

736. *The Preparation of Uniformly ^{14}C -Labelled Substances on a Laboratory Scale, with Special Reference to L-Tryptophan.*

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A comparison is reported of two methods of biosynthesis, on a laboratory scale, of substances uniformly labelled with ^{14}C . A two-stage "heterotrophic" approach used biosynthesis of carbohydrate by tobacco leaves and subsequent growth of the food yeast, *Torulopsis utilis*, on labelled substrate. This method is potentially adaptable to preparation of a wide range of labelled substances. A one-stage "autotrophic" approach, suitable for the commoner cell constituents, used the green alga, *Chlorella vulgaris*. Uniformly ^{14}C -labelled L-tryptophan can be obtained in an overall yield (based on initial $\text{Ba}^{14}\text{CO}_3$) of 0.2—0.3% by either route. Improved procedures are reported for many of the steps involved. "Infinite thinness" counting was found most suitable for determining, without wastage, the radioactivity of highly active materials available in only limited amount.

THE complexity and stereochemical specificity of many substances required for metabolic investigations make the chemical synthesis of isotopically labelled specimens time-consuming or impracticable, and biological synthesis becomes the method of choice. We have therefore investigated methods of biological synthesis suitable for equipment in a small laboratory.

Only in rare cases can biological synthesis from simple labelled precursors lead to a complex molecule labelled in a specific and predictable position. The choice therefore lies between uniform labelling, the uniformity being ensured as far as possible by requiring all carbon atoms to be derived from a single simple labelled source (preferably $^{14}\text{CO}_2$), and

random labelling, *i.e.*, multiple labelling without uniformity. Random labelling is often easier to achieve, *e.g.*, by allowing an organism to incorporate a labelled precursor whilst using another unlabelled substrate as major energy source, but the substances produced are of far less value for metabolic investigations, and we have rejected this approach. Uniformly labelled substances can be obtained with high specific activities, their use makes the results of metabolic experiments much easier to interpret, and moreover all reactions which the substances undergo become theoretically detectable.

Micro-organisms are potentially the most useful agents for preparative laboratory biosyntheses. Two approaches are available. An autotrophic organism can be grown with ¹⁴CO₂ as sole carbon source. This approach is useful for the more universal tissue constituents such as amino-acids, but has the disadvantage that the growth of most suitable autotrophs is slow. Alternatively a heterotrophic organism can be grown on a uniformly labelled substrate (conveniently glucose). This approach has the disadvantages that an additional step is involved in preparing the labelled substrate, and that a proportion of this substrate is lost by respiration (though this respired activity is recoverable without appreciable dilution). It has the advantages that more rapidly growing organisms can be used and that the range of substances potentially obtainable is greatly extended, as it becomes possible to grow a wide range of wild-type or mutant organisms producing different substances in unusual amount. We have used both approaches, and have compared their relative merits for the preparation of uniformly ¹⁴C-labelled L-tryptophan, a substance conveniently prepared by either route and not previously available. The results are, however, much more widely applicable.

Three problems are involved: (1) the growth of a suitable biological system using, as ultimate source of all carbon, ¹⁴CO₂ from the commercially available barium [¹⁴C]carbonate; (2) the isolation of the required substance; and (3) measurement, without wastage, of the activities of materials of high specific activity available in only small amounts. We have studied the first and third aspects and have confined ourselves largely to published procedures for isolation. For the two-stage "heterotrophic" approach we used tobacco leaves for the photosynthesis of labelled carbohydrate from ¹⁴CO₂ in the apparatus described in the accompanying paper.¹ This carbohydrate was then used as sole carbon substrate for growth of the food yeast *Torulopsis utilis*. For the direct "autotrophic" approach we have grown the alga *Chlorella vulgaris* on ¹⁴CO₂ in the same photosynthesis apparatus.

DETERMINATION OF ACTIVITIES

The high activities, and small quantities, of metabolites available severely limit the methods for activity determination. Counting of samples at infinite thickness with standard geometry is inapplicable except after dilution with unlabelled substance, and this involves loss of both time and material. We have therefore investigated other techniques, and compared the values obtained with those derived from "infinite thickness" counting of diluted samples. Of the available methods, gas counting was considered too laborious, as well as requiring a high dilution of samples of the activities encountered. Scintillation counting of samples co-dissolved with a phosphor, and gas-flow counting of solutions, seemed of too limited application. Estimation of *Bremstrahlung* should be speedy and accurate, but with the counters available the activities were insufficiently high. Thus, when a "Coronet" counter² was used count rates of only 200 counts min.⁻¹ mc⁻¹ were obtained, but we consider this might be developed into a suitable method by using other types of counter. We have therefore relied principally on "infinite thinness" counting though, as shown below, this has limitations.

EXPERIMENTAL

Apparatus.—Activities were determined in a counter with a thin mica end-window. Counts were taken for times sufficient to give a standard error of count better than $\pm 3\%$, and in most cases of $\pm 1\%$. All figures quoted are corrected for background and coincidence.

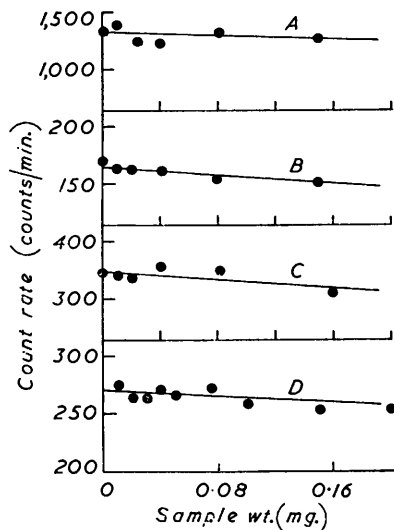
¹ Dutton and Dalgliesh, following paper.

² Veal and Baptista, *Brit. J. Radiol.*, 1954, **27**, 198.

Barium [^{14}C]carbonate, supplied by the Radiochemical Centre, Amersham, was taken as the ultimate standard, and the figures for activity supplied with the samples were accepted as correct. A standard 1 sq. cm. disc of poly([^{14}C]methyl methylacrylate) was used as substandard, and was accurately calibrated in terms of barium [^{14}C]carbonate.

Back-scattering Factors.—To relate the activities of organic substances to barium carbonate the relative back-scattering factors must be known. Yankwich and Weigl³ found, for 12% geometry, that the back-scattering factors for air, barium carbonate, and paper or wax, were respectively 1.00, 1.30 ± 0.01 , and 1.04 ± 0.015 . On the assumption that the factor for paper and wax can be generally applied to organic materials the ratio of back-scattering factors for barium carbonate and organic substances should be 1.25. Yankwich and Weigl's figures are not universally accepted⁴ and we considered it desirable to make our own determination.

An annular mount was constructed which fitted closely over a standard 2 sq. cm. Polythene planchette. A thin film of "Formvar" was then spread on a glass slide, floated off in water on to the mount, and dried. A sample of active barium carbonate on a 2 sq. cm. planchette



The variation of count rate with sample weight for (A) leucine, (B) arginine, (C) phenylalanine, and (D) glucose. The samples for each substance contain a constant activity in a variable weight of material.

was then counted with and without the mount and film in position. The values obtained were $13,184 \pm 115$ and $13,466 \pm 116$ counts/min. respectively. The small difference of the two means indicated that the film was sufficiently thin. Glucose of specific activity $10 \mu\text{c}/\text{mg}$. was dissolved in water, and 0.05 ml. of the solution was applied to the film and allowed to evaporate so as to give $3 \mu\text{c}$ in $0.3 \mu\text{g}$ of material. The work described below shows that such a sample has no appreciable self-absorption. Inactive barium carbonate and inactive glucose were then compacted in the usual way on 2 sq. cm. Polythene planchettes so that the material was flush with the Polythene surface. Each planchette was then counted with the active source in position over it, and 0.5 mm. from the backing. The means of five determinations, made alternately with each backing, were: barium carbonate 1132 ± 16 ; glucose 909 ± 13 counts/min. The ratio of these is $(1.25 \pm 0.025) : 1$, in agreement with the results of Yankwich and Weigl.

Measurement of Thin Films.—In early experiments the material was distributed on lens tissue, but considerable variations of count rate occurred with small variations in thickness. As the specific activities encountered in the present work were high it was decided to try the counting of films so thin as to approach zero thickness. For such a purpose the lens tissue would have to be omitted, and solutions were therefore placed in several small spots on the surface of nickel planchettes, which were then dried in a vacuum-desiccator or in an oven. Several spots were applied to ensure a greater total perimeter should the material dry out mainly at the edge of the spot. Known weights of glucose were then counted and compared with the calculated count, on the assumption that the glucose had been uniformly spread. This showed that sample weights above 0.2 mg. were undesirable. Below 0.1 mg. the activities

³ Yankwich and Weigl, *Science*, 1948, **107**, 651.

⁴ Glascock, "Isotopic Gas Analysis for Biochemists," Academic Press Inc., New York, 1954, p. 3.

did not vary with sample weight by much more than the standard error of the count, and these conditions were used for counting solutions of highly active materials encountered in the present work. In the Figure are shown the variations of count rate with sample weight for four compounds, determined by diluting aliquot parts of solutions of high activity with known weights of unlabelled material. The activity of the undiluted radioactive material is plotted at zero weight. The method allows determination of activity of solutions of materials containing more than 10 $\mu\text{c}/\text{mg}$. with an overall statistical and sample preparation error of $\pm 5\%$. This lowest desirable level of specific activity was appreciably lower than that of most samples described in the present work.

PHOTOSYNTHETIC PRODUCTION OF LABELLED CARBOHYDRATE

Of the various plant materials used by previous workers, tobacco leaves^{5,6} seemed most suitable. Bean leaves (*Phaseolus vulgaris*: cf. ref. 7) were less satisfactory. Our photosynthesis apparatus¹ was used, which freed the method from dependence on sunlight. As monosaccharide, rather than starch or sucrose, was the desired end-product, published procedures were unnecessarily laborious. After removal of soluble sugars by aqueous or aqueous-ethanolic extraction, starch has previously been isolated by differential sedimentation of homogenates,⁷ by dissolution in aqueous potassium hydroxide and reprecipitation with ethanol,⁸ by homogenisation of leaf material with water and toluene, followed by differential sedimentation,⁶ by gelatinisation, extraction with aqueous calcium chloride, and precipitation as the starch-iodine complex,⁵ or by a similar process of extraction with perchloric acid in place of calcium chloride.⁹ All these methods were found to be laborious. A much simpler technique is described below. Soluble sugars are extracted with water. The starch in the leaf debris is then simultaneously hydrolysed and extracted by an aqueous enzyme preparation, and hydrolysis of the extract to monosaccharide is completed by dilute mineral acid. Under our conditions 70–80% of the activity of the initial $\text{Ba}^{14}\text{CO}_3$ was recovered in the final monosaccharide extracts, the mean value for 7 runs being 74%. This overall yield compares well with other published procedures, and the technique is appreciably simpler.

EXPERIMENTAL

Plant Material.—Seed of *Nicotiana virginica*, Burley, was obtained from Messrs. Thompson & Morgan, Ipswich. It was sown in shallow trays, and seedlings were planted out at 2–4 weeks. “National Growmore” fertiliser was applied monthly, or whenever symptoms of nitrogen deficiency became apparent. Plants were grown beside a south-facing window. In winter they were placed above heaters maintaining a temperature of 15–25°, and the period of illumination was artificially extended to 14–16 hr./day. Under these conditions suitable plants could be raised throughout the year, but winter-raised plants tended to give somewhat lower yields of carbohydrate and occasionally failed to take up carbon dioxide at all.

Plants were used when 6–10 weeks old. They were placed in the dark for 24–60 hr. before use, to deplete the leaves of starch. When complete depletion of starch was demonstrable, three recently emerged leaves, 15–25 cm. in length, of young plants were chosen, their surface area measured, and photosynthesis then carried out (cf. ref. 1).

Isolation of Carbohydrate.—The leaves were cut into small pieces and transferred to an Atomix blender. The contents of the beakers into which the leaves dipped in the photosynthesis apparatus were added and the volume made up to 80–100 ml. with water. The leaves were homogenised for 5 min., and the homogenate was centrifuged for 30 min. at 3000 r.p.m. The supernatant “aqueous fraction” was decanted, and the debris was washed with 100 ml. of water and re-centrifuged. The aqueous fractions were pooled. The debris was transferred, with water, to a 250 ml. beaker, the volume made up to 100 ml., and the suspension boiled for 2 min. to gelatinise the starch and inactivate enzymes. After cooling to 65°, a bacterial amylase (10 mg.) (“Bacterase”; Norman, Evans, & Rais, Ltd., Manchester) was added and the mixture incubated at 70° for 1 hr., during which the starch was converted into dextrans and maltose. The temperature was again raised to 100° for a short period and when cool the mixture was

⁵ Putman, Hassid, Krotkov, and Barker, *J. Biol. Chem.*, 1948, **173**, 785.

⁶ Porter and Martin, *J. Exp. Bot.*, 1952, **3**, 326.

⁷ Livingston and Medes, *J. Gen. Physiol.*, 1947, **31**, 75.

⁸ Villee and Hastings, *J. Biol. Chem.*, 1949, **179**, 673.

⁹ Nielsen, *Ind. Eng. Chem., Analyt.*, 1943, **15**, 176.

centrifuged at 3000 r.p.m. for 30 min. The supernatant liquid was decanted and the residue submitted to a second enzymic extraction. The final debris was not further worked up. The efficiency of the extraction is illustrated in Table 1, the mean recovery of activity in soluble form being almost 95%.

TABLE 1. *Efficiency of enzymic hydrolysis and extraction of starch. Percentages relate to the total activity in the leaf material after extraction of soluble sugars.*

Run	1st extract		2nd extract		Final debris	
	μC	%	μC	%	μC	%
1	550	88	55	9	18.3	3
2	45	83	4.8	9	4.5	8
3	278	86	29	9	16.1	5
4	2250	84	257	9	220	7
5	3460	90	230	6	135	4

The two enzymic extracts were pooled to give a clear yellow solution, which was evaporated under reduced pressure to 100 ml. Concentrated sulphuric acid (1 ml.) was added and the solution refluxed for 1 hr. to complete hydrolysis. A slight excess of barium carbonate was added to the cooled solution, and the precipitated barium sulphate (which carried most of the colour down with it) was removed and washed with boiling distilled water (50 ml.). The solution was concentrated, and subsequently purified as described by Porter and Martin,⁶ salts being removed by precipitation with ethanol, and the crude sugar purified by chromatography on a charcoal-celite column and finally desalted on a mixed-bed ion-exchange resin (Amberlite M.B.1).

The original aqueous extract of the leaf homogenate was similarly submitted to acid hydrolysis before purification, as the reducing-sugar content was found to rise after such treatment.

In most cases the purified de-ionised monosaccharide solutions were used directly when preparing medium for the subsequent stage. Adequate purity was confirmed by paper chromatography and autoradiography. It was not thought necessary rigorously to purify the product, which would involve a considerable loss of yield without compensatory advantage.¹⁰

Uniformity of labelling in the product was assumed on the basis of the work of, e.g., Gibbs¹¹ and Vittorio, Krotkov, and Reed.¹²

CONVERSION OF LABELLED CARBOHYDRATE INTO YEAST PROTEIN

Little attention has been paid to the use of heterotrophic organisms for the biosynthesis of a range of ¹⁴C-labelled substances. We have used the food yeast, *Torulopsis utilis*, about which much information has been acquired in other connections. Previously this, or related organisms, have been used for preparing [¹⁵N]amino-acids¹³ and [³⁵S]amino-acids,¹⁴ but have been rejected¹⁵ for ¹⁴C-labelling on the grounds of loss of substrate activity as respiratory carbon dioxide. But by suitable choice of growth conditions anaerobic metabolism can be minimised and the loss of activity by respiration reduced to ~30%, this respired activity in any case being recoverable (as Ba¹⁴CO₃) without appreciable dilution.

Conditions for obtaining maximum yields of yeast protein, based on the carbohydrate used as sole carbon source, were determined. Secondly, a method for hydrolysis of the synthesised protein and the separation of the amino-acids without destruction or racemisation of the tryptophan was worked out. An apparatus was designed for the efficient aeration of the growing yeast cultures, but proved no more satisfactory than very rapid "shake cultures" which were finally used. The effect of sugar-substrate concentration on yield was reinvestigated, as there seemed some conflict in the literature, and the effect of additional nitrogen, trace elements, and growth factors was also studied. From a number of enzyme preparations tried, pancreatic was selected for hydrolysis of yeast protein. The results of three syntheses are summarised in Table 2.

¹⁰ Udenfriend and Gibbs, *Science*, 1949, **110**, 708.

¹¹ Gibbs, *Plant Physiol.*, 1951, **26**, 549.

¹² Vittorio, Krotkov, and Reed, *Proc. Soc. Exp. Biol. Med.*, N.Y., 1950, **74**, 775.

¹³ Aqvist, *Acta Chem. Scand.*, 1951, **5**, 1031.

¹⁴ Williams and Dawson, *Biochem. J.*, 1952, **52**, 314.

¹⁵ Schieler, McClure, and Dunn, *J. Biol. Chem.*, 1953, **203**, 1039.

TABLE 2. *Distribution of activity in the fractions obtained after yeast syntheses. (Expressed as % of radioactivity supplied.)*

	Run :		
	1	2	3
Activity supplied in μC :	820	4180	4480
Synthesised yeast	53%	50%	54%
" Cold TCA extract "	4	4	3.5
" Hot TCA extract "	8	8	13
Enzymic hydrolysate of protein	22	28	27
aliphatic amino-acids	18.5	18	21
phenylalanine	—	0.65	0.68
tyrosine	0.96	0.85	0.89
tryptophan	0.25	0.20	0.50
Recovered Ba ¹⁴ CO ₃	44	30	29

EXPERIMENTAL

Materials.—A culture of *Torulopsis utilis* var. *major* was obtained from Messrs. Barclay, Perkins Ltd., London.

Apparatus.—The cultures were grown in 250 ml. conical flasks carrying a B 19 Quickfit joint. Into this socket was inserted a cone carrying an inlet and an exit tube for the slow circulation of air through the flask. Air was drawn through a wash-bottle containing 40% aqueous sodium hydroxide before entering the culture flask and the radioactive carbon dioxide in the respired air was recovered in barium hydroxide traps. The flask was very rapidly shaken in a "microid" laboratory shaker (Griffin & Tatlock, Ltd., London) and the ambient temperature maintained at 30°. The culture medium was as follows: diammonium hydrogen phosphate 0.56 g.; ammonium sulphate 0.47 g.; potassium sulphate 0.29 g.; magnesium sulphate heptahydrate 0.21 g., the whole made up to 1 l. with distilled water and adjusted to pH 4.4 with hydrochloric acid. Ammonia was added to the culture at intervals throughout the growth period to maintain the initial pH. The pH of the medium was followed by using bromocresol-green as internal indicator (0.5 ml. of a 0.2% aqueous solution per 100 ml.). The medium was boiled for 2 minutes before use, which provided adequate sterilisation of the medium with the large inocula used. The growth of the culture was followed by turbidity measurements, calibrated in terms of dry weight, on small aliquot parts removed at intervals. Nitrogen determinations were made by the micro-Kjeldahl method.

The yield and nitrogen content of the yeast were measured in a series of experiments in which the appropriate additions were made to the medium described. The following results were established: (1) The presence of internal indicator had no effect on yield or growth rate (provided it was not added as an alcoholic solution). (2) The yield increased as the sugar concentration was lowered, to reach a maximum when the sugar concentration was 0.5%, below which no further increase was obtained. (3) To obtain this yield at 0.5% sugar concentration the flask must be shaken at or above 1000 cycles/minute. (4) The addition of nitrogen supplements (ammonium sulphate), growth factors (riboflavin, calcium pantothenate, thiamine, pyridoxine, nicotinamide), or trace elements (Zn, Mn, Cu, Na, Mo, and Fe) had no effect on the yield. (5) The addition of synthetic detergent to increase aeration, or the use of an oxygen atmosphere, did not improve the yield.

Preferred Experimental Procedure.—A pilot culture, inoculated from agar slope cultures, was grown overnight and harvested in the logarithmic phase of growth. Fresh medium (50 ml.) was inoculated with washed *Torulopsis utilis* from the pilot culture to give an initial dry weight of ca. 20 mg. The gas train was started and 10 ml. of a solution containing 2–4 mc in 200–500 mg. of [¹⁴C]monosaccharide were added portionwise at intervals throughout the growth period to maintain the concentration of monosaccharide at about 0.2%. The addition of sugar, adjustment of pH, and measurement of turbidity necessitated opening the flask with consequent loss of respired carbon dioxide, and were therefore carried out as swiftly as possible.

Growth was followed by turbidity readings, and the culture harvested at the end of the logarithmic phase. The yeast cells were twice washed in distilled water and dried by washing with acetone and then ether. The respiratory ¹⁴CO₂ trapped as Ba¹⁴CO₃ was recovered in the usual way and stored dry for re-use.

Fractionation of the ¹⁴C-Yeast.—Acid hydrolysis was unsuitable because of resultant destruction of tryptophan. Alkaline hydrolysis was unsuitable because of the resultant racemisation of amino-acids. Various proteolytic enzyme preparations were therefore examined. The yeast was first extracted with 5% trichloroacetic acid (TCA) solution at room temperature to

give a "cold TCA extract." The yeast was recovered by centrifugation and the process repeated. The acid was removed from the combined extracts by ether extraction, and the aqueous fraction was then freeze-dried and stored. The nucleic acids were removed from the yeast residue in a similar fashion by two extractions with 5% trichloroacetic acid solution at 90° ("hot TCA fraction"); the extracts were similarly treated and stored. The yeast residue was washed twice with acetone and twice with water and then submitted to proteolysis. The criterion of hydrolytic efficiency was the amount of tryptophan found in the supernatant fraction after hydrolysis and centrifugation, expressed as a percentage of that obtained by alkaline hydrolysis. The results are summarised in Table 3. Pancreatin alone was as efficient

TABLE 3. *Yield of tryptophan after various enzymic procedures.*

Enzyme or enzyme mixture	Yield (% of yield obtained by alkaline hydrolysis)			Additional yield (%) by alkaline hydrolysis	Total yield (%)
Pancreatin (Pancreatin powder U.S.P.XI, Armour Laboratories)	97	90, 98	Mean, 95	7	104
Pepsin [Pepsin, crystallised pepsin (Porcine mucosa), Armour Laboratories]	74, 76, 56	..	69	31	105
Trypsin (Trypsin, B.D.H.)	69, 77	..	73	27.5	96.5
Papain (Papainum B.P., Allen & Hanburys Ltd.)	76, 78, 74	..	76	36	112
Pepsin and trypsin	99	—	—	5	104
Pepsin, trypsin, and pancreatin	101	—	—	7	108
Papain, pepsin, and trypsin	118	—	—	—	—
Pepsin, trypsin, pancreatin, and duodenal mucus (Duodenin, Armour Laboratories)	115, 110	..	113	—	—

The figures for additional yield obtained by alkaline hydrolysis apply only to the first column of figures under the previous heading. Values greater than 100% are due to autolysis, or alkaline hydrolysis, of enzyme protein.

as any of the combinations of enzymes examined. The yeast was suspended in distilled water (100 ml.) and the pH adjusted to 8.2. Pancreatin (25 mg.) and a few drops of toluene were added. The mixture was incubated at 37° for 3 days with frequent shaking and occasional adjustment of pH when necessary. Although 90—100% of the tryptophan was recovered in the soluble fraction after centrifugation, a considerable proportion of this was still peptide-bound. It was found that only 50—60% of the theoretical was recovered in the tryptophan peak after chromatography on a starch column (see below). Thus, making allowance for a column recovery factor, it was calculated that two-thirds of the soluble tryptophan was free and one-third peptide-bound, agreeing with findings later reported by Werner.¹⁶ The supernatant hydrolysate was decanted from the unhydrolysable debris after centrifugation, and evaporated to dryness. It was then taken up in 0.1N-hydrochloric acid (2—3 ml.) and put through a starch column,¹⁷ the aromatic amino-acids being recovered as isolated peaks, detected by following radioactivity and ultraviolet absorption of the eluate. The appropriate fractions were pooled and freeze-dried. The purity of the isolated amino-acids was investigated by chromatography and autoradiography. The tryptophan was generally obtained pure after the one step, but the tyrosine and phenylalanine peaks sometimes contained other amino-acids and required further purification on suitable columns.

AUTOTROPHIC SYNTHESIS USING *CHLORELLA*

The cultures of *Chlorella* were grown on inorganic media with ¹⁴C₂ as sole carbon source. It was necessary to maintain rapid growth rates to obtain a product of high protein content. This was carried out as described in the accompanying paper.¹

TABLE 4. *Typical distribution of activity in the fractions obtained from the ¹⁴C-Chlorella (expressed as % of activity supplied).*

Activity supplied :	Run 1	Run 2	Activity supplied :	Run 1	Run 2
	10 mc	27 mc		10 mc	27 mc
Medium and washings	2.6	0.6	Ether extracts of TCA extracts	0.1	0.2
Alcohol-ether extract	20.8	16	Enzymic hydrolysate of protein	32.5	30
"Cold TCA extract"	9	5.9	aliphatic amino-acids	24	17
"Hot TCA extract"	10	6.2	tryptophan	0.26	0.33

¹⁶ Werner, *Int. Z. Vitaminforsch.*, 1954, **25**, 315.¹⁷ Moore and Stein, *J. Biol. Chem.*, 1949, **178**, 53.

The ¹⁴C-*Chlorella* obtained was fractionated in a manner similar to that already described for *Torulopsis utilis*, and the aromatic amino-acids were isolated. The activities residing in each of the fractions obtained are summarised, for two high-activity runs, in Table 4.

EXPERIMENTAL

Materials.—A culture of *Chlorella vulgaris* was obtained from Professor Pearsall (University College, London) and maintained on agar slopes. The medium used was essentially that described by Pearsall and Loose,¹⁸ but sodium citrate and glucose were omitted to exclude alternative carbon sources.

Fractionation of the Synthesised ¹⁴C-Chlorella.—The *Chlorella* was harvested, washed twice with distilled water by centrifugation, and dried to constant weight at 60°. The dried material was ground under alcohol and continuously extracted (Soxhlet) for 24 hr. with alcohol-ether (4 : 1). The extract was evaporated to dryness and the residue extracted first with cold and then with hot 5% trichloroacetic acid solution, as described for yeast. A comparison of enzymic hydrolyses was made. Pancreatin hydrolysis was again found the most satisfactory and this was used as described above. The hydrolysate was concentrated and the aromatic amino-acids were isolated on starch columns as before.

DISCUSSION

The immediate aim of these syntheses has been the production of uniformly labelled L-tryptophan. Similar yields were obtained by both the one- and the two-stage syntheses. In the sugar-yeast synthesis 74% of the initial Ba¹⁴CO₃ activity was converted into monosaccharide suitable as yeast substrate. Of the monosaccharide activity supplied, 19% was recovered as aliphatic amino-acids, 0.7% as phenylalanine, 0.9% as tyrosine, and 0.32% as tryptophan, giving overall yields of 14.5%, 0.5%, 0.7%, and 0.24% respectively. A further 25% of the initial activity was recovered as Ba¹⁴CO₃ from the respired carbon dioxide, while 7.5% was obtained as crude nucleic acid and 3.1% in the "cold TCA extract." Thus some 28% of the initial Ba¹⁴CO₃ was converted into useful fractions, or 39% allowing for the activity of the recovered respiratory ¹⁴CO₂.

In the *Chlorella* syntheses some 60% of the activity appeared in useful fractions. The activity contained in the alcohol-ether extract corresponds to the fat content of *Chlorella* grown under these conditions.¹⁹ Although much of the pigment is also removed, some, presumably degraded, material is left which eventually appears in the aliphatic amino-acid fraction. The yield of aliphatic amino-acid was about 20% and of tryptophan 0.3%. The activity in the "cold TCA fraction" was similar to that obtained with *Torulopsis utilis*, but that in the "hot TCA extract" was only half the yeast value, reflecting the much lower nucleic acid content of *Chlorella*.

The yield of total amino-acid and of tryptophan in the case of *Chlorella* was somewhat lower than predicted from the nitrogen content. It is possible that this yield might be raised by an improved hydrolysis. The lower yields resulting from enzymic hydrolysis were, however, accepted as it was necessary neither to destroy nor to racemise the L-tryptophan.

The *Chlorella* syntheses represent a considerable economy of time and effort over the tobacco-yeast syntheses and result in a higher overall amino-acid yield. The *Chlorella* are also a good source of labelled fat. However, the yeast is probably more suitable for the production of labelled nucleic acids and certain vitamins, and analogous use of suitable micro-organisms makes a whole range of substances potentially preparable.

The apparatus and techniques described are considered to be suitable for the economical production of a wide range of uniformly ¹⁴C-labelled compounds on a small scale.

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¹⁸ Pearsall and Loose, *Proc. Roy. Soc.*, 1936, B, 121, 451.

¹⁹ Pearsall, *Times Science Review*, Spring, 1954, p. 13.