Excretion of cystine and the dibasic amino acids by the 2 daughters of a Type I–III cystine stone former

Source	mg/day					
	Sample	Cystine	Lysine	Arginine	Ornithine	Homocysteine Cysteine
C.	1st	8	37	2.9	1.5	3.3
	2nd	13	62	3.4	2.5	
E.	1st	15	73	3.5	2.9	3.9
	2nd	13	47	4.4	2.1	
Normal, up to		37	28	4.0	2.0	2.3

the families of record were recognized as 'double heterozygotes' through the different excretion patterns of cystine and the dibasic amino acids by 2 or more presumed carriers in each pedigree, either by the 2 parents or by 2 or more children of the stone formers. The 'double heterozygotes' resembled homozygotes clinically and biochemically and were not distinguished without the family studies.

Our analysis of amino acid excretion rates in 2 children of a Type I–III 'doubly heterozygous' stone former[3] adds more data to the evidence for allelism in cystinuria and broadens the interpretation of the carrier state in the Type III form.

Materials and methods. Urine from the 2 children of a Type I–III stone former, both healthy daughters, aged 4 (C) and 7 years (E) when first sampled, was collected for 24 h with HCl preservative and was assayed for amino acids by nitroprusside reaction[7], paper chromatography[8,9], and ion exchange chromatography in a Beckman 120C analyzer[10]. The daily excretion rates of cystine and the dibasic amino acids were calculated from the column chromatographs and were compared with published values[11] and with rates from 9 children of laboratory personnel, aged 3 to 13 years. The mixed disulfide of cysteine-homocysteine was identified in assays in which the buffer change in the 59 cm column of the analyzer was postponed until 340 min[12]. Second samples were obtained from the girls 2 years later and were assayed by similar procedures.

Results. Positive nitroprusside-cyanide reactions and paper chromatography suggested that the first samples of urine from both girls contained excessive concentrations of cystine. The daily excretion rates of cystine, however, were within normal ranges. The excretion rates of lysine and of the mixed disulfide, cysteine-homocysteine, at the first sampling, were greater than normal. Those of ornithine and arginine by the older girl were in the 'high normal' ranges (Table). Methionine sulfoxide excretion by both was normal.

The rates of cystine excretion by the girls when they were 2 years older were again normal. Lysine excretion rates were still greater than normal, however, and those of ornithine and arginine were in the 'high normal' ranges for both (Table).

Discussion. The offspring of either homozygous or 'doubly heterozygous' cystine stone formers are heterozygotes, if the other parent is normal. They may or may not express the gene defect biochemically, depending upon the form of cystinuria of the affected parent. If the genes for the 3 known disease forms are alleles[2], the biochemical characteristics of one or another form should be found in the individual offspring, not those of 2 forms combined. The 2 daughters of the Type I–III stone former, a 'double heterozygote', should excrete, accordingly, either normal amounts of the amino acids, as do Type I carriers (and their grandmother), or slight excesses of cystine and the dibasic amino acids, as do Type III carriers (and their grandfather).

We consider both girls heterozygotes of the Type III form of cystinuria, as they excrete excesses of lysine and of the mixed disulfide and 'high normal' amounts of ornithine and arginine. Excessive excretion of cystine is not a sine qua non, apparently, of Type III carriers, as several, excreting excesses of lysine only, have been described in 'incompletely recessive' families or in those with Type III cystinuria[3,13,14].

Résumé. L'analyse des acides aminés de la cystinurie du type I–III dans l'urine de deux enfants d'un hétérozygote double a révélé que les quantité d'acides aminés dibasiques excrétées en 24 h sont celles qui furent trouvées chez les hétérozygotes de la cystinurie du type III. Les données expérimentales s'accordent sur ce point que les types biochimiquement hétérogènes de la cystinurie sont règlés par des allèles.

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Replication of Intranucleolar DNA in Smittia (Diptera, Chironomidae)

One of the major functions of nucleolus is the production of ribosomal RNA and the genes which code for this class of RNA are localized in or near the nucleolar organizer in *Drosophila*[1] and *Xenopus*[2]. In animal and plant cells, it has been known for a long time that the organizer is the specific site of formation of the nucleolus but various aspects, structural and functional, of the nucleolus-organizer chromatin complex still remain to be elucidated. In recent years it has come to be widely recognized that the chromatin of the organizer may

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exist not only around the nucleolus as perinucleolar, associated chromatin but also within the nucleolar body as intranucleolar chromatin. The occurrence of intranucleolar DNA has been convincingly demonstrated in various vertebrate cells (see BERNHARD and GRANBOULAN[3]) and in the polytenic nuclei of several dipterans[4-8]. A suggestion has also been made that in the chironomid *Smittia*, the nucleolar organizer may be divisible on the basis of morphological as well as functional criteria into an internal and an external organizer[9]. The present note is on the replicative behaviour of the intranucleolar DNA in the same material and the observations bear on the functional relationship between this DNA and that of the nucleolar organizer band situated on the polytenic chromosome. The organizer in *Smittia* is in chromosome II and the compound band which extends to about 2 µm[10] passes through the nucleolus[11].

Material and methods. The salivary glands of actively growing, premetamorphic larvae were dissected out and incubated in vitro at room temperature for 30 min in tissue culture medium 199 (Glaxo) containing 50 µCi/ml of thymidine-methyl-^{3}H (4.24 Ci/m*M*; Radiochemical Centre, Amersham). The glands were then fixed in 5% trichloroacetic acid, squashed in 45% acetic acid and autoradiographs made with Kodak AR-10 stripping film. The preparations were exposed for 13–18 days at 4 °C, developed for 3 min at 20 °C in Kodak D19-b, fixed in Johnson's acid hardening fixer, washed in water and stained with methyl green-pyronin.

Results. The 4 types of nuclear labelling that have been observed are the following: 1. Chromosomes labelled throughout their length including the organizer band and the nucleolus (N°) also labelled (Figure 1). 2. Chromosomes well labelled as above but the nucleolus (N°) not labelled (Figure 2) – the few grains on the nucleolus close to the organizer band may be due to short projections from the organizer that are known[5] to penetrate the nucleolus or may be due to cross-fire from the well labelled organizer proper. 3. Chromosomes lightly and discontinuously labelled and although the organizer band is labelled, the nucleolus (N°) is not (Figure 3). 4. Unlabelled chromosomes with labelled nucleolus (N°) (Figures 4 and 5) – the silver grains over the nucleoli are distributed more or less uniformly.

The relative frequency of the different types of labelling described above has not been estimated, but instances of nucleolar labelling as in Figures 1, 4 and 5 are not very common. An inspection of our preparations shows that all the chromosomes of a given nucleus display the same pattern of labelling and grain density. Further, it

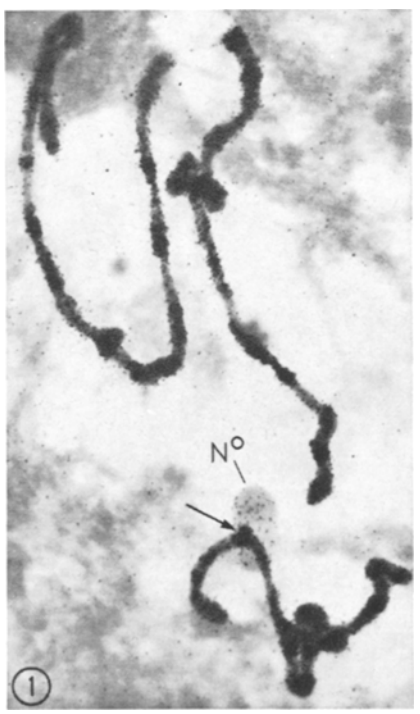

Fig. 1. Autoradiograph of a squashed nucleus that had incorporated ^{3}H-thymidine. The chromosomes are labelled throughout their length and the nucleolus (N°.) is also labelled. The arrow points to the location of the organizer band.

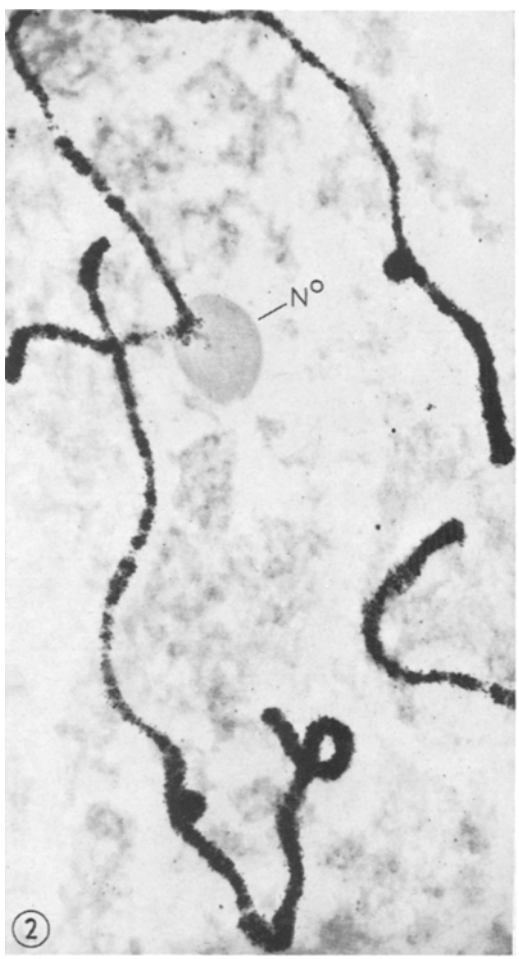

Fig. 2. Autoradiograph showing unlabelled nucleolus (N°.) but continuously labelled chromosomes.

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may be noted that in the replicating nuclei shown in Figures 1–3, the organizer band is always labelled although the intensity of labelling varies, as would be expected, depending on the phase of replication at the site. But what is of particular interest is the finding that there is no correlation between the patterns of chromosomal labelling including the organizer band and the labelling of the intranucleolar DNA.

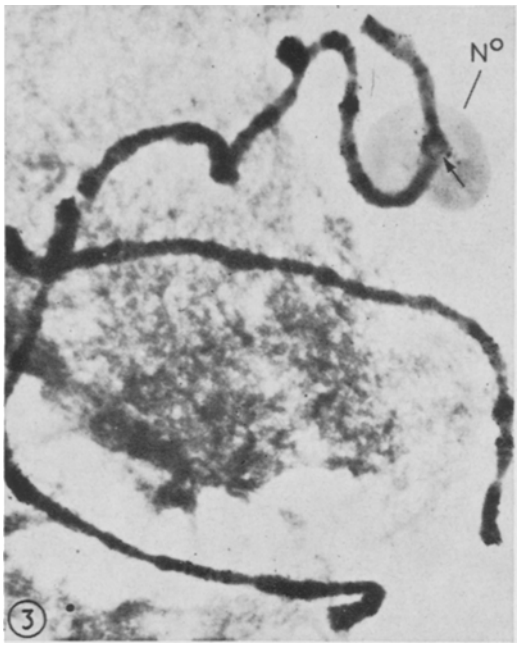

Fig. 3. In this preparation the chromosomes show discontinuous labelling. The organizer band (arrow) is labelled but the nucleolus (N°.) is unlabelled.

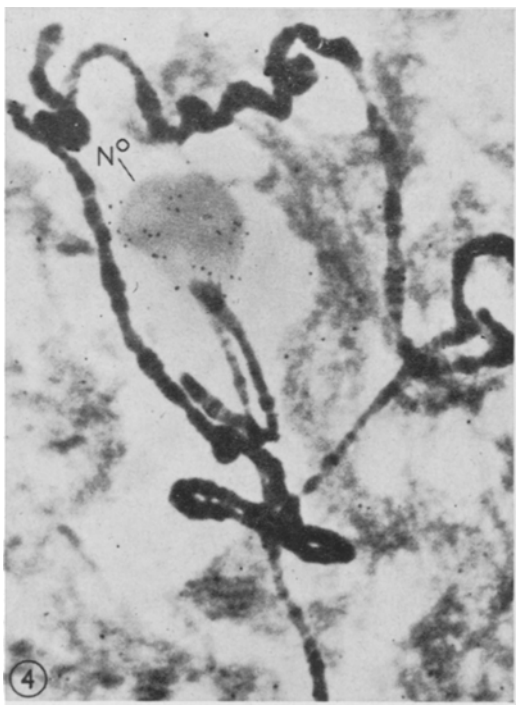

Fig. 4. In this nucleus of the salivary gland, incubated in ^{3}H-thymidine, the only labelled entity is the nucleolus (N°.).

Discussion. Continuous and discontinuous patterns of chromosomal DNA synthesis have been previously reported in the polytenic chromosomes of several dipterans after short pulses of tritiated thymidine. These patterns are believed to represent different phases in the normal replication of chromosomal DNA[12–15]. In *Drosophila melanogaster* nucleolar DNA labelling was initially thought to be associated with dense and continuous chromosomal labelling but this correlation was not found to be valid in interspecific hybrids[4]. More recently, also in *D. melanogaster*, the density of intranucleolar DNA label was shown[8] to be related to the density of chromosomal label, but there seemed to be no strict correlation between the frequency of intranucleolar DNA labelling and any particular pattern of chromosomal labelling. In *Smittia*, intranucleolar DNA replication appears to be infrequent since in our experiments most nucleoli were unlabelled as in Figures 2 and 3. Our observations also indicate that the synthesis of intranucleolar DNA, when it occurs, is unrelated to DNA replication in the chromosomes. Figures 4 and 5 further reveal that intranucleolar DNA synthesis may also occur at a time when the rest of the genomes including the organizer region is inactive in this respect. Such a finding has not so far been reported in *Drosophila*[8] and it recalls to mind the differential synthesis of ribosomal DNA during pachytene stage in the oocytes of the toad *Xenopus*[16]. This does not

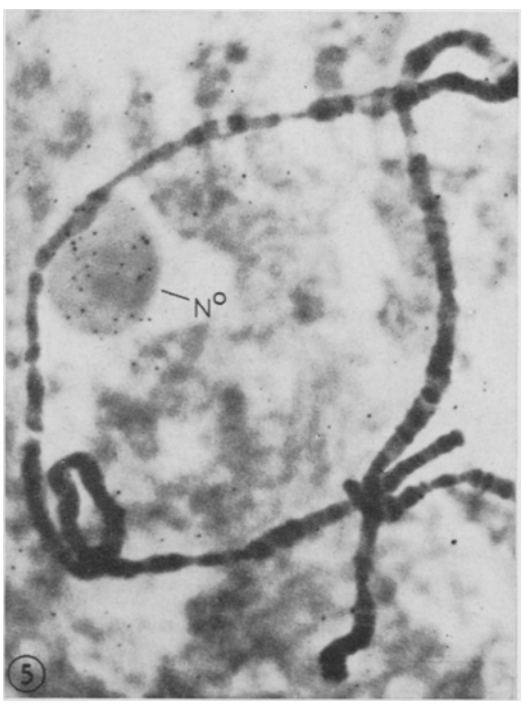

Fig. 5. Another example of nucleolar (N°.) labelling when all the chromosomes are free of label.

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mean that the phenomenon of ribosomal gene amplification is necessarily involved in the present material. The above mentioned findings in *Smittia* may be interpreted simply as indicating that the intranucleolar DNA constitutes units of replication which are autonomous of the chromosomes and behave quite asynchronously with the chromosomal cycles of duplication. An amplification of the ribosomal DNA within the nucleolus cannot, however, be ruled out in such cases since, in a recent molecular hybridization study at the chromosome level[17] in *D. hydei*, ribosomal RNA was seen to hybridize specifically and in considerable amounts with the DNA within the nucleolus and not at all with the DNA of any band in the chromosomes. This problem remains to be tested biochemically in dipteran material keeping in mind the possible variability of the genome fraction set apart for ribosomal RNA in the diploid cell[18] and also the repression of replication of certain chromosome segments of diploid tissue during development of polytenic nuclei[19,20].

Riassunto. Ghiandole salivari di larve di *Smittia* (Chironomidae) sono state incubate in vitro in presenza di timidina tritiata. Le modalità di marcatura dei cromosomi e dei nucleoli dimostrano che in questo materiale non esiste correlazione tra frequenza di marcatura del DNA intranucleolare e modalità o intensità di marcatura del DNA cromosomico. In particolare sono stati riscontrati casi in cui il DNA intranucleolare appare marcato mentre il DNA cromosomico non è in fase di replicazione. I risultati ottenuti sembrano indicare che il DNA intranucleolare in *Smittia* sia costituito da una o più unità di replicazione autonome.

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Indefinite in vivo Life Span of Serially Isografted Mouse Mammary Gland[1]

Whether mammary parenchymal tissue may survive in vivo indefinitely or not if environmental conditions are favourable[2] is controversial[3]. Mammary grafts from a quiescent gland of a 734-day-old virgin female mouse regenerated normal looking glands and secreted milk following parturition in host mice[4]. The quiescent status of mammary glands of old mice seems not to be due to the ageing of the glands themselves. Mammary tissues of the original donor CBA mouse were serially isografted by Hoshino's quantitative transplantation technique[5], and the longest in vivo life span of grafted mammary tissues observed was 1379 days[2], which is much longer than the life span of a mouse. From these results, we postulated in 1967 that the capability of mammary parenchymal tissue to survive in vivo is indefinite if the favourable environmental conditions were renewed. In 1968, Daniel et al.[3] made a contradictory report that the maximal time that any normal mammary transplant series could be carried was 24 months, which is a period within the life span of a mouse, and he concluded that normal mammary glands have a limited ability to proliferate in vivo even under favourable conditions. Recently, we obtained additional cases which seem to support our previous postulation and would like to report these results in this communication.

Materials and methods. The 3 new lines of serial isografts of mammary glands (designated as Lines 3, 4, and 5) were established and the data obtained from them were compared with the 2 lines previously reported by us[2] (referred to as Lines 1 and 2). All transplanted tissues were excized from the 3rd pair of mammary glands of the donor mice. For the 1st generation, 0.6 mm mammary duct-segments were isografted from virgin female donors into the 4th mammary gland-free fat pads[6,7] of the virgin female hosts by Hoshino's quantitative transplantation technique[5]. Identical techniques were used for transplantation of successfully grafted mammary tissues to succeeding generations from the preceeding one. Serial transplantation of the mammary tissues derived from the original donors were undertaken: from a 135-day-old CBA mouse to (♀ BC_B × ♂ CBA)F-1 mice (Line 1), and to (♀ CBA × ♂ BC_B)F-1 mice (Line 2), from a 268-day-old C3H mouse to (♀ C57BL × ♂ C3H)F-1 mice (Line 3) and to (♀ C3H × ♂ C57BL)F-1 mice (Line 4), and from a 206-day-old (♀CBA × ♂ C57BL)F-1 mouse to (♀CBA × ♂C57BL)-F-1 mice (Line 5). The interval between the serial transplantation ranged from 34 to 222 days. Following mammary transplantation, the hosts were neither mated nor given any other treatment. Throughout the experiments, all animals were maintained under uniformly controlled environment and provided with Purina Lab Chow and water ad libitum. The BC_B, CBA, C3H, and C57BL mice are all pedigreed inbred strains which have been raised by sister-to-brother mating and maintained in our laboratory[8,9].

Results. The incidence of successful transplants at each transplantation generation in all the 5 lines is shown in the Figure. Except for Line 5, serial transplantation was discontinued after varying periods of transplantation instead of carrying on indefinitely. The longest periods of in vivo life span of the mammary glands derived from the respective original donors within the experimental limitation were 1414 days (6 generations) in Line 1, 1379 days (7 generations) in Line 2, 491 days (5 generations) in Line 3, 394 days (3 generations) in Line 4, and 748 days

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