ALTERATION OF CARBON METABOLISM BY A BASE ANALOG

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ABSTRACT The study of carbon metabolism by cultures of the yeast *C. utilis* exposed to 5-fluorouracil revealed that the growth rate and synthesis of macromolecules was altered. The amino acid composition of the metabolic pool of amino acids was vastly altered, but the protein composition was unchanged. It is suggested that the analog may exert a selective action on certain amino acids, and that this action may be related to a template-like mechanism.

The flow of sugar and other exogenous supplements into the macromolecules of the yeast, *Candida utilis,* has been described in a series of papers by Cowie and coworkers (1-5). They reported that precursor materials (amino acids, purines, and pyrimidines) were held within the cells in two different metabolic pools. One of these, the "expandable" pool, exists only when the culture medium is supplemented with precursor materials. This pool may be emptied either by loss to the external medium or by passage to the second pool, the "internal" pool. The latter is always present in the cell in fixed quantity. It may be removed by extracting the cells with cold 5 per cent trichloroacetic acid (TCA), or by boiling the cells in water. After such extraction, the internal pool contents are found to be principally amino acids, with small quantities of nucleotides and other small biochemical intermediates. These internal pool compounds comprise 13 per cent of the cellular carbon and 29 per cent of the phosphorus. This carbon level is unaltered by carbon starvation, abrupt temperature changes, cessation and initiation of macromolecular synthesis, or by alteration of the external osmotic or ionic strength.

Isotopic studies showed that the internal pool contents were the obligatory precursors to protein and nucleic acid synthesis within the cell. The amino acids found within this pool were derived from the products of sugar metabolism, and also from the expandable pool and external medium when the synthetic culture medium was so supplemented. Exogenous sugar was taken up by the cells and converted to those products associated closely with the Krebs cycle (glutamic acid, alanine, aspartic acid, pyruvate, etc.). These materials, referred to as the "biochemical family heads," were fed directly to the internal pool where they were then converted to the "daughter" or "product" amino acids. These were the only sources of internal pool carbon.

The only loss from the pool was to the proteins and nucleic acids in the case of pool nucleotides.

The amino acid content of the internal pool was found to have certain remarkable characteristics (1, 3). As has already been pointed out, the amino acids in the internal pool are insensitive to osmotic shock or to competition with exogenous amino acids. In the internal pool, amino acid molecules in a parent-daughter relationship (such as glutamic acid and arginine) are each independently unaffected by the addition of either compound to the medium.

The distribution of amino acids in this pool is also remarkably constant. More than 50 per cent of the molar content of the internal pool is in the form of alanine and glutamic acid. Appreciable quantities of arginine, valine, histidine, and lysine are also found. The remaining amino acids are present in small quantities. However, this pool distribution does not correspond to the amino acid content of the cellular proteins. This can be accounted for by assuming that different amino acids in the pool turn over into protein at different rates.

Some information concerning the specificity of the pool for amino acids has been obtained by supplying the yeast with exogenous mixtures of parafluorophenylalanine and phenylalanine in a known molar ratio (4). The cells were found to incorporate both compounds into the expandable pool, showing a preferred selection for the natural compound. Passage of these labeled molecules from the medium and the expandable pool into the internal pool was accompanied by a second selection in which there was a marked preference for the natural amino acid. However, once inside the internal pool, the relative amounts of analog and natural compound were not altered in passage out of this pool and into the final protein. It appears, therefore, that the last step in selection between analog and phenylalanine occurred at the time of entrance into the internal pool. It was suggested that amino acids and analogs in the internal pool might be already selected by a protein-forming "template" but not yet linked into polypeptide strands.

Because of the current belief that ribonucleic acid (RNA) is in some way related to protein synthesis, and may possibly be one of the molecular components of a template, it seemed of interest to see what effects a nucleic acid base analog would have on the process of protein synthesis. In *Candida utilis,* the incorporation of 5-fluorouracil (FU) was found to be exclusively into RNA (5), with no incorporation into the DNA. This analog therefore offers the possibility of a distinct perturbation of the process of protein synthesis.

MATERIALS AND METHODS

Cultures of *Candida utilis* (American Type Culture Collection No. 9226) maintained on agar slants were grown overnight at 30°C in minimal medium[1] (6) with fructose as a

[1] Minimal medium: 2 gm NH_4Cl, 6 gm Na_2HPO_4, 3 gm KH_2PO_4, 3 gm NaCl, 0.01 gm Mg as $MgCl_2$, 0.026 gm S as Na_2SO_4, 100 ml 10 per cent fructose, and 900 ml distilled H_2O.

sole source of carbon and energy. Exponentially growing cultures were subcultured into fresh medium supplemented with radioactive (C^{14}) fructose and appropriate carrier to bring the tracer to the desired concentration. Radiofructose (uniformly labeled, 11.0 μc/mg) was obtained from New England Nuclear Corporation, Boston, Massachusetts. Some cultures were supplemented with 5-fluorouracil at a level of 10 μg analog/ml culture medium.

The growth of cultures was followed turbidometrically in a Beckman model DU spectrophotometer at 6500 A.

50 or 100 ml samples of culture were centrifuged at appropriate times and washed with minimal medium. The pellet of cells was treated with 20 ml distilled water and recentrifuged. The yeast were then extracted in the cold with 5 ml of 5 per cent TCA. After 2 hours, the cells were centrifuged and the supernatant was saved. The pellet was then extracted with 5 ml of ethanol-ether (1:1 v:v) at 45°C for 20 minutes. The nucleic acid was removed from the cells by treating the pellet with 10 per cent TCA at 100°C for twenty minutes, and the final protein precipitate was dissolved in hydrochloric acid. Aliquots of each fraction were evaporated to dryness on stainless steel planchets and assayed for radioactivity under a thin-window Geiger tube with 15 per cent efficiency for carbon 14.

Orcinol determinations of RNA were made according to the method of Hurlbert *et al.* (7). DNA was detected by the method of Ceriotti (8). Protein measurements were made using the Lowry modification of the Folin-Ciocalteu test (9).

Amino acid analyses were performed on a Spinco amino acid analyzer.

RESULTS

In the experiments described here, cultures of *C. utilis* were grown in an inorganic medium with radiofructose as the sole source of energy and carbon; experimental cultures were also supplemented with a small quantity of FU (10 μg/ml culture medium). Under these conditions, the water-soluble "expandable" pool will contain only very small quantities of the free FU base (5); almost all of the low molecular weight compounds of the cell will be in the internal pool (1-5). At the concentration of FU used here, cellular growth will continue for about 6 hours. The mass increase proceeds linearly with time, and doubles in about 300 minutes (5).

The Internal Pool

Unlabeled cultures of *C. utilis* were harvested, washed in fresh medium, and resuspended in C^{14}-fructose medium with or without the addition of FU. During a 300- to 400-minute growth period, samples were taken and chemically fractionated as described in Materials and Methods. Typical incorporation curves of radiocarbon into the internal pool of normal and FU-treated cells are shown in Fig. 1. Although the initial rates of incorporation are similar, the final pool saturation was found to be 20 per cent greater in the analog incubated cells. 2-dimensional chromatograms of the pool contents revealed no new radioactive spots that might be accumulating.

A second yeast culture was grown in C^{14}-fructose medium for 24 hours. Under these conditions, more than 99 per cent of the cellular carbon will have been derived

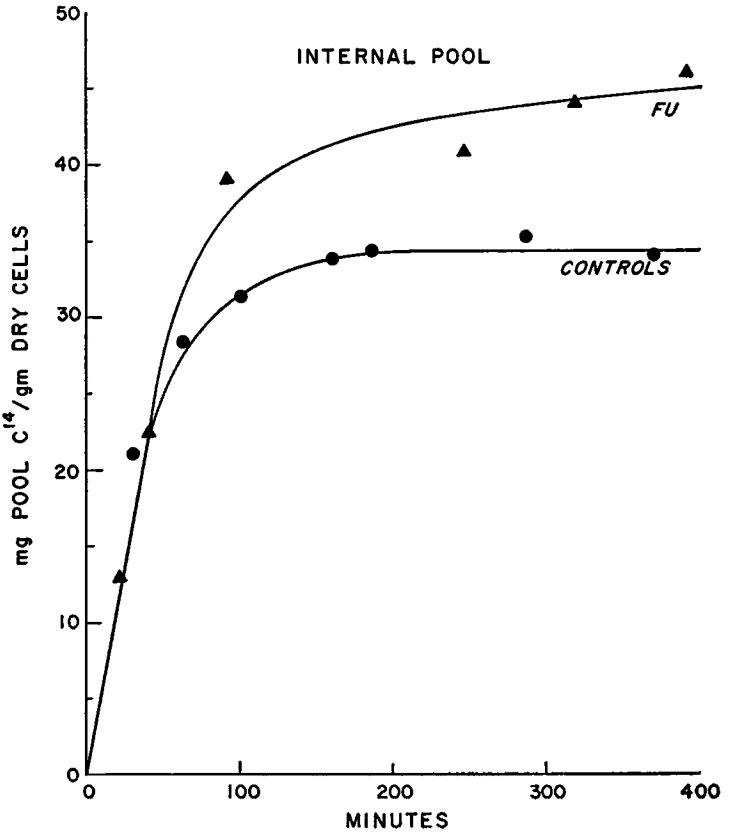

FIGURE 1 Appearance of radiocarbon in the internal pool. The absolute pool size reaches a steady value within 200 minutes in control cells. In FU-supplemented cultures, the pool continues to rise to a value larger than that of the controls.

from the radioactive source. The culture was divided in two, and 10 μg FU/ml culture medium was added to one. Both cultures were then grown in the continued presence of the labeled sugar. The internal pool, which was now totally labeled, was extracted and measured during a 300-minute growth period. The size of the pool (in milligrams C per gram of dry cells) at the time of addition of the analog was taken as 100 per cent; the pool size at succeeding times was expressed in terms of this. In Fig. 2 it is seen that there is a 20 per cent rise in the absolute size of the internal pool in the presence of FU.

In order to investigate more thoroughly this effect of the analog, cells were grown in carrier-free radiofructose with and without FU for 100 minutes. The yeast were then centrifuged, washed with unlabeled medium, and resuspended in a medium containing the same quantities of FU and a 10,000-fold increase in non-radioactive sugar. During the succeeding growth period, the loss of radioactive pool materials

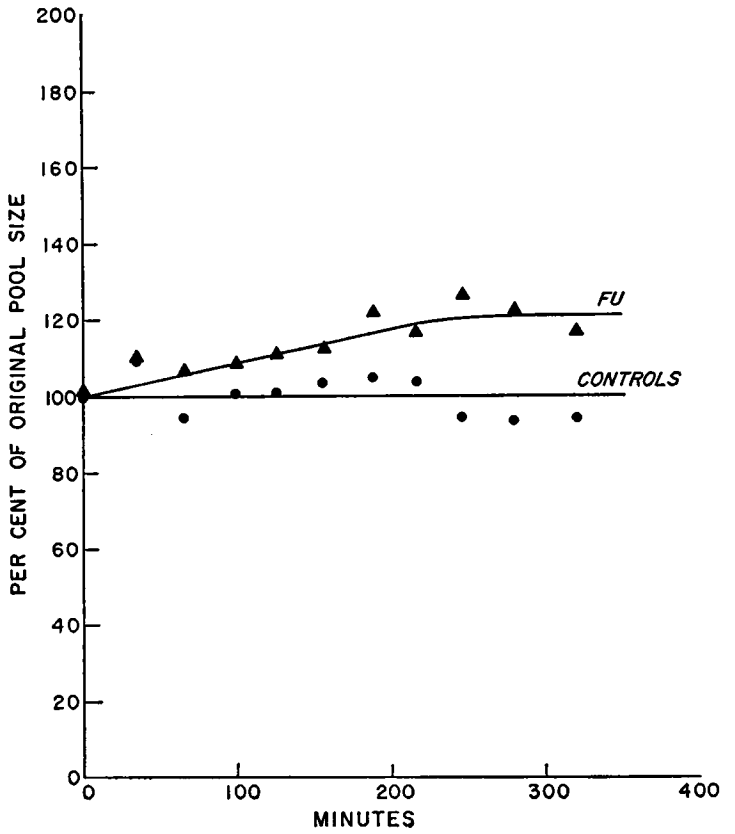

FIGURE 2 Pool size of totally labeled cells incubated with or without FU. The analog-formed pool becomes 20 per cent larger than normal.

was followed. The results of these experiments are shown in Fig. 3. The initial loss of pool materials is independent of the analog, but a small fraction of the pool formed in the presence of FU is seen to be kinetically less labile than in the controls. It is not known whether these inhibited compounds are amino acids or the other intermediates found in the pool.

Since 85- to 90 per cent of the pool materials are amino acids (3), and since 2-dimensional chromatography revealed no new radioactive spots in the analog-formed pool, it is necessary to look at the distribution of amino acids contained in the internal pool under normal and analog conditions. Three separate preparations of the pool from cultures grown with FU for 300 to 400 minutes and three normal cultures were analyzed on a Spinco amino acid analyzer. The results of a typical analysis are given in Table I. The total amino acid pool as measured by the ninhydrin reaction in the analyzer reflects the increase in pool size found by radiocarbon determinations.

The contents of the normal yeast pool are in reasonable agreement with that reported by Cowie and McClure (3); however, this distribution is dramatically altered by the analog FU. Alanine, which is normally one of the largest components of the pool, is reduced by a factor of two. All of the other amino acids are either unaltered (methionine, serine, glutamic acid, and ornithine) or increased in abundance. The most striking change in distribution is the fivefold increase in the quantities of some of the more acidic amino acids (aspartic acid, threonine, tyrosine, and proline). The basic amino acids (hydroxylysine, lysine, histidine, and arginine together with cysteine) seem to be two to two and one half times more abundant, while the more neutral amino acids (glycine, isoleucine, γ-aminobutyric acid, leucine, phenylalanine and valine) are only slightly enriched. No tryptophan could be detected in the internal pool of either control or experimental samples.

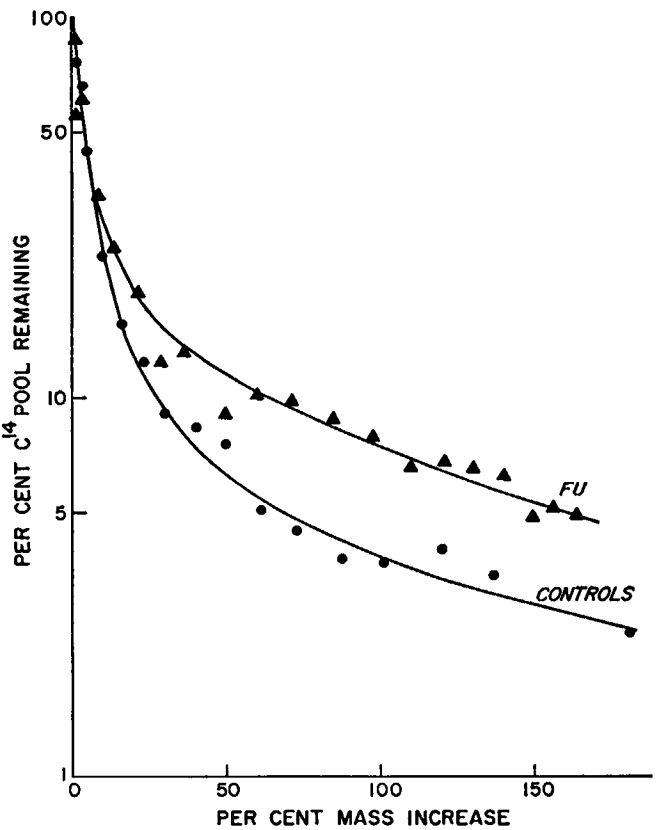

FIGURE 3 Internal pool of yeast cells labeled with radiofructose (both normal and FU-incubated) for 100 minutes and transferred to non-radioactive media. The loss of C^{14} from the pool as a function of mass increase shows a small component of the pool formed in the presence of the analog to be less labile than in controls.

TABLE I

AMINO ACID DISTRIBUTION OF INTERNAL POOL AND PROTEIN OF *CANDIDA UTILIS*

Micromoles per gram of dry cells

Amino acid	Pool Controls	Pool FU-treated	Ratio FU-treated/Controls	Protein*
Alanine	103.5	51.3	0.50	175.5
γ-aminobutyric acid	0.25	0.45	1.80	0
Arginine	22.7	48.1	2.12	82.5
Aspartic acid	0.27	1.43	5.30	183.8
Cysteine	0.28	0.61	2.18	
Glutamic acid	82.5	102.5	1.24	215.6
Glycine	1.2	2.24	1.87	140.4
Histidine	3.09	7.89	2.55	34.5
Hydroxylysine	0.17	0.49	2.88	
Isoleucine	0.48	0.89	1.85	78.1
Leucine	0.44	0.66	1.50	144.6
Lysine	3.25	6.89	2.12	146.7
Methionine	0.24	0.19	0.79	25.1
Ornithine	2.40	2.69	1.12	0
Phenylalanine	0.13	0.19	1.46	61.8
Proline	0.17	0.77	4.55	78.5
Serine	1.54	1.76	1.14	134.5
Threonine	1.46	7.21	4.94	113.6
Tryptophan	0	0		
Tyrosine	0.26	1.35	5.19	34.5
Valine	11.3	16.4	1.45	136.2

* Amount recovered from acid hydrolysis; about 30 per cent of total.

Ethanol-Ether–Soluble Fraction. The lipid material extracted in the ethanol-ether–soluble samples was measured for radioactivity. The rates of incorporation were found to be unaltered by the presence of the analog. In all succeeding experiments of "steady-state" and "wash-out" labeling this fraction was similarly unaffected. No further analysis of these lipids was undertaken.

Cells grown in C^{14}-fructose with and without FU were transferred to non-radioactive media containing the same concentration of analog. There was no loss of C^{14} from any fraction other than the cold TCA-soluble pool, indicating that the lipids, nucleic acids, and proteins synthesized normally or under analog conditions are stable and are not degraded to any appreciable extent.

Hot TCA–Soluble Fraction. The RNA and DNA extracted in hot TCA-soluble materials were detected by radioactivity and by color reactions. The orcinol determinations were somewhat variable, but the DNA measurements made by the method of Ceriotti were more reproducible.

It has already been shown that the rate of incorporation of radiouracil into RNA is reduced in the presence of FU (5). The rate of radiofructose incorporation is

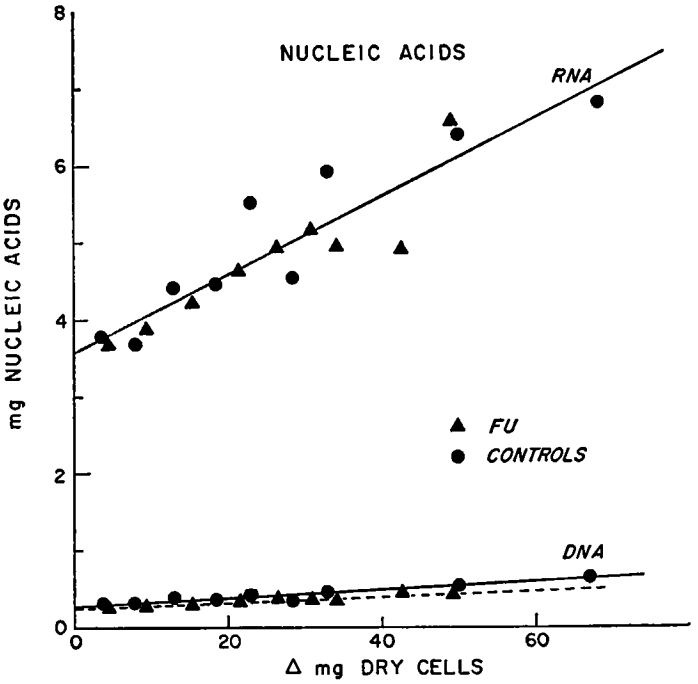

FIGURE 4 Nucleic acid synthesis in yeast in the presence of FU. Circles represent normal cells; triangles represent cells exposed to analog. RNA determinations by orcinol reaction. DNA measured by the method of Ceriotti.

similarly depressed. Both these rates were expressed as micrograms of labeled material incorporated as a function of time. Since the growth of analog-incubated cultures is altered, it is preferable to express this as μg of material per change in dry weight of cells. This is done in Fig. 4 for the RNA and DNA color determinations. Although the orcinol-RNA data scatter somewhat, the data indicate that the synthesis of RNA is proportional to growth, independent of the presence of the analog. In the case of DNA, there may be a slight reduction in the rate of synthesis in the presence of FU.

Hot TCA–Insolube Fraction. The proteins found in the residue pellets after the above extractions were analyzed by radioactivity measurements and by the Folin-Ciocalteu-Lowry reactions. The total protein measurements indicated that the rate of synthesis was proportional to growth: exponential increases in protein content were found in the control cultures (where growth was exponential) and linear increases in cells exposed to FU (linear growth). In Fig. 5, the incorporation of radiocarbon from fructose is shown to be directly proportional to the increase in total mass of cells and independent of the presence of the analog.

The proteins synthesized under both control and experimental conditions of growth were hydrolyzed in hydrochloric acid and analyzed for amino acid content

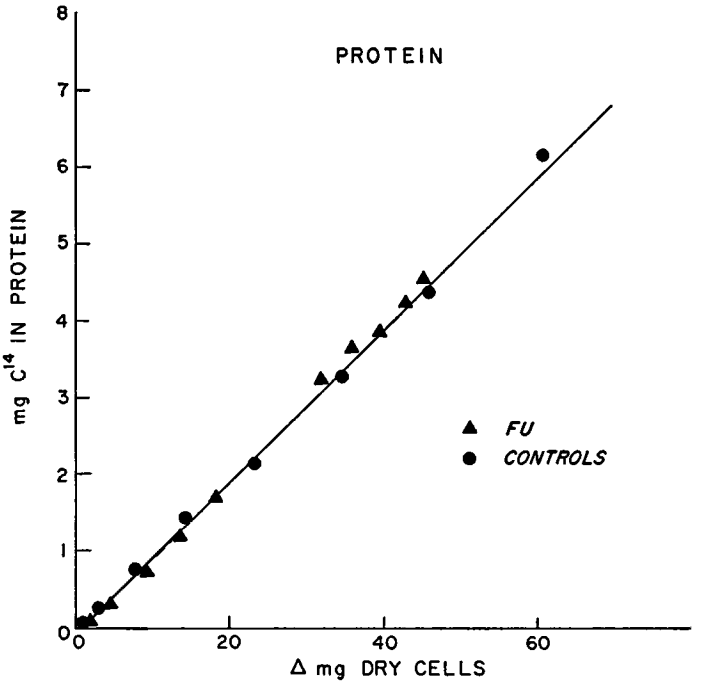

FIGURE 5 Incorporation of radiocarbon into the proteins of yeast. Circles represent controls; triangles represent cells grown in FU.

as in the case of the internal pool. The amino acid composition of the proteins from control cultures is shown in Table I. The distribution of amino acids in FU culture proteins was the same within experimental error (7 per cent in one experiment, 5 per cent in a duplicate analysis).

DISCUSSION

The most obvious effect of the analog 5-fluorouracil on cultures of *Candida utilis* is the alteration in growth (5). As is the case with amino acid analogs, the mass increase changes from an exponential to a (apparently) linear function of time. The kinetic flow of carbon into macromolecules is similarly disturbed.

The most extreme change in cellular metabolism was observed in the internal pool. Two of the major characteristics of this pool changed in the presence of the analog. There was a 20 per cent increase in the absolute carbon content in the form of amino acids, and the distribution of these amino acids was sharply perturbed. The more acidic amino acids were found to be five times more abundant than normal. The basic amino acids increased two to two and one half fold. The neutral amino acids accumulated about 60 per cent more than normal. A few amino acids were essentially unaltered in abundance, and alanine was depressed to half of the control

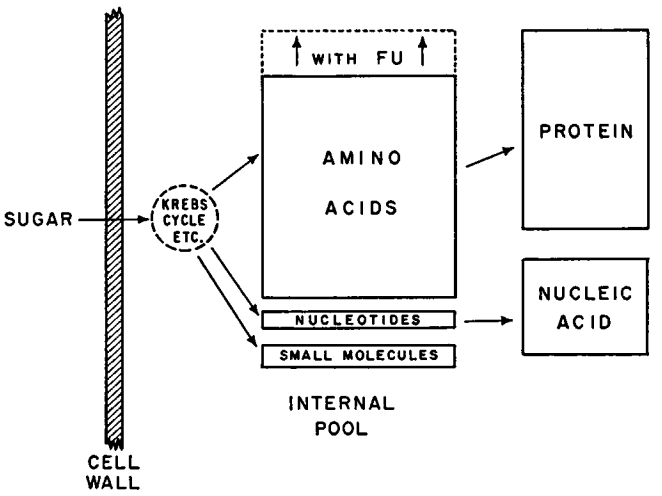

FIGURE 6 Flow of exogenous carbon in yeast. Pool size of amino acids increases by 20 per cent in the presence of FU.

value. No tryptophan could be found in either control or FU-formed pools. The flow of the internal pool carbon into proteins and nucleic acids was only slightly altered, as if a small component of the pool was kinetically inhibited. In Fig. 6, the model of carbon flow (4) has been modified to show the action of 5-fluorouracil.

In a previous report (5) it was shown that the incorporation of radioactive uracil in the presence of FU was accompanied by an abnormal distribution of particulate to non-particulate RNA. The data indicated, however, that the total quantity of uracil incorporated into RNA per unit mass was unaffected by the analog. Both radiocarbon and the orcinol determinations reported here support this finding. The primary action of the analog is on the growth rate; the synthesis of macromolecules remains proportional to mass.

The protein content of yeast similarly parallels mass increase. In the presence of FU, both the relative quantity of protein carbon and Folin-reactive material increase linearly with mass with the same slope found in the control cultures. The amino acid analysis of the gross protein preparations revealed no differences between FU and normal cells. This agrees with the results obtained by Aronson (10) on *E. coli* grown in the presence of the same analog, but differs from that of Naono and Gros (11). It had already been shown that *E. coli* could synthesize protein in the presence of the analog (12).

The alterations of amino acid concentrations in the pool on exposure to FU is caused by some unknown mechanism. Uracil and its derivatives have not been reported as cofactors in the synthesis of amino acids. It therefore seems unlikely that the effect of FU is involved with any biochemical step previous to the amino acid itself. Because of the specificity of the internal pool for phenylalanine and para-

fluorophenylalanine, it has been suggested (4) that the internal pool contains a template-like selective mechanism in which the amino acids have been chosen but not yet linked in polypeptide chains. If this suggestion is correct, it indicates that FU is being incorporated into a template RNA and then exerting an abnormal selection process on some of the amino acids. However, since the final proteins produced have a normal amino acid distribution, these analog templates must be incapable of passing the (incorrectly) selected amino acids into the end product.

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