In approximately a quarter of the rats studied, an additional procedure included measurement of radioactivity in the blood. One and a half hours after injection of the labelled precursor, the animals were sedated by intramuscular injection of sodium pentobarbital (30 mg kg^{-1}). Rats were anaesthetised in an ether chamber just before death 2 h after injection and 3.0 ml of blood was obtained by a direct cardiac tap using a 20 gauge needle and a heparinised syringe. Aliquots of 0.1 ml of plasma were placed in 10.0 ml of Bray's solution to determine radioactivity. The maternal liver was homogenised and prepared as described earlier. The level of blood radioactivity measured in the plasma was relatively constant throughout pregnant and non-pregnant animals with only a slight decrease at 21 d. With orotic acid and uridine the radioactivity measured 40,000 to 55,000 c.p.m. ml^{-1} of plasma; whereas, cytidine showed only 9,000 to 11,000 c.p.m. ml^{-1} of plasma.

Figure 2 illustrates the differences in incorporation of 16-d pregnant rats using precursors with varying specific activities. Orotic acid showed a slight rise in radioactivity with increasing specific activities, but cytidine exhibited a marked increase. In direct contrast, uridine showed no change with even greater alterations in specific activities suggesting a rapid saturation of its system. Although the actual measured radioactive counts were considerably lower, a similar pattern of response was observed in 21-d pregnant rats with all three precursors.

Figure 3 demonstrates the marked differences in the incorporation of ^{3}H-uridine and ^{3}H-cytidine into the nuclear and cytoplasmic portions of the liver during various stages of pregnancy. Although the actual amount of radioactive labelling is relatively small by comparison, ^{3}H-uridine is incorporated rapidly into both the nuclear and cytoplasmic RNA and then falls off within 30 min. The 21-d pregnant rat shows poor uptake of radioactivity throughout the 2 h period of observation. The incorporation of ^{3}H-cytidine differs in that the isotopic incorporation in both the nucleus and cytoplasm increases during the 2 h period, in a manner similar to that observed using labelled orotic acid. The 8 and 18-d pregnant rats reveal enhanced labelling compared with the non-pregnant rat, but the 21-d pregnant rat displays a markedly depressed level of incorporation as noted in the ^{3}H-uridine experiments.

To exclude the possible effects of changes in the intraperitoneal absorption of isotopes, the three labelled precursors were administered intravenously through the tail vein to another group of 16-d and 21-d pregnant animals. Again a comparable labelling of the 16-d pregnant and the depressed incorporation of the 21-d pregnant rats were noted as the results show in Fig. 1.

Table 1 summarises our studies on the nuclear, nucleolar and extranucleolar DNA-dependent RNA polymerases from the livers of 16-d and 21-d pregnant rats. The results indicate that there is no drastic decrease in the specific activity of DNA-dependent RNA polymerases in the 21-d pregnant rat in spite of the marked depression in the incorporation of RNA precursors. There is a measurable drop only in the extranucleolar DNA-dependent RNA polymerase.

The specific causes for the changes in the incorporation of the various RNA precursors during pregnancy remains unresolved although endocrine influences must play a dominant role. The work reported here, however, clearly indicates that pregnancy and the duration of pregnancy have profound effects on the degree of RNA incorporation with different RNA precursors.

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Table 1 Comparison of Specific Activity of Liver RNA Polymerases in 16-d and 21-d Pregnant Rats

	16-d pregnant rats	21-d pregnant rats
	Mg^{2+}-activated	
Nuclear	131	253
Nucleolar	158	161
Extranucleolar	2,199	1,306
	Mn^{2+} $(NH_4)_2SO_4$-activated	
Nuclear	116	220
Nucleolar	217	165
Extranucleolar	2,716	1,623

Nuclei were isolated from liver homogenate according to Widnell and Tata[4], and nucleolar and extranucleolar (nucleoplasmic) suspensions were prepared as described by Yu and Fiegelson[5]. The RNA polymerase assay was essentially the method of Weiss[6]. The reaction mixture contained 50 μmol of Tris-HCl buffer, *p*H 8.5; 2.5 μmol of magnesium chloride, 3 μmol of sodium fluoride and 10 μmol of cysteine; 0.3 μmol each of GTP, CTP and ATP and 0.015 μmol of UTP; 2.0 μCi of 5-^{3}H-UTP (Schwarz-Mann, 14.0 Ci $mmol^{-1}$) and 0.1 ml of enzyme suspension. For the ammonium sulphate assay 2.0 μmol of $MnCl_2$ and 0.2 mmol of $(NH_4)_2SO_4$ were used instead of $MgCl_2$ and the *p*H of the buffer was 7.5 (ref. 7). After incubation for 15 min at 37° C the reactions were stopped, adding 0.10 M sodium pyrophosphate containing RNA and bovine serum albumin[8]. The RNA was collected on Whatman GF C filters and radioactivity measured with liquid scintillation counter using 10.0 ml Bray's solution. RNA determinations were done according to Fleck and Munro[9]. Values represent an average of three determinations and indicate pmol of UMP incorporation per mg DNA.

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Slightly Deleterious Mutant Substitutions in Evolution

Recent advances in molecular genetics have had a great deal of influence on evolutionary theory, and in particular, the neutral mutation-random drift hypothesis of molecular evolution[1,2] has stimulated much interest. The concept of neutral mutant substitution in the population by random genetic drift can be extended to include random fixation of very slightly deleterious mutations which have more chance of being selected against than of being selected for[3,4]. If this class of mutant substitution is important, we can predict that the evolution is rapid in small populations or at the time of speciation[5]. Here I shall organize the observed facts which indicate that this class is in fact important.

Fitch and Margoliash[6] have shown that the frequency of double fixation of mutants in a leg of their cytochrome c phylogenetic tree is higher than predicted from chance. The

most plausible interpretation is that the first mutant is slightly deleterious and that the second compensates for the first. This interpretation makes Fitch's concept of the covarion[7] most easily understood. If the first mutant disturbs very slightly the normal function of the molecule, there are probably many ways of compensating, and so the first mutant provides a new possibility for evolutionary change. This is considered to correspond to Fitch's observation of unstable covarions[7].

The evolution of tRNA follows the same pattern. It is known that evolution is more rapid in the paired region than in the non-paired region of tRNA[8]. In most tRNAs, the paired region is almost exactly paired, therefore there must have been coupled substitutions in evolution as first mentioned by Jukes[9]. Dayhoff and McLaughlin[8] state "this could be explained by an oscillatory sequence of evolutionary events, involving first a single base change that weakens the bonding, followed by a second change which restores the hydrogen-bond pairing". It is hard to conclude from this that each sequence has evolved so as to fit better into its environment. It indicates rather that the first substitution is slightly deleterious. This idea can be extended to other protein molecules, particularly if the polypeptide-folding mechanisms are important, as discussed by Barnard *et al.*[10]. Any amino acid substitution of a well organized molecule is likely to disturb the organization, but once it spreads in the population by random drift, it opens the way for further evolutionary change.

This hypothesis is compatible with a different point of view, that is, frequency distribution of multiple alleles detected by electrophoresis. Several investigators have been trying to prove or disprove the neutral theory of protein polymorphisms by looking at the pattern of allelic distribution[11,12]. In such attempts, it is usually assumed that every mutant appearing in the population is one not pre-existing, that is to say, the so called "infinite allele model" of Kimura and Crow[13] has been used extensively. The convenient statistics often used in the analysis are the effective number of alleles (n_e; reciprocal of the sum of squares of allelic frequencies) and the actual number of alleles observed (n_a). Recently, Yamazaki and Maruyama[14] made an extensive compilation and statistical analysis of published data on enzyme polymorphisms. In particular, they showed that the average ratio of the effective to the actual number of alleles ($\overline{n_e/n_a}$) from the observed enzyme polymorphisms agrees well with the neutral hypothesis according to Kimura and Crow's infinite allele model. Here the ratio, n_e/n_a, represents a sort of evenness of allele distribution and, the higher the ratio, the more uniform the distribution expected. But this model seems to be inappropriate for electrophoretically detectable polymorphisms, for this method can only detect a difference in the electric charges of molecules as discrete bands on the gels. In fact Bulmer[15] noticed non-random arrangements of electrophoretic bands of common and rare alleles. In view of these points, Maynard Smith[16] and Kimura and Ohta[17] have suggested that the model of stepwise allele production is more appropriate. Using this mutational scheme but assuming an infinite number of possible states, we have shown that the effective number of neutral alleles maintained in a finite population becomes, $n_e = \sqrt{(1+8N_e v)}$, where N_e is the effective population size and v is the mutation rate per gamete[18]. When $N_e v$ is large this formula gives a smaller effective allele number than that predicted by Kimura and Crow's model. Furthermore, the frequency distribution of multiple alleles is influenced significantly. We have performed extensive Monte Carlo experiments and have shown that the ratio of the effective to the actual number of alleles (n_e/n_a) is larger in the step allele model, particularly when n_a is large (T. O. and M. Kimura, unpublished). Then the agreement between the observation and Kimura and Crow's model shows that there exist more rare alleles than expected from the strict neutral hypothesis. From this, I conclude that rare alleles are on the average selected against. The alleles, which can increase their frequency up to several per cent in very large populations like *Drosophila* species, can replace the previous allele in small populations by random drift.

Table 1 Relationship between Substitution Rate in Small Populations and Equilibrium Frequency in Large Populations of Slightly Deleterious Mutations

Homozygous disadvantage	Equilibrium frequency in large populations	Rate of substitution in proportion of the completely neutral case $N_e=10^2$	$N_e=10^3$
Recessive deleterious			
10^{-2}	0.032	0.43	0.8×10^{-7}
10^{-3}	0.1	0.94	0.43
10^{-4}	0.32	0.99	0.94
Semidominant deleterious			
10^{-2}	0.002	0.31	0.5×10^{-7}
10^{-3}	0.02	0.90	0.31
10^{-4}	0.2	0.99	0.90

The mutation rate is assumed to be 10^{-5}.

O'Brien *et al.*[19] reported that the "null" alleles at the α-glycerophosphate dehydrogenase-1 locus of *Drosophila melanogaster* are deleterious. As their null alleles were induced by EMS, they may be different from those observed in low frequencies in natural populations. It is likely, however, that the null alleles are generally deleterious. They can replace the previous allele in a bottleneck population. Vitamin requirement is in fact considered to be the result of such procedures. King and Jukes[2] noticed that lack of vitamin C is always associated with the kind of food and they consider that, when selective constraints are reduced, the genes for vitamin C have deteriorated.

As an example, suppose that the homozygous disadvantage of the recessive deleterious mutant is 10^{-3}. If the mutation rate is 10^{-5}, the equilibrium mutant frequency is 10% in large populations. By using Kimura's[20] formula for the fixation probability of mutant genes, the rate of substitution of this slightly deleterious mutant becomes 0.43×10^{-5} per generation if the effective population size is 10^3. This corresponds to 43% of the substitution rate of the strictly neutral case. Table 1 illustrates such examples in terms of the proportion of the substitution rate of the completely neutral case.

I have previously suggested the possibility of slightly deleterious mutant substitutions in cistrons in view of the apparently contradictory observation of generation-dependent DNA evolution against year-dependent cistron evolution[4]. Together with the supporting observations presented here, I suggest that very slight genetic deterioration might play an important role in molecular evolution. Here, organization at a much lower level in each molecule or in several interacting molecules could provide a clue to understanding the evolutionary change at the molecular level, rather than cohesive interaction of the pool often postulated by orthodox neo-Darwinism[21]. It seems from the report of Mukai *et al.*[22] that there is no evidence that alleles at different loci constitute such interaction systems or "super genes". I thank Dr M. Kimura for discussions.

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Antitumour Activity of Iodine in Acidic Medium with Calcium

THE basal metabolism is increased in many patients with lymphatic and myelogenous leukaemia. Elevated levels of plasma iodine have been found in various leukaemic cases, possibly indicating involvement of the thyroid gland[1]. It is thought that at lower *p*H values there is more free iodine, and so greater penetrability into cells and greater iodine activity[2]. Iodine fixation by human leukocytes requires energy[3]. Iodine alters the redox state of the cytoplasm and mitochondria, because the couple $2\ I^- \rightarrow I^2 + 2\ e$ has a rather high redox potential: +0.535 V; iodine seems to be one of the oxidizing agents which can be used for ATP formation[4]. Flavin is the most likely oxidizing agent for a couple with such a high redox potential[5].

The importance of calcium ions in controlling the permeability of cells[6] and in regulating cellular activity[7] has been established. The penetration of isolated ascites tumour cells by calcium ions depends on the metabolic state of the cells[8]; calcium accumulation, like iodine accumulation, requires energy and alters the redox state of the respiratory chain components.

It has been suggested that iodine in acidic medium with calcium has antitumour activity (L. Heinonen, personal communication). We therefore studied the effect of these substances against myelocytic chloroleukaemia[9]. This leukaemia was induced in 2 to 3-week-old Sprague-Dawley and Wistar rats by intraperitoneal or subcutaneous inoculation. Chloroleukaemic cells were suspended in physiological saline and 0.1 ml of the cell suspension, containing 10,000–20,000 cells, was injected into the rats. Survival time, histological examination and lactic acid dehydrogenase (LDH) activity were used to evaluate antitumour activity.

Treatment was started 24 h after the intraperitoneal inoculation by giving each rat about 500 μg of iodine daily as a 5% iodine-ethanol solution in cultured milk. Fourteen days after the inoculation the treatment with iodine was continued by subcutaneous injection. Treatment of the subcutaneously induced local tumours was by subcutaneous injection of iodine, starting about two weeks after inoculation, when the tumour was clearly visible and was usually about 2 cm×3 cm, sometimes larger. The subcutaneous injections consisted of 500 μg of iodine in ethanol solution mixed with 1 ml of liver extract (Pernaemon forte, N.V., Organon, Oss, Netherlands). Immediately afterwards 1 ml of calcium gluconolactobionate solution (about 9 mg Ca^{2+}) (Calcium Sandoz, Sandoz AG, Basel, Switzerland) was injected intramuscularly. The injections were made once (intraperitoneally inoculated rats) or twice (subcutaneously inoculated rats) daily. Eleven days after inoculation the intraperitoneally inoculated rats were injected with 25 mg of thiamine chloride (Neuramin forte, Orion Ltd., Espoo, Finland).

The rats' food consisted of vegetables, including cabbage and carrots, and fresh liver, with 3 mg riboflavin (Orion Ltd., Espoo, Finland) per rat per day. The control rats were given all the nutrients, liver extract, calcium and vitamins but not iodine.

Tissue samples were taken at autopsy not only from the visible tumours but also as a routine procedure from liver, spleen, kidney, lung, heart and bone marrow. After fixation in formalin the tissue samples were cut into sections 6 μm thick and stained with haematoxylin-eosin. The smear samples of femoral bone marrow were stained by the May-Grünwald-Giemsa method. The preparations were examined under a light microscope. LDH activity was estimated by measuring the extent of oxidation of NADH (at 340 nm).

Table 1 shows the effect of treatment on the survival time of the rats bearing a tumour inoculated intraperitoneally or subcutaneously. The index $T/C \times 100$ [(mean survival time of treated rats)/(mean survival time of untreated control rats)×100] was calculated to be >415 for the intraperitoneally inoculated rats. This is three to four times higher than the ratio reported by other workers[10–12]. Our result might have been even better if the iodine administration had been by injection from the start of the experiment, as we found that the rats did not drink all of their iodine-containing cultured milk. The index was not calculated for the subcutaneously inoculated rats because the tumours had disappeared before the rats were killed (Tables 1 and 2). The average survival time

Table 1 Influence of Iodine Treatment on Survival Time

Intraperitoneally inoculated rats

Rat	Survival time T (d after inoculation)	$T/C^* \times 100$	Cause of death	No. of treatments	Dose of iodine (μg d^{-1})
Treated rats					
a	>61	>555	Killed	25	500
b	28	255	Died	17	500
c	17	155	Died	3	500
d	>61	>555	Killed	25	500
e	>61	>555	Killed	26	500
Untreated control rats					
f	12		Died	—	—
g	12		Died	—	—
k	9		Died	—	—

Subcutaneously inoculated rats (local tumour)

Rat	Survival time (d after inoculation)	Cause of death	No. of treatments	Dose of iodine (μg d^{-1})	Disappearance of the local tumour (d)
Treated rats					
II†	>198	Killed	68	625	14
q‡	>33	Killed	35	1,000–4,500	‡
r	>33	Killed	24	1,000	3
s	>33	Killed	13	1,000	2
v	>33	Killed	12	1,000	3
Untreated control rats					
p	>33	Killed	—	—	
XI	24	Died	—	—	
j	35	Died	—	—	
ö	34	Died	—	—	
y	35	Died	—	—	
z	41	Died	—	—	

* C, Mean survival time of untreated control rats (f–k)=11 d and of untreated control rats (XI–z)=34 d.
† Rat II received iodine in cultured milk during the whole experimental period.
‡ Rat q received a more concentrated iodine-liver extract daily (1,000 to 4,500 μg of iodine ml^{-1}). The tumour underwent alteration to fatty necrosis.