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Serum 'uracil + uridine' levels in normal subjects and their possible significance

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SYNOPSIS A microbiological method for the assay of uracil is described. The growth of the test organism is supported by uracil and also by uridine but not by uridylic acid. The method therefore measures uracil and uridine together. The 'uracil + uridine' level, expressed as uracil, has been measured in blood from 144 normal subjects ranging in age from cord blood to the eighth decade. The mean level of 22 μ mol/l (0·25 mg%) in cord blood decreases to 15 μmol/l (0·17 mg%) in adults over the age of 20. There is no difference between the sexes.

Uracil is of interest because (a) it is a constituent base of RNA, (b) it is the precursor of two of the bases thymine and cytosine that enter into the composition of DNA, and (c) under certain circumstances it has mutagenic properties. The last is dependent upon the existence of two tautomeric forms of uracil, the common keto form which pairs normally with adenine and the rare enol form which pairs with guanine. A mistake in base pairing which allows uracil in its enol form to enter the DNA molecule and pair with guanine can result in a G = C → A = T base transition in the DNA molecule. The molecular mechanism involved as well as the possible bearing on somatic mutation are discussed.

Few attempts have been made to measure blood pyrimidine levels although microbiological assay methods were described by Merrifield and Dunn in 1948. We have recently applied these to human sera but preliminary experiments showed that neither cytosine nor thymine could be satisfactorily measured. A suitable microorganism is not available for the assay of cytosine on its own and thymine could not be demonstrated even when a concentrated ultrafiltrate equivalent to 8·0 ml of serum was added to the assay tubes. It was readily demonstrated however with *Streptococcus* sp, NCIB 8191 (*Str. faecalis* ATCC 9790) as test organism using the conventional amounts of protein-free filtrate (≡ to 0·66 ml of serum) after thymine had been ingested by mouth. We calculated that if thymine is present in serum its concentration is of the order of 0·5 μ mol/litre (0·006 mg%) or less. After testing 123 different organisms Merrifield and Dunn found three with a specific growth requirement for uracil. These were *Lactobacillus helveticus* 335, *L. helveticus* 6345, and *Str. lactis* 7963. We abandoned the first after exhaustive trials because we failed to obtain a satisfactory and reproducible growth curve. We have no experience of the second but *Str. lactis* 7963 (which was kindly obtained for us from the United States by the Torry Research Station, Aberdeen) behaved more consistently and within well defined limits (see below) gave satisfactory growth curves.

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Materials and Methods

CHEMICALS

Uracil, uridine and uridine-2′(3′)-monophosphoric acid (mixed isomers) all chromatographically homogenous, and uridine-5′-monophosphoric acid, disodium salt (U.M.P.), were obtained from BDH Chemicals Ltd. In addition uridine (crystalline), uridine 2′ + 3′-monophosphoric acid (mixed isomers), and uridine 5′-monophosphoric acid, disodium salt (crystalline) were also obtained from Sigma London Chemical Co. Ltd. All solutions were made up in distilled water of analytic reagent purity (BDH, AnalaR water).

THE TEST ORGANISM

Streptococcus lactis (National Collection of Industrial Bacteria no. 10769, American Type Culture

Collection no. 7963) is on first isolation strictly uracil dependent. After several subcultures it has in our hands undergone a fairly sudden change whereupon it grows profusely in the absence of uracil. This has happened with three separate freeze-dried subcultures and occurs after daily subcultures for about three months. Bacterial contamination could not be demonstrated and this, together with the fairly constant pattern, led us to conclude that the organism had undergone mutation with the emergence of a uracil-independent mutant; we consider the time interval too long for a simple selection from the original culture. As an important factor in inducing mutation appears to be the number of times the organism is subcultured, we now, as soon as the initial freeze-dried culture grows well in maintenance broth and gives a satisfactory growth curve, make several 'master' cultures on maintenance agar slopes. These are kept at 4°C in a refrigerator. One master culture is used at a time and a maintenance agar plate is inoculated from it at weekly intervals and incubated anaerobically for 48 hours. The same single colony from the plate is inoculated into both maintenance broth (containing uracil) as well as into a uracil-free broth and incubated aerobically. There should be no growth in the latter when measured in the spectrophotometer. The inoculum for the actual assay is obtained from a 24-hour growth in maintenance broth as previously described for amino acids (Blackmore and Parry, 1972). A master slope can be used in this manner for three or four weeks when it is subcultured onto another agar slope. It is discarded and another master culture used at the earliest sign of mutation which is heralded by increased growth in the maintenance broth, erratic growth in the standard tubes, and finally by growth in the uracil-free broth. We hope in this manner to prolong the useful life of the uracil-dependent culture and the method has already improved the reproducibility of the standard graph.

THE ASSAY MEDIUM

The single-strength amino acid assay medium of Dunn, Shankman, Camien, and Block (1947) was modified in the following way: (a) Ammonium chloride was omitted and hypoxanthine (0·001%) added as suggested by Merrifield and Dunn (1948). (b) Because microorganisms generally do not respond to the D- isomers of amino acids (Blackmore and Parry, 1972), all the amino acids listed in the DL-form with the exception of nor-valine and nor-leucine, the L-forms of which were not available commercially, were incorporated in the medium in the L-form in one half of the recommended quantities, ie, 334 instead of 667 mg/litre. This facilitated the solution of otherwise fairly large quantities of amino acids. (c) L-aspartic acid (334 mg/litre) was incorporated in the medium. (d) Cytosine and thymine (0·001%) were added but uracil was omitted.

The medium is initially made up at double strength and glucose (4%) is added immediately before use. For the maintenance of the test organism this is diluted with an equal volume of water and uracil (0·001%) added. One per cent agar powder is added to provide maintenance agar. Penicillinase to a final concentration of 1/250 is added to each assay tube immediately before inoculation.

PROTEIN PRECIPITATION

To 3·0 ml of serum is added 3·0 ml of 0·05 N acetic acid and 3·0 ml of water. The tube is placed in a boiling water bath for three to five minutes and the precipitated protein removed by centrifugation. After adjusting the pH of the supernatant to 6·8, 2·0 ml ($\equiv$ 0·66 ml of serum), together with 2·0 ml of double-strength medium are added to the assay tubes giving a final volume of 4·0 ml.

THE ASSAY PROCEDURE

This is done in triplicate as previously described for amino acids (Blackmore and Parry, 1972) except that only one dilution of serum is employed and the assay tubes are incubated for two nights instead of one, ie, for 42 to 48 hours. A stock standard solution of uracil is prepared by dissolving 0·112 g of uracil in 200 ml of water ($0{\cdot}5 \times 10^{-2}$M) and a working standard by making 0·8 ml of this up to 200 ml with water ($0{\cdot}2 \times 10^{-4}$M). This, using the volumes given in the amino acid method, gives a final concentration in the assay tubes of 1, 2, 3, 4, 6, 8, and 10 μmol/l. For optical density readings we found incubation at 37°C more satisfactory than at 32°C which was recommended by Merrifield and Dunn for their acid titration method.

All cultures are killed by adding one drop of 40% formaldehyde to each tube and after standing for five to 10 minutes the optical densities are read at 600 nm in an Optica CF 4 spectrophotometer using 1·0 cm cuvettes.

Normal Subjects

The normal subjects included in this study comprised (a) members of the hospital and laboratory staff; (b) healthy blood donors; (c) children admitted to hospital, usually for some minor surgical procedure such as repair of a hernia or orchidopexy but were otherwise normal and in whom a venepuncture was indicated for some other purpose, eg, for blood grouping and crossmatching; (d) cord blood from

six normal full-term infants (three at 40 weeks', two at 39, and one at 41 weeks' gestation); (e) normal elderly subjects. An old people's home was visited and a number of volunteers were bled. Blood samples from a number of elderly subjects were also obtained through their general practitioners. A full blood count as well as serum B_{12} and folate assays were done whenever possible and particularly so on the elderly. Only those with normal values were included.

Results

Although the original authors investigated the growth requirement of microorganisms for the free pyrimidine bases only, we have found that the growth of *Str. lactis* is supported equally by uracil and by uridine but not by uridine-2'(3')-monophosphoric acid (uridylic acid) (fig 1). The method therefore measures 'uracil + uridine' and this is expressed as uracil. Uridine and uridine-2'(3')-monophosphoric acid obtained from the two different sources behaved alike. Moreover the disodium salt of uridine-5'-monophosphoric acid obtained from the two sources gave results similar to those obtained with uridine-2'(3')-monophosphoric acid.

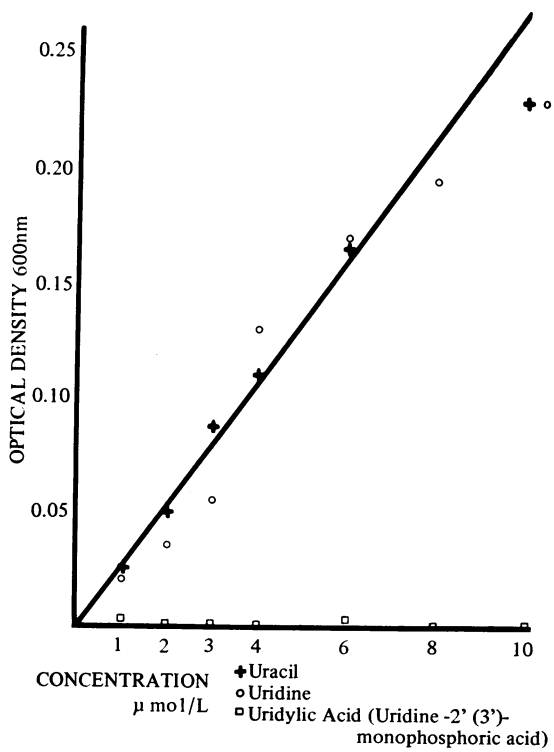

Fig 1 *Typical growth curve for uracil. Uridine supports the growth of* Str. lactis *to the same degree as uracil. Uridine-2' (3')-monophosphoric acid does not support the growth of the organism.*

Recovery Experiments

The recovery rates after adding 10, 20, and 30 μmol/l to three sera in five different experiments are shown in table I. The fairly wide scatter with the two smaller quantities is considered to be due to the very small amount of uracil added (= 0·12 and 0·22 mg% respectively). A minute error in the recovery would, under these circumstances, result in a fairly large percentage error.

Serum Sample	*Uracil Added (μmol/l)*		
	+10	*+20*	*+30*
1	96	86	94
	101	116	94
2	95	95	106
	130	87	92
3	120	114	89
Mean	108	100	95

Table I *Recovery rates (%) after adding uracil to normal serum*

A number of normal sera were included as controls in successive assays and were repeatedly assayed from four to 11 times. Altogether seven sera were assayed a total of 45 times. This enabled the reproducibility of the method to be calculated. The mean of the means of the seven samples was 14·64 and the standard deviation between different assays of the same serum, pooled over all samples, was 2·73 giving a coefficient of variation of 19%.

The level of 'uracil + uridine' has been measured in the sera of 70 males and 68 females ranging in age from early childhood to the eighth decade as well as in the cord blood of six normal full-term infants. The mean values for males and females in each decade is shown in table II. Analysis of variance

Age	*Male*	*Female*	*Combined Sample*		
			No.	*Mean*	*SD*
Cord blood	—	—	6	22·0	7·7
0- 9	17·5 (13)[1]	18·0 (6)	19	17·6	7·6
10-19	19·0 (7)	13·8 (6)	13	16·6	5·4
20-29	14·2 (7)	17·2 (6)	13	15·5	3·2
30-39	13·7 (6)	14·2 (12)	18	14·0	4·0
40-49	15·1 (8)	13·8 (9)	17	14·4	4·0
50-59	16·0 (8)	15·2 (9)	17	15·6	4·4
60-69	13·5 (12)	15·7 (11)	23	14·5	3·2
70-79	16·8 (9)	14·1 (9)	18	15·4	5·9
All ages	15·8	15·1	144	15·4	—

Table II *Mean levels of uracil + uridine in serum*

[1]Number of sera examined.

shows that the level does not depend on sex and decreases ($P < 0.01$) during childhood up to the age of 20 years; it is particularly high in cord blood where the mean value is 22 μmol/l (= 0·25 mg%). It levels off to about 15 μmol/l (= 0·17 mg%) in adults over the age of 20, the mean value for this group being 14·9 μmol/l. The best estimate for the standard deviation is 4·96 $\simeq$ 5·0. Approximately 5·0 mg of 'uracil + uridine' therefore circulates in the blood throughout adult life.

Discussion

Uracil is of interest because it is itself a component of RNA and the precursor of two of the bases—thymine and cytosine—which enter into the composition of DNA. Its main interest, however, is that under certain circumstances it has mutagenic properties (Freese, 1959; Vielmetter and Schuster, 1960; Freese, 1963). The molecular basis of the latter is illustrated in figs 2a and 2b (modified from Freese, 1963) and is dependent upon its ability to pair in its rare enol form with guanine in addition to its usual pairing in its common keto forms with adenine (fig 2a). When this occurs a G = C[1] pair can on replication become G = U (U representing uracil in its enol form). On further replication uracil can undergo tautomeric or ionic shift and pair normally with A, G = U → A = U, and in a subsequent replication A can pair normally with T; A = U → A = T. In this manner a G = C → A = T base transition can be brought about in the DNA molecule (fig 2b).

The demonstration of a substance in measurable quantities in human serum which is capable of inducing mistakes in base pairing is of considerable interest. As far as the authors are aware no other substance with similar properties has hitherto been described in blood. Burnet's 'forbidden clone' as well as the normal process of aging, to mention only two, are important phenomena which have been attributed to somatic mutation (Burnet, 1962; Burnet, 1965; Comfort, 1963; Curtis, 1968), and, although the factors inducing and controlling them are still unknown, the demonstration of a mutagenic substance (uracil) in blood presented here provides at least one possible mechanism by which such mutations can be brought about at the molecular level.

[1]A = adenylic acid, C = cytidylic acid, G = guanylic acid, T = thymidylic acid and U = uridylic acid.

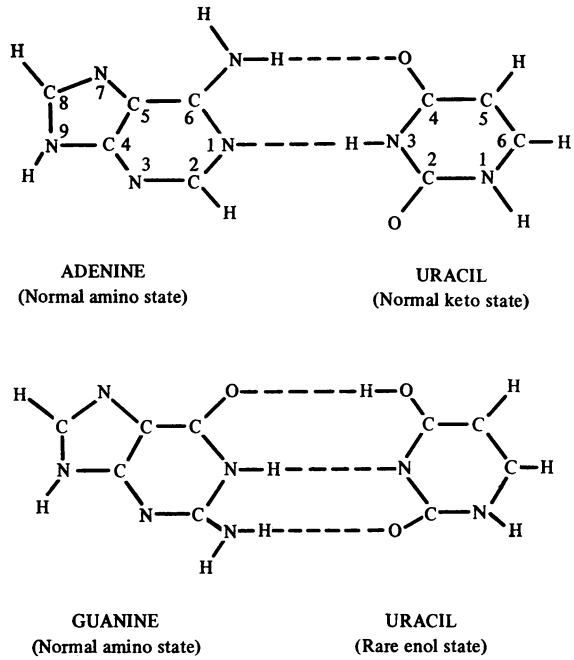

Fig 2a *The pairing of uracil in its normal keto state with adenine and in its rare enol state with guanine.*

We wish to thank Professor H. Campbell for the statistical analysis, the Curator of the Torry Research

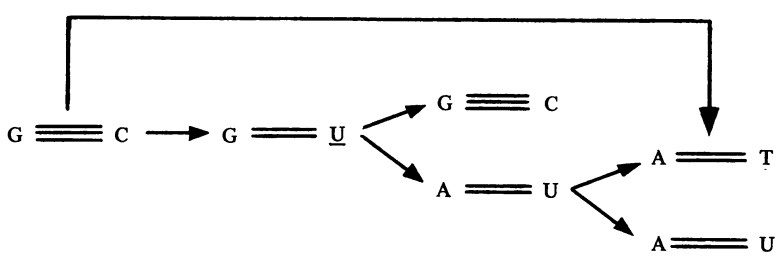

Fig 2b *The subsequent base pairing following the entry of uracil in its enol form into the DNA molecule to pair with G resulting in a G = C → A = T base transition.*

Both figs 2a and 2b are modified from Freese (1963). In the original of fig 2a, 5-bromuracil is illustrated in its keto and enol forms. Its base pairing properties, however, are the same as those of uracil.

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