1. Some substances can inhibit D-amino acid oxidase by combining with the FAD in competition with the apo-protein.

2. A new kinetic method using indicators has been applied for the analysis of the behaviour of an inhibitor in its competition with FAD.

3. Positive interaction has been demonstrated between the binding sites of the apo-protein.

These results may be useful not only for the elucidation of the mechanism of action of several drugs reported in this series, but also for the demonstration of the actual method of complex formation involved in the enzyme action of D-amino acid oxidase.

REFERENCES

1 K. YAGI, J. OKUDA, T. OZAWA AND K. OKADA, *Biochim. Biophys. Acta*, 34 (1959) 372.
2 K. YAGI, T. OZAWA AND K. OKADA, *Biochim. Biophys. Acta*, 35 (1959) 102.
3 K. YAGI AND T. OZAWA, *Biochim. Biophys. Acta*, 39 (1960) 304.
4 K. YAGI, T. NAGATSU AND T. OZAWA, *Nature*, 177 (1956) 891.
5 K. YAGI, T. OZAWA AND T. NAGATSU, *Nature*, 184 (1959) 982.
6 H. LÖW, *Biochim. Biophys. Acta*, 32 (1959) 11.
7 E. NEGELEIN AND D. BRÖMEL, *Biochem. Z.*, 300 (1939) 225.
8 K. YAGI, Y. MATSUOKA, S. KUYAMA AND M. TADA, *J. Biochem.*, (Tokyo), 43 (1956) 93.
9 N. TAKAHASHI, K. YAGI AND F. EGAMI, *J. Chem. Soc. Japan, Pure Chem. Sect.*, 78 (1957) 1287.
10 N. TAKAHASHI AND F. EGAMI, *Bull. Chem. Soc. Japan*, 9 (1955) 666.
11 K. YAGI AND T. ARAKAWA, *Vitamins (Japan)*, 6 (1953) 523.
12 K. YAGI, J. OKUDA, T. OZAWA AND K. OKADA, *Biochem. Z.*, 328 (1957) 492.
13 K. YAGI, T. OZAWA, M. ANDO AND T. NAGATSU, *J. Neurochem.*, 5 (1960) 304.
14 B. GRABE, *Biochim. Biophys. Acta*, 30 (1958) 560.

A RAPID ASSAY METHOD FOR TRITIUM IN BACTERIAL CELLS

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SUMMARY

1. The use of a simple bacterial filtration technique involving collodion membranes has permitted a precise means of assaying tritiated compounds. Radioassays at infinite thickness were readily converted to relative total count values. It is suggested that the method has general applicability.

2. Bacterial cultures rapidly converted exogenous tritiated thymidine to thymine. [^{3}H]diaminopimelic acid served as a more stable biochemical precursor, enabling accurate studies on the incorporation of tritium into bacterial cells.

Abbreviation: TCA, trichloroacetic acid.

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INTRODUCTION

The ready availability of relatively inexpensive tritium-labeled biochemicals has made their use particularly attractive for investigation of metabolic processes. However, the difficulties associated with detection and measurement of radioactivity from such a weak β-emitter have discouraged many workers. Unquestionably, the most sensitive and precise method for measurement of this isotope is the internal liquid scintillation method[1]. This method requires the availability of expensive equipment beyond the reach of most laboratories, and consequently several investigators have applied the method of conventional windowless gas-flow counting[2-4]. Reproducibility is not very satisfactory because of the difficulty of obtaining reliable geometry of distribution. A simple procedure was sought, therefore, for the assay of tritium that would be particularly suitable for existing windowless gas-flow equipment.

The membrane filtration procedure has been employed in this laboratory for the rapid and quantitative recovery of bacterial[5] and tumor[6] cells for the radioassay of ^{14}C. In this technique, cells were removed from their suspensions and were spread quite evenly on the filters. This method has now been applied to the assay of ^{3}H in bacterial cells. Infinite thickness levels were reached readily, and the uniform geometry allowed reproducible count rates. From the specific activity values derived by this technique, highly reproducible values for relative total activity were obtained with a minimum of manipulation or expense.

METHODS

Bacillus cereus, strain 569 H, was grown in a casein hydrolysate-salts medium, as described[5]. Growth was assayed by turbidity measurements at 540 mμ in a Beckman spectrophotometer, Model DU. 2-ml samples of the culture to which a radioactive compound had been added (approx. 0.15 μC/ml of medium) were removed for turbidimetric assay and immediately afterward mixed with an equal volume of 10% TCA. The acid-treated cells were filtered through collodion membrane filters (Schleicher and Schuell Co., Keene, New Hampshire, U.S.A.), and washed with two 5-ml portions of 1% TCA. The filters were mounted in aluminum planchettes with rubber cement and were assayed for radioactivity in a windowless gas-flow counter using natural gas for counting. The membrane method has been described by BRITTEN *et al.*[7] of the Carnegie Institution of Washington, Washington, D.C.

Bacteria washed with saline were fractionated by extraction with cold TCA, aqueous ethanol, ethanol–ether and hot TCA, as described[8]. The residue, termed "total protein fraction" was hydrolyzed with 6 *N* HCl in a pressure cooker at 8 lbs/in^2 (112°) for 3 h.

[^{3}H]thymidine (360 μC/μmole) was obtained from Schwarz Laboratories, Mount Vernon, N.Y., and [^{3}H]diaminopimelic acid was kindly furnished by Dr. J. L. STROMINGER, Department of Pharmacology, Washington University School of Medicine, St. Louis, Mo. (U.S.A.). [Me-^{14}C]thymidine (14 μC/μmole) was obtained from the Cancer Chemotherapy National Service Center, National Institutes of Health, Bethesda, Md. (U.S.A.).

RESULTS

Experiments with [^{3}H]thymidine

Thymidine was used in these experiments because the compound was known to

be utilized for DNA synthesis by *B. cereus*[9]. Varying quantities of a cell suspension of *Bacillus cereus* which had been grown in the presence of [^{3}H]thymidine were filtered by the usual procedure. A self-absorption curve (Fig. 1) indicated that constant count rates were obtained, once infinite thickness levels of radioactive cells had been plated. In these experiments, maximal radioactivity assays were observed

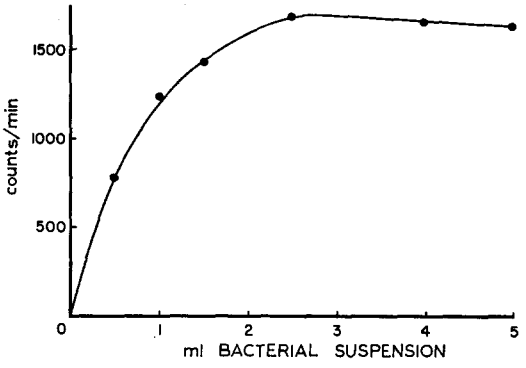

Fig. 1. Radioactivity, in counts/min, on membranes after filtration of varying volumes of suspensions of *B. cereus* grown in the presence of [^{3}H]thymidine. At OD_{540} of 0.4, 1 ml of suspension contained approx. $2 \cdot 10^8$ cells or 0.3 mg dry wt. Area of grid, 2.85 cm^2.

after 2.5 ml of bacterial suspension had been filtered, corresponding to 0.25 mg dry wt./cm^2. The absolute area density of ^{3}H labeled compounds for infinite thickness has been shown to be somewhat variable by HANNGREN[4], depending upon the nature of the compound and the means of plating it. Since it is difficult to reliably correct measurements of ^{3}H radioactivity for self-absorption, one must therefore depend upon specific activity measurements, which are obtained simply and precisely by the technique described.

Kinetic experiments on cellular incorporation of exogenous [^{3}H]thymidine revealed that the rapid bacterial uptake of radioactivity ceased after a short period of incubation, and specific activities of the isolated cells began to decrease with time. Although the growth medium, separated from the cells by centrifugation, was still highly radioactive, and newly inoculated cells proliferated in a normal fashion in this medium, the cells produced were essentially devoid of radioactivity. Each additional supplementation of [^{3}H]thymidine resulted in a period of renewed incorporation of label, and it was concluded that in the presence of the growing cells, the isotopic compound was rapidly transformed into a less readily incorporated product. Addition of large quantities of carrier thymidine with the isotope slowed the rate of destruction, and a longer period of isotopic uptake was observed.

Paper chromatography (70 % aqueous isopropanol saturated with NH_3)[10] of the bacterial medium after the uptake of [^{3}H]thymidine had terminated, revealed the presence of a metabolite which corresponded in its u.v. absorption characteristics at pH 7 and pH 13 and in R_F value to those of thymine. The slower rate of uptake of the free base, in comparison with that of the deoxyriboside, would explain the drop in specific activities observed if, after the decomposition of exogenous thymidine, thymine of DNA were synthesized from a source other than the labeled free base present, such as by methylation of deoxyuridine[11]. The instability of thymidine in

the presence of bacteria was also observed with a wild strain of *E. coli* (ATCC 11303), as well as a thymine requiring mutant of *E. coli*[12]. PRUSOFF[13] has reported a similar cleavage by *Streptococcus faecalis*.

Parallel experiments with [Me-^{14}C]thymidine confirmed that the incorporation of the compound into bacterial cells almost ceased after a period of incubation.

Experiments with [^{3}H]diaminopimelic acid

The biological instability of thymidine in the bacterial medium led to examination of another tritiated compound, diaminopimelic acid. Cultures of *B. cereus* grown in the presence of this labeled amino acid were harvested and fractionated as described. Chromatography of the hydrolyzed total protein fraction in isopropanol (680 ml)–conc. HCl (176 ml) made up to 1 l with water[8] revealed that all of the radioactivity was recovered in the isolated diaminopimelic acid. This compound has been shown to be a constituent of cell walls[14]. Radioautography of the chromatograms led to no darkening of Eastman Kodak blue-sensitive medical X-ray film, whereas radiocarbon chromatograms with counts 0.05 of that from the tritium compounds produced noticeable darkening of the film.

The incorporation of exogenous tritiated diaminopimelic acid into exponentially growing cells of *B. cereus* was followed by periodic sampling of the culture using the membrane technique. The turbidity of the culture was recorded simultaneously. The results, shown in Fig. 2, are represented in terms of the incorporation of radioactivity as a function of increase in turbidity. Relative total radioactivity has been computed by multiplying the specific activity values obtained from direct count of the filters by the turbidity of the bacterial suspension, the latter being a function of cell mass. As apparent from Fig. 2, the incorporation of the isotopic precursor was directly related to the increase in cell mass, thus validating the present procedure with tritium.

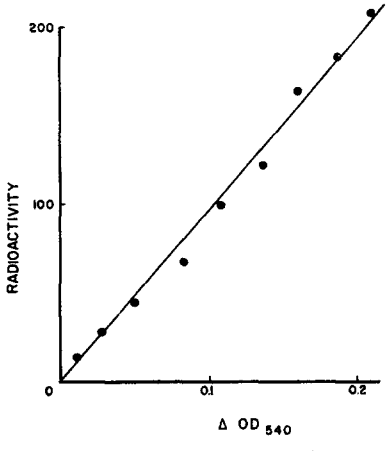

Fig. 2. Incorporation of radioactive diaminopimelic acid into *B. cereus* as a function of bacterial growth. Total activity units computed from counts and mass of cells for the sample filtered. Growth (ΔOD_{540}) measured turbidimetrically at 540 mμ.

DISCUSSION

The method described here may be applied to the assay of tritium in any system of an insoluble, filterable precipitate which contains the radioactivity of the material being

assayed. It is ideally suited for assay of bacterial cells or of dispersed animal cells such as ascites tumor cells or blood cells. It is also applicable to the measurement of an insoluble intracellular component such as nuclear DNA. It must be born in mind that the measurement of specific activity alone is not limiting if an independent measure of concentration, such as turbidimetric measurements or cell counts are available. The product of specific activity and the concentration measurement gives a suitable relative estimate of total activity. The simplicity, reproducibility, and inexpensive applicability of the method permit the use of tritium labeled compounds in almost any biochemical laboratory.

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REFERENCES

[1] J. D. DAVIDSON AND F. FEIGELSON, *Intern. J. Appl. Radiation and Isotopes*, 2 (1957) 1.
[2] M. L. EIDINOFF AND J. E. KNOLL, *Science*, 112 (1950) 250.
[3] F. J. BOLLUM AND V. R. POTTER, *J. Biol. Chem.*, 233 (1958) 478.
[4] A. HANNGREN, *Acta Radiol. Suppl.*, 175 (1959).
[5] H. G. MANDEL, *J. Biol. Chem.*, 225 (1957) 137.
[6] F. U. BROWN AND H. G. MANDEL, *Proc. Am. Assoc. Cancer Research*, 2 (1958) 284.
[7] R. J. BRITTEN, R. B. ROBERTS AND E. F. FRENCH, *Proc. Natl. Acad. Sci. U.S.*, 41 (1955) 863.
[8] H. G. MANDEL AND R. L. ALTMAN, *J. Biol. Chem.*, 235 (1960) 2029.
[9] D. B. ROODYN AND H. G. MANDEL, *Biochim. Biophys. Acta*, 41 (1960) 80.
[10] R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 52 (1952) 552.
[11] M. FRIEDKIN AND DEW. ROBERTS, *J. Biol. Chem.*, 220 (1956) 653.
[12] B. GANDELMAN, S. ZAMENHOF AND E. CHARGAFF, *Biochim. Biophys. Acta*, 9 (1952) 399.
[13] W. H. PRUSOFF, *J. Biol. Chem.*, 215 (1955) 809.
[14] M. R. J. SALTON AND J. M. GHUYSEN, *Biochim. Biophys. Acta*, 24 (1957) 160.