which *Chlorella* has been envisaged as a possible source of dietary protein. When the results in Table 3 are examined from this angle, it is evident that the quality of the protein is nearly independent of culture age. Thus harvesting of the cells could be arranged at a time which from an industrial viewpoint would be most economical. The ten 'essential' amino-acids of Rose account for 53% of the total protein nitrogen, or 50% of the total weight of all amino-acids released during protein hydrolysis. Therefore, although the content of cystine is low, the remaining essential amino-acids are present in *Chlorella* protein in proportions comparing very favourably with those for other plant proteins.

SUMMARY

1. The unicellular alga *Chlorella vulgaris* was grown in pure culture. The utilization by the cells of NH_3-N and NO_3^--N supplied in the medium was examined, growth being observed to continue until the supplies of nitrogen became exhausted.

2. Bulk protein samples representative of the whole cell proteins were isolated from cultures of different ages, and their amino-acid compositions determined by a paper-chromatographic technique. It was found that culture age had little effect on the protein composition except for a significant increase in the histidine contents with increasing age of the cells, and slight changes in alanine, lysine, arginine and amide N contents.

3. The 'essential' amino-acids accounted for 50% by weight of the total amino-acids released during hydrolysis of the proteins, although the cystine contents were uniformly low.

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The Biosynthesis of L-Cystine and L-Methionine Labelled with Radioactive Sulphur (^{35}S)

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The sulphur-containing amino-acids labelled with radioactive sulphur (^{35}S) are usually administered in biological experiments as a racemic mixture of their optical isomers. As the metabolism of L-amino-acids in animals generally differs from that of the D forms (Neuberger, 1948; Graham *et al.* 1950; Camien, Malin & Dunn, 1951), it may be desirable in certain experiments to use the labelled amino-acids as their naturally occurring isomers. The chemical synthesis of cystine or methionine from labelled sulphate ions followed by the resolution of the isomers is a somewhat tedious procedure and a biological synthesis may be preferred.

During the growth of baker's yeast, inorganic sulphate is easily assimilated and is a better sulphur source than organically combined sulphur (Sugata & Koch, 1935; Schultz & McManus, 1950). It seemed likely therefore that the proteins of yeast grown in a medium containing labelled sulphate ions would serve as a convenient source for the isolation of labelled L-cystine and L-methionine. This paper gives details of the preparation of these

labelled amino-acids from the proteins of yeast grown in a radioactive medium. Glutathione labelled with radioactive sulphur has also been prepared from a similar yeast preparation, but as the method followed closely one published during this investigation (Woodward, 1951) no description will be given.

METHODS AND RESULTS

Growth of yeast and preparation of crude yeast protein

Baker's yeast (*Saccharomyces cerevisiae*) was grown in a synthetic medium similar to that used by Olsen & Johnson (1949). 1 l. of the medium contained the following: glucose, 20 g.; $(NH_4)_2HPO_4$, 3·5 g.; KH_2PO_4, 0·2 g.; $MgSO_4.7H_2O$, 0·25 g.; sodium citrate, 1·0 g.; L-asparagine monohydrate, 2·5 g.; biotin, 10 μg.; calcium pantothenate, 0·5 mg.; inositol, 10 mg.; thiamine, 6 mg.; pyridoxin, 1 mg.; Zn^{++} (acetate), 400 μg.; Fe^{+++} (chloride), 150 μg.; Cu^{++} (chloride), 25 μg.

The medium was adjusted to pH 5 with H_3PO_4 and warmed to 30°. Radioactive sulphur (1 mc./l. as carrier-free SO_4^{2-}) was then added. Sterilization of the medium was found to be unnecessary if it was inoculated immediately after preparation with 10 mg. of pressed baker's yeast/l. (Distillers Co. Ltd.).

It was convenient to grow the yeast in a Winchester quart bottle containing 2 l. of the medium. This was incubated for 40 hr. at 30° and during the incubation a vigorous stream of air (1·5–2 l./min.) was drawn through the medium. The air was previously passed through water at 30° to minimize evaporation of the medium. At the end of the incubation period the yeast was centrifuged, dehydrated with ethanol and refluxed for 30 min. with light petroleum to remove lipid material. The residue was filtered and washed once with ether. The yield of crude protein residue was consistently between 3·0 and 3·5 g./l. medium. After drying at 60–70° the residue was dust-like and was easily disturbed by slight air currents. The dry weight was therefore taken in a small flask (250 ml.) into which the still ether-damp residue was placed. The next stage of the isolation was carried out in this flask, thus avoiding pollution of the air with radioactive dust.

Preparation of radioactive L-*cystine*

Initial experiments showed, in agreement with Csonka (1935), that during the hydrolysis of yeast protein with hydrochloric acid there occurred considerable destruction of cystine. An attempt to block the breakdown of the liberated labelled L-cystine was made by conducting the hydrolysis in the presence of added L-cystine, but it was found that the recovery of cystine from the hydrolysate was low.

Bailey (1937) has obtained evidence that the destruction of cystine during the hydrolysis of plant proteins is associated with the presence of pentoses; these are presumably converted into furfural which then condenses with the amino-acids. As yeast protein is known to contain a considerable quantity of pentoses combined in nucleoproteins, the protein residue was extracted with hot trichloroacetic acid solution before the hydrolysis with hydrochloric acid. Schneider (1945) has shown that hot trichloroacetic acid solution quantitatively extracts the nucleic acids from animal tissues. This extraction of the yeast protein residue coupled with the addition of L-cystine enabled a satisfactory yield of labelled L-cystine to be obtained.

The lipid-free yeast residue from 2 l. of medium was extracted with 70 ml. of 5% (w/v) trichloroacetic acid solution for 15 min. at 90°, centrifuged, and washed once with cold 5% trichloroacetic acid solution. The damp residue was hydrolysed with 50 ml. boiling 5N-HCl in the presence of L-cystine (50 mg./g. yeast residue) which had been recrystallized from commercial samples. The time of hydrolysis was kept short (5·5 hr.), as experiments showed that the specific activity of the product was no higher after a longer hydrolysis, which at the same time would increase the degree of racemization (Hoffman & Gortner, 1922) and homocystine formation (Butz & Du Vigneaud, 1932). The hydrolysis was conducted in a fume chamber to remove any volatile sulphur compounds which might be released during the reaction (Grossfeld & Young-Yen, 1941). The hydrolysate was filtered from acid-insoluble humin and the filtrate boiled for a few min. with 200 mg. of activated charcoal (calcium phosphate-free). After removal of the charcoal the light-brown solution was evaporated to a viscous residue under reduced pressure. The residue was taken up in 50 ml. water and adjusted to pH 4·5 with 10N-NaOH solution. After standing for 24 hr. at 5° the crude cystine was filtered off and washed with water. The crude cystine was suspended in 60 ml. of warm water and HCl added until it had just dissolved. This solution was boiled with 40 mg. of activated charcoal until colourless, filtered and the cold filtrate adjusted to pH 4·5 with a saturated aqueous solution of sodium acetate. After standing 2 hr. at 0° the precipitated cystine was filtered off and re-precipitated from acid solution using NH_3 to neutralize the solution. Yield: approximately 50% based on the added L-cystine.

The L-cystine isolated as described consisted of the typical irregular and hexagonal plates of the precipitated compound. It gave a correct analysis for N and S, and $[\alpha]_D^{22°}$ in a typical experiment was $-210·7°$ in N-HCl and did not change on recrystallization. The product was free from tyrosine when examined by chromatography and by the method of Arnow (1937).

The cystine had a counting rate of approximately 3×10^7 counts/min./m-mole when measured under a mica-window Geiger-Müller counting tube (mica, 1·7 mg. sq.cm.). The absence of traces of sulphur-containing contaminants of a high specific activity was demonstrated by the following observations. On twice precipitating at the isoelectric point and then crystallizing from a 50% (v/v) ethanol-water mixture, the specific activity of the cystine was unchanged. On conversion of the product to dibenzoylcystine, m.p. 180° (Gortner & Hoffman, 1921), the specific activity of the sulphur remained constant. Similarly, when thiazolidine-4-carboxylic acid, m.p. 196°, was prepared from the cystine (Rachelle, Reed, Kidwai, Ferger & Du Vigneaud, 1950) no change in the specific activity of the sulphur was observed. The cystine obeyed the constant-solubility test of Gutmann & Wood (1949) for the detection of radioactive impurities using water as the test solvent.

The absence of labelled homocystine from the product was tested for by dissolving excess DL-homocystine in a HCl solution of the product and precipitating the homocystine at pH 5·5. After thirteen such precipitations the specific activity of the homocystine was low enough to indicate that even if it were uncontaminated with cystine, less than 0·1% of the radioactivity of the cystine sample could be in the form of homocystine.

Isolation of labelled methionine

Radioactive methionine was isolated from the mother liquor of the cystine precipitation after the addition of L-methionine. Preliminary experiments showed that it was not advantageous to add the methionine before the hydrolysis of the yeast protein, nor was the specific activity of the isolated methionine increased by prolonging the hydrolysis to 18 hr. In an initial series of experiments the methionine was isolated from the hydrolysate by the method of Hill & Robson (1934), using their mercuric acetate reagent to precipitate the methionine directly from the hydrolysate. It was found that the product always contained traces of cystine and that this contamination was extremely difficult to prevent. Preliminary removal of the cystine from the hydrolysate by precipitation with cuprous oxide removed large quantities of the active methionine even after the hydrolysate had been boiled with zinc dust previous to the precipitation (see, Bailey, Chibnall, Rees & Williams, 1943; Beveridge & Lucas, 1944). An attempt was made to remove the cystine from a sulphuric acid hydrolysate with mercuric sulphate, but the subsequently isolated methionine was still impure, showing that the cystine precipitation was incomplete, although the reagent appears to precipitate cystine nearly quantitatively from a pure solution (Lavine, 1937). It became necessary, therefore, to prevent radioactive contamination by reducing the specific activity of the cystine remaining in the hydrolysate to negligible proportions by the addition of cystine before isolating the methionine (Williams & Dawson, 1952).

It was then found that when the method of Pirie (1932) was used for the isolation of the methionine the product was free from detectable amounts of cystine. Although in this method the precipitate formed with mercuric acetate is extracted with hot baryta the resulting racemization of the L-methionine appears to be very small. In the original method for the isolation of methionine from casein (Mueller, 1923) which involves several similar extractions with hot baryta, the product had a specific optical rotation very close to the correct value for L-methionine. The butanol extraction of the methionine from the hydrolysate used in Pirie's method probably leaves a considerable portion of the cystine in the acid layer (Hess & Sullivan, 1935). Moreover, the removal of barium ions with sulphuric acid during the methionine isolation would remove cystine possibly as a double salt with barium sulphate (Chibnall, Rees & Williams, 1943).

The mother liquor from the cystine precipitation was acidified with 5 ml. conc. HCl, and evaporated under reduced pressure to a viscous residue. The hydrochlorides of the amino-acids were dissolved in ethanol, and the solution was filtered from a residue of salt. The filtrate was evaporated *in vacuo* and the residue re-dissolved in 50 ml. of 0·7N-HCl. L-Methionine (Nutritional Biochemicals Corp.: Cleveland) was added; 100 mg./g. of yeast protein residue hydrolysed. The method of isolation then followed closely the procedure of Pirie (1932), with a number of minor modifications. The hydrolysate was extracted with 200 ml. *n*-butanol which had previously been saturated with aqueous N-HCl. After a double extraction with aqueous NaOH solution as described by Pirie (1932), the combined alkaline extracts were adjusted to pH 5 with HCl and evaporated under reduced pressure until free of butanol. Water was added to 250 ml. and saturated mercuric acetate in 3% (v/v) aqueous acetic acid was added until no further precipitation occurred. The precipitate was centrifuged and washed once with water, suspended in 50 ml. of water and added to 200 ml. of boiling 0·33N-$Ba(OH)_2$. The mixture was filtered hot, and the residue again extracted with 200 ml. of boiling 0·14N-$Ba(OH)_2$. The combined baryta extracts were freed from Ba ions with H_2SO_4 and the solution evaporated to dryness under reduced pressure. The residue was taken up in 10 ml. of hot water, and the methionine precipitated by the addition of a boiling solution of 5 g. $HgCl_2$ in 60 ml. water. After boiling for a few minutes the mixture was cooled, and stored at 0° for 2 hr. when the supernatant was decanted from the putty-like mercury complex. The latter was kneaded with a small quantity of ice-cold water, and then transferred to about 70 ml. warm water and decomposed with H_2S. The HgS was removed and thoroughly extracted with boiling water. The combined filtrate and washings were re-filtered and evaporated to dryness under reduced pressure. The residue was taken up in 5 ml. of ethanol and the methionine precipitated by adjusting to pH 5 with pyridine and standing at 0°. The precipitate was dissolved in 2 ml. of hot water, filtered and crystallized by adding 7 ml. of ethanol. Yield: approximately 30% of the added L-methionine, as shining plates giving a correct analysis for N and S.

Two-dimensional paper chromatography failed to reveal the presence of other amino-acids. The counting rate with the counter previously described was approximately 2×10^7 counts/min./m-mole. The specific activity of the methionine did not change on recrystallizing twice from 80% (v/v) ethanol. The specific activity of the sulphur remained constant on conversion of the methionine into the α-naphthyl isocyanate derivative, m.p. 187–188° (Mueller, 1923), this being recrystallized from ethanol-water. The absence of cystine in the methionine was shown by running 0·2 mg. of it on a one-dimensional paper chromatogram using a water-saturated phenol solvent, after which the dried chromatogram was sprayed with ninhydrin solution. The methionine was initially oxidized with H_2O_2 according to the method of Dent (1948). It was observed that when this amount of methionine was treated on the paper with

H_2O_2 for a prolonged period, oxidation to the sulphone was far from quantitative. Even when ammonium molybdate was also present during the oxidation, two main spots could always be seen on the chromatogram. Moreover, it was found that pure samples of synthetic methionine on chromatography gave a faint ninhydrin spot with a small R_F value indistinguishable from homocysteic acid. It is assumed that during the oxidation of methionine with H_2O_2 a small portion is demethylated and oxidized to homocysteic acid. No cysteic acid spot could be observed on the chromatogram of the labelled L-methionine (<0·5% cystine). The absence of homocystine from the product was shown by adding DL-homocystine to a sample of the methionine dissolved in dilute HCl and isolating the homocystine at the isoelectric point. After purification the homocystine was found to have negligible radioactivity (<0·2%).

DISCUSSION

The requirements of a satisfactory method for the preparation of a labelled amino-acid are that the procedure should be reasonably simple, the starting materials easily obtained, the product of a sufficiently high specific activity and free from impurities either inactive or active. The preparation may need to be repeated several times during an investigation especially when the tracer used decays relatively quickly. The methods described in this paper for the preparation of ^{35}S-labelled L-cystine and L-methionine have proved in practice to be very suitable for the intermittent preparation of these amino-acids. They require only apparatus and materials normally found in a small biological laboratory. During a series of preparations the unused radioactive sulphate ions in the culture medium can be recovered by precipitation with barium. The barium sulphate is then fused with eight equivalents of potassium carbonate which quantitatively converts it into potassium sulphate. The fusion mass is then extracted with water and the filtered extract, after neutralization with phosphoric acid, is added to the next culture medium which is adjusted accordingly.

Yeast was found to grow well in a medium 25 times more radioactive than the one described in the present series of experiments, with no diminution in the yield of crude yeast protein. The activity of the medium could probably be increased considerably further before the β-radiation affected the growth of the yeast cells.

Although Pirie's (1932) method for the isolation of methionine was better than that of Hill & Robson (1934) in that it gave a product free from detectable amounts of cystine it suffers from the disadvantage that a very small racemization of the amino-acid may occur during the isolation. In the intact animal and in isolated tissue preparations, methionine can be rapidly converted into cystine (Tarver & Schmidt, 1939; Melchior & Tarver, 1947), so that in animal experiments a trace of cystine in labelled methionine is not likely to be of great significance.

SUMMARY

1. A simple biosynthetic method is described for preparing L-cystine and L-methionine labelled with radioactive sulphur (^{35}S).

2. Baker's yeast was grown in a synthetic medium containing labelled sulphate ions, and the amino-acids were isolated from the yeast proteins.

3. The amino-acids isolated had high specific activities.

4. No impurities, either inactive or radioactive, were detected in the isolated products.

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