final volume of the reaction mixture was made to 2 ml. with water. The centre well contained 0.1 ml. of 20% (w/v) KOH. The gas space contained O_2 . The incubation at 37° was extended generally to 3 hr. but in some experiments to 5 or 17 hr.

With the concentration of enzyme used, the theoretical O_2 uptake for the pure amino acids was usually reached in 3 hr. The other substrates usually showed an almost complete stop in O_2 uptake by this time. The contents of each flask were subsequently analysed chromatographically as below.

Chromatographic analysis for amino acids

Two-way paper chromatograms on large squares of filter paper (18 × 22 in.) were always used (Consden, Gordon & Martin, 1944). Phenol was used as first solvent in the presence of NH_3 and HCN vapour and collidine; lutidine in the presence of diethylamine as second solvent (Dent, 1948).

With the casein hydrolysate, a solution containing 510 μ g. of original casein was taken for each analysis. With plasma and cerebrospinal fluid the equivalent of 625 μ l. of original fluid was taken. The volume of urine taken was that which contained 250 μ g. N (Kjeldahl).

An additional one-way chromatogram in *tert.*-amyl alcohol in the presence of diethylamine was also carried out on similar quantities, to distinguish methionine, leucine and isoleucine (Work, 1949).

RESULTS

Casein hydrolysate

The L-amino acid oxidase brought about the complete disappearance from the casein hydrolysate of histidine, leucine, isoleucine, methionine, phenyl-

alanine and tyrosine. In addition, about half of the valine disappeared. These are the amino acids which induce the highest oxygen uptake in experiments with single amino acids (Zeller & Maritz, 1944, 1945). Incubation for 2–3 hr. gave an almost maximal oxidation; very little further oxygen uptake or change in amino acid pattern was induced by continuing to 5 or 17 hr. The observations of Zeller & Maritz (1944) and of Krebs (1935) on the influence of the concentration of the substrate on the enzymic activity of L- and D-amino acid oxidases led us to determine the effect of the L oxidase on amounts of casein hydrolysate containing 815, 1630 and 3260 μg . of original casein. In each case the oxygen uptake was directly proportional to the quantity of substrate added and the final observed pattern of amino acids was the same.

Ultrafiltrate of normal plasma

The L-amino acid oxidase brought about the complete disappearance from the plasma sample of the histidine, isoleucine, leucine, phenylalanine and tyrosine, i.e. all the amino acids detectable in plasma by our methods and known to be oxidized readily by the enzyme. In addition, about half of the valine disappeared. Quantities of plasma assumed to contain 70–140 μg . α -amino N were treated with the enzyme. The oxygen uptake was roughly proportional to the amount of α -amino N. The final pattern of amino acids was the same in each case. There was, however, a slight oxygen uptake with the substrate alone without enzyme. Experiments were also carried out adding L-tryptophan (in amounts containing 75 μg . α -amino N) and L-tyrosine (in amounts containing 25 μg . α -amino N) to the plasma (containing 140 μg . α -amino N). In these cases the enzyme decomposed the added amino acid as well as the above plasma constituents, so there is no evidence that under these conditions appreciable substrate competition for the enzyme was taking place.

In all the above experiments the test simultaneously run with D-amino acid oxidase showed no appreciable oxygen uptake or decomposition of amino acids.

Cerebrospinal fluid

The fluid from two patients with phenylketonuria (phenylpyruvic oligophrenia) has been examined. In the volume (0.5 ml.) taken for chromatographic analysis only phenylalanine and glutamine were detectable as strong spots, the other amino acids being present at much lower concentrations than in plasma. A 2 hr. incubation with L-amino acid oxidase produced in the one case a complete disappearance of the phenylalanine, in the other an almost complete disappearance. From the oxygen uptake the concentrations of amino acid oxidized

(calc. as phenylalanine) were 8.1 and 13.3 mg./100 ml., respectively. The D-amino acid oxidase produced no appreciable oxygen uptake and no apparent decomposition of the phenylalanine.

Urine

The urine passed by four patients with Fanconi syndrome was tested. Two of these were adult cases, the other two were children who also showed cystinosis. All the specimens showed gross aminoaciduria. The L-amino acid oxidase treatment resulted in complete disappearance of histidine, isoleucine, leucine, phenylalanine and tyrosine, and also of about half the valine present. These were the only amino acids detectable in the urine that are known to be readily oxidized by the enzyme. The D-amino acid oxidase was applied to one of the adult and to one of the child cases. It had no effect on the amino acids present. Among those easily detectable which, if in the D form, would have been oxidized were (in decreasing order of rate of reaction) proline, isoleucine, phenylalanine, alanine, valine, serine, leucine and tyrosine.

The urine from a case of 'Hart's syndrome' was tested with the L-amino acid oxidase only. This syndrome (E. W. Hart, to be published) has not yet been described clinically, but among its features there is found a gross aminoaciduria of renal origin. The L oxidase was shown to destroy completely the histidine, isoleucine, leucine, phenylalanine, tryptophan and tyrosine, and also over half of the valine present.

DISCUSSION

The method described here for determination of amino acid configuration involves paper-chromatographic analysis of the original mixture before and after allowing it to react with both D- and L-amino acid oxidases. The reactions were mostly carried out in Warburg flasks to check the rate of oxygen uptake and control experiments with the enzymes alone and with added substrate were also run. For routine purposes, however, it is probably unnecessary to do this, since with complicated biological fluids it has been impossible to interpret quantitatively the figures for oxygen uptake. The Warburg technique remains useful only in cases of negative reaction and to detect the end point when reaction is taking place.

As proof of configuration we have found that with isoleucine, leucine, methionine, phenylalanine and tyrosine, all being substances that react with both oxidases, there is obtained a convincing double check, namely that the amino acid is totally destroyed by the one enzyme and quite unaffected by the other. In the case of valine, however, the action with the L oxidase is much slower and tends to be incomplete even after 17 hr. incubation. It

has only been possible to show, therefore, that the destruction of valine in the biological fluids was of the same order as is obtained with the L-valine of a casein hydrolysate and was not effected at all by the D oxidase. We presumed, therefore, that most, if not all, of it was of L configuration. In the case of alanine, histidine, proline and tryptophan, only one or other of the oxidases reacts readily. The evidence of configuration for these substances has to be based only on survival or decomposition by the one enzyme in question. We may further conclude, from the data of Krebs (1935), that a similar single check probably will provide evidence for configuration for further amino acids than the ten mentioned above. These are, in decreasing order of their rates of reaction with the D oxidase: serine, α -aminobutyric acid, cysteine, arginine, aspartic acid and glutamic acid. We have not, however, investigated the reaction under our conditions with the D substrates in question. There is scope for trials of oxidases from other sources, as from *Neurospora crassa* (Horowitz, 1944; Bender & Krebs, 1950), since the amino acid specificity of these is quite different (see also Stumpf & Green, 1944).

The preparation from sheep's kidney cortex of D-amino acid oxidase, sufficiently free of amino acids for our purposes, is now simplified. The L oxidase from snake venom is not, however, likely to be generally available. Unfortunately the commercial Russell viper venom was found to have no L oxidase activity.

When the method was applied to human plasma no evidence for the presence of D-amino acids was obtained. In addition the phenylalanine at elevated concentration (8–13 mg./100 ml. according to the oxygen uptake) in the cerebrospinal fluid in phenylketonuria was found to be all of L configuration. A similar conclusion has recently been reached by Prescott, Borek, Brecher & Waelsch (1949), who used microbiological assays. The urine from four cases of Fanconi syndrome and from one case of 'Hart's syndrome' all showing gross aminoaciduria in the presence of normal plasma levels of α -amino nitrogen likewise appeared to contain only L-amino acids, as far as we could ascertain. This confirms that the aminoaciduria is due to a true lowered renal threshold for the normal blood amino acids (Dent, 1947), and is not due to the presence in the plasma of D-amino acids. These latter are already known to be excreted more easily by the kidney than the L-forms (Silber, Seeler & Howe, 1946; Crampton & Smyth, 1953) and, if present, could have caused an apparent 'renal aminoaciduria' of the type occurring in the Fanconi and other syndromes.

From these preliminary results we therefore conclude that the method is likely to be applicable to most biological fluids. The difficulties we anticipated, namely, inactivation of the enzyme by

unknown poisons or by high concentrations of salts, inability of the enzymes to react with minute concentrations of substrate, etc., have not occurred or have been circumvented.

SUMMARY

1. A micro method for the determination of the optical configuration of amino acids in biological fluids is described. In its present form the only common amino acids to which it has been shown to be applicable are alanine, histidine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, tyrosine and valine.

2. The method involves incubation of the fluid with D-amino acid oxidase of sheep's kidney and with L-amino acid oxidase of snake venom. The extent of the reactions is followed by paper-chromatographic analysis.

3. D-amino acid oxidase, free from amino acids and active enough for the present purposes, has been prepared without the need to split off and subsequently to add the coenzyme.

4. The method has been applied to acid hydrolysate of casein, to a human plasma ultrafiltrate, to the cerebrospinal fluid from two cases of phenylketonuria (phenylpyruvic oligophrenia) and to the urine from two cases of Fanconi syndrome (adult type), two cases in children of Fanconi syndrome with cystinosis, and one case of a syndrome involving renal aminoaciduria, not yet fully described clinically. No evidence was obtained of the presence in these fluids of D forms of the above ten amino acids.

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Respiration Rate of Bacteria as a Function of Oxygen Concentration

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The respiration rate of an organism is measured by determining the rate at which it absorbs oxygen. It is usual to relate this value to some measurement of the size of the organism such as weight or surface area. Variations in the rate of respiration have been determined in this work, so absolute measurements will not be mentioned. In the absence of oxygen, respiration cannot take place and it is known that when the concentration of oxygen surrounding the organism is progressively reduced, the respiration rate falls as the concentration becomes sufficiently low. In this paper is described a study of the respiration rate of some bacteria as a function of oxygen concentration.

When bacteria are suspended in a medium containing a suitable supply of nutrient they use up the oxygen in solution. If the solution is exposed to the air, oxygen will go into solution, tending to bring the system back to equilibrium. After a time a steady state is set up when the rate of solution of oxygen equals the rate at which the organisms are respiring. By using a manometric device the rate of solution can be measured and thus the respiration rate. It is possible to determine the concentration of oxygen in solution by techniques of this sort. One method is to employ very small numbers of bacteria so that the rate of uptake of oxygen is so slow that in the steady state the oxygen in solution is very nearly in equilibrium with that in the gas phase.

In papers published some years ago (Warburg & Kubowitz, 1929; Meyerhof & Schulz, 1932; Shoup, 1929; Cook, 1930) manometric methods of determining the oxygen concentration at which yeast and various bacteria respire at half their maximum rates were described. The figures so obtained were for a few organisms only and there appeared to be some disagreement, even allowing for the large

temperature coefficients. It seemed advisable to redetermine this oxygen concentration for a number of organisms in the hope of finding some fundamental relationship so that the measurements could be predicted for any organism at any temperature and pH.

An attempt was made to repeat the previous manometric techniques and an improved method suggested by Dr D. Herbert was also tried. It was soon apparent that while these methods might yield results in the hands of highly skilled workers, in fact they involved working at the limits of usefulness of manometric techniques where diffusion from the gas to the liquid phase becomes limiting (Dixon, 1943). It was therefore decided that direct measurement of the oxygen concentration in solution by a polarographic method should be tried.

The use of the conventional dropping-mercury electrode was first investigated. It was found that a slowing of the respiration rates of bacterial suspensions at low concentrations of oxygen could be shown, but that this occurred at the limits of sensitivity of the instrument.

Harris & Lindsey (1948) described a vibrating platinum electrode which gave very much higher diffusion currents for the same concentration of oxygen when compared with the dropping-mercury electrode. This was tried, but for mechanical reasons it could not be used conveniently in a cell free from a gas phase.

During a visit here, Prof. Chain suggested a very ingenious rotating platinum electrode, which easily overcame this difficulty. Several experiments showed that measurements of the concentration of oxygen at which bacteria respire at half their maximum rates could be obtained with this apparatus. Duplicate determinations, however,