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Acid-soluble Nucleotides of the Earthworm (*Lumbricus terrestris*)

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The acid-soluble nucleotides of the body wall musculature of the earthworm have been analysed by chromatographic methods. The following nucleotides were identified:* AMP, ADP, ATP, ADPR, DPN, IMP, GMP, GDP, GTP, UMP, UTP and CTP. The identifications are based on elution positions in ion-exchange, paper chromatography before and after hydrolysis, ultraviolet absorption spectra and estimation of the phosphorus, pentose and base contents. Two fractions, a CMP derivative and an UDP derivative containing ninhydrin-positive components have not been fully identified.

In recent years the isolation of ATP from muscle [1,2] has been supplemented by the isolation of further nucleoside polyphosphates. It was shown [3,4] that the muscle of vertebrates contains beside AMP, ADP and ATP, the corresponding derivatives of guanosine, uridine and cytidine. In the muscle of invertebrates, however, only adenosine nucleotides have hitherto been demonstrated [5–8]. An investigation of the acid-soluble nucleotides in invertebrate material was therefore undertaken. The earthworm is a suitable object for such work as it has a well defined body wall musculature which is easy to handle, and furthermore it is easy to obtain large numbers of the animal. In the present work, therefore, earthworm was chosen for the analysis of the acid-soluble nucleotides of invertebrate muscle.

* AMP = adenosinemonophosphate
ADP = adenosinediphosphate
ATP = adenosinetriphosphate
ADPR = adenosinediphosphoribose
DPN = diphosphopyridine nucleotide
IMP = inosinemonophosphate
GMP = guanosinemonophosphate
GDP = guanosinediphosphate
GTP = guanosinetriphosphate
UMP = uridinemonophosphate
UTP = uridinetriphosphate
CTP = cytidinetriphosphate

MATERIAL AND METHODS

Preparation of trichloroacetic acid extract. The earthworm species *Lumbricus terrestris* was used and the animals were handled immediately after collection. Before extraction the animals were dipped into cold alcohol (−60°C) for a few seconds. They could then easily be gutted, and the muscle was immersed into cold alcohol. After enough tissue had been collected (usually 100−125 g) it was homogenized with one volume of ice-cold 10 % trichloroacetic acid in a Turmix blendor and pressed through madapolam. The extraction was repeated three times with 5 % trichloroacetic acid and the combined extracts filtered through Hyflo Supercel. The filtrate was shaken six times with ether to remove the bulk of the trichloroacetic acid, neutralized with a drop of ammonia and freed from ether by aeration. The protein free extract thus obtained was used for phosphorus analysis and paper chromatography.

The crude extract could also be used directly for ion-exchange chromatography. The presence of non-nucleotide constituents (amino acids etc.) results, however, in high background absorption and impairs on the separation and quantitative analysis of the smaller nucleotide fractions. These disadvantages could be avoided by treatment of the extract with norite prior to chromatography.

Purification of the nucleotide fraction by the norite method. The extract containing the nucleotides was acidified with hydrochloric acid to pH 1 and shaken for 30 min with an appropriate amount of norite. After filtration the norite was washed free from acid and sucked dry. The nucleotides were eluted with 50 % ethanol containing 1 % ammonia. In order to obtain a quantitative yield several elutions were necessary. Table 1 shows the nucleotide content of consecutive eluates as measured by ultraviolet absorption at 260 mμ. No differences in the type of ultraviolet absorption were seen between the first and the seventh eluate (Fig. 1).

Ion-exchange chromatography. Dowex-1 (200−400 mesh) in the formate form was used and elution was carried out with a modification of the formate system used by Bergkvist and Deutsch [9]. Fractions were collected by a time-operated fraction collector and every fraction was read at 250, 260 and 270 mμ in a Beckman DU spectrophotometer. Appropriate fractions were pooled and the nucleotides concentrated by the norite method.

Paper chromatography. Two dimensional chromatography [10] was used to resolve the nucleotides in the crude trichloroacetic acid extract, with *n*-propanol-ammonia (*d* 0.880)-water (60:30:10) in the first direction and saturated ammonium sulphate solution-water-*iso*propanol (79:19:2) in the second.

One dimensional chromatography in *iso*propanol-hydrochloric acid-water (97:25:28) was used for the separation of the products obtained on hydrolysis of the different nucleotide fractions from ion-exchange chromatography. Hydrolysis was carried out in N hydrochloric acid for one hour at 100 °C.

For determination of the individual nucleotides before hydrolysis the fractions were run in the *n*-propanol-ammonia-water system, together with the authentic substances.

Descending chromatography on Whatman No. 1 paper was used throughout and the spots on the finished chromatogram were located by ultraviolet photography.

For quantitative estimations, the spots were cut out together with blank areas and eluted with 0.1 N hydrochloric acid over night; the eluates were used for ultraviolet spectrophotometry and phosphorus analysis.

Analytical procedures. Phosphorus was determined by the method of Allen [11] using a Klett Summerson photoelectric colorimeter with a red filter and was differentiated in

Table 1. Elution of nucleotides from norite. Elution with 50 % ethanol containing 1 % ammonia. Every eluate *ca* 6 ml.

Elution-number	1	2	3	4	5	6	7
ΣE_{260}	1 485	909	455	241	153	109	56

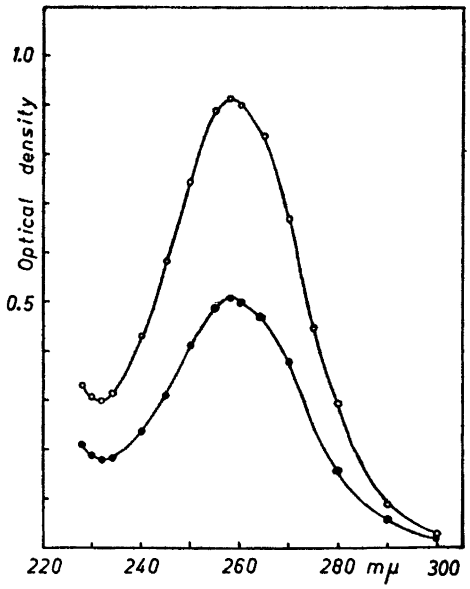

Fig. 1. Ultraviolet absorption curves for norite-eluates. O—O—O Eluate No. 1. 10 μl eluate + 3.0 ml 0.1 M hydrochloric acid. ●—●—● Eluate No. 7. 150 μl eluate + 3.0 ml 0.1 M hydrochloric acid.

separate analyses into total phosphorus (P_T) inorganic phosphorus (P_i) and acid-labile phosphorus (P_{10}) determined after 10 min hydrolysis in N hydrochloric acid at 100°C.

Pentose was determined by the method of Mejbaum as modified by Albaum and Umbreit [12]. A Klett Summerson photoelectric colorimeter was used with a red filter.

Optical densities were measured in a Beckman Universal Spectrophotometer Model DU in 0.1 N hydrochloric acid, the molecular extinction coefficients of 14 200 at 260 mμ for adenosine phosphates, 13 200 at 250 mμ for inosine phosphates, 10 850 at 256 mμ for guanosine phosphates, 10 050 at 262 mμ for uridine phosphates and 13 000 at 280 mμ for cytidine phosphates, being used.

Cyanide complex formation for the identification of DPN was carried out in M NaCN according to Colowick, Kaplan and Ciotti [13], using the increase in absorption at 325 mμ as a measure for N-substituted nicotinamide derivatives.

Oxidation with periodate for the estimation of 5′-substitution was carried out by the method of Dixon and Lipkin [14].

Table 2. Phosphorus analysis of crude and norite-purified trichloroacetic acid extracts of *Lumbricus* muscle expressed as μg P per gram muscle wet weight.

	P_i μg / g	P_{10} μg / g	P_T μg / g	$P_{10}-P_i$ μg / g	P_T-P_{10} μg / g
Crude extract	215	583	858	368	275
Crude extract	188	398	787	210	389
Norite eluate	0	172	283	172	109

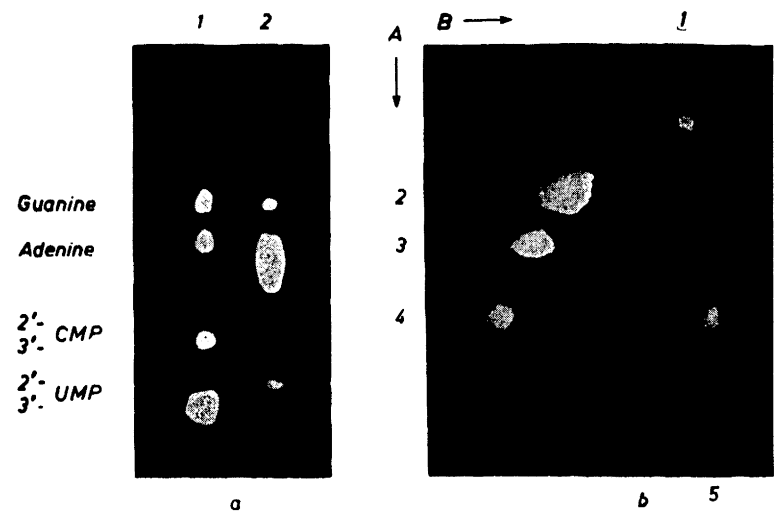

Fig. 2. (a) paper chromatography of the extract from *Lumbricus* muscle after hydrolysis for 1 h in N hydrochloric acid at 100 °C. Solvent: *iso*propanol-hydrochloric acid-water (97:25:28). Time 24 h. Whatman No. 1 paper. (1) RNA-hydrolysate, (2) hydrolysed extract. (b) twodimensional chromatography of the unhydrolysed extract. Solvent: (A) propanol-ammonia-water (60:30:10) 72 h, (B) saturated ammoniumsulphate-*iso*-propanol-water (79:2:19) 8 h, Whatman No. 1 paper. (1) GTP, (2) ATP, (3) ADP, (4) AMP, (5) uridinederivative.

RESULTS

Phosphorus analysis of the trichloroacetic acid extract showed that *Lumbricus* muscle contains considerable quantities of acid-soluble phosphorus both as inorganic phosphate and as acid-labile and acid-stable phosphates (Table 2). Nearly all acid-labile phosphate was adsorbed on norite and recovered in the norite eluate indicating that most of this phosphorus fraction consists of nucleotides. Only about one-third of the acid-stable phosphorus, however, was adsorbed on norite; the phosphate fraction not adsorbed was probably composed of lombricine [15] and of sugar phosphates. Lombricine was isolated from the norite filtrate by precipitation of the barium-salt with three volumes of alcohol and was identified as guanidoethylserylphosphoric acid by paper chromatography of the compound and its products of hydrolysis, as previously described by Thoai and Robin [15].

Paper chromatography of the hydrolysed extract (Fig. 2a) gave a large spot for adenine besides smaller spots for guanine and uridylic acid, indicating that the main ultraviolet-absorbing components of the extract before hydrolysis were adenine derivatives besides guanine- and uridine nucleotides. The slightly lower R_F-value of the uridylic acid spot as compared with that from RNA may possibly indicate the presence of 5′-derivative in the extract (Fig. 2a). Two dimensional chromatography of the unhydrolyzed extract comfirmed these results. The major components as shown by Fig. 2b were

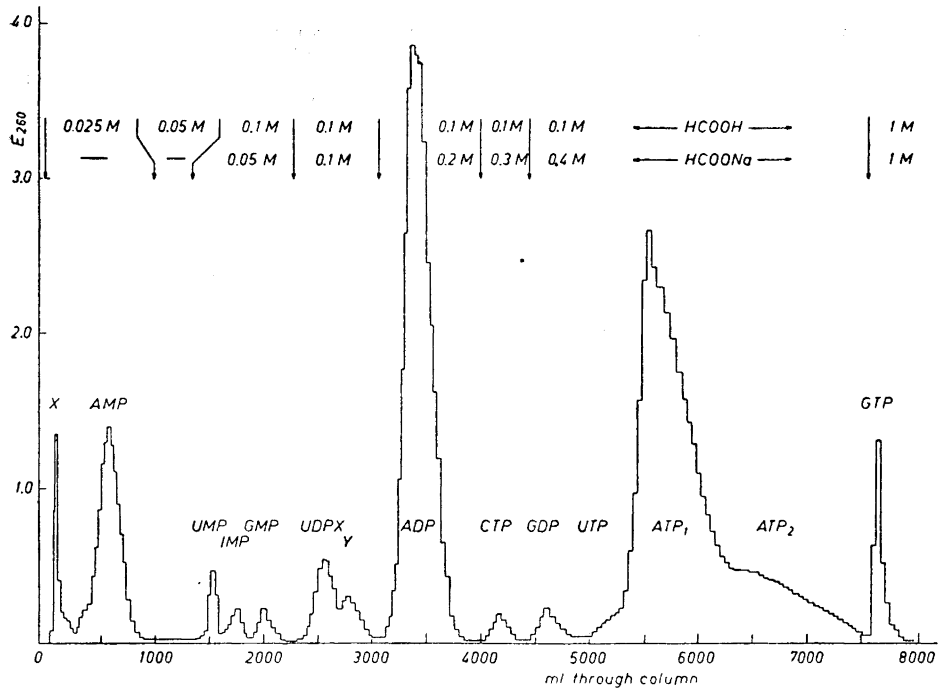

Fig. 3. Ion-exchange chromatography of a purified trichloroacetic acid extract from earthworm muscle. Dowex-1 (200–400 mesh) column, 1 cm^2 × 13 cm in the formate system. Adsorbed material: extract with optical density E_{260} = 3 900. Recovery 101 %.

ATP, ADP and AMP besides weak spots indicating the presence of other nucleotide compounds.

Paper chromatography of a complex mixture like that in the trichloroacetic acid extract, however, gave only a rough picture of the main components. For the detailed analysis of all the different nucleotides ion-exchange chromatography is the method of choice. Therefore, both the crude trichloroacetic acid extract and the purified norite-eluates were analysed by chromatography on Dowex-1 in a formate system, giving in the main the same results. However, the purified extract gave — for the reason mentioned above — the clearer separation. Fig. 3 shows the results of ion-exchange analysis of the acid-soluble nucleotides from the body wall musculature of the earthworm.

The first tentative identification of the components was made according to elution positions in ion-exchange and the ultraviolet absorption curves. For further identification, the appropriate fractions were pooled and concentrated *via* norite adsorption-elution as described above. In the next step the base components were determined by chromatography of the fractions after hydrolysis in N hydrochloric acid at 100°C for one hour. The R_F-values of the products of hydrolysis are shown in Table 3 together with those for the authentic compounds.

Table 3. R_F-values of ion-exchange fractions from earth-worm muscle after hydrolysis in N hydrochloric acid at 100 °C for one hour, compared with the R_F-values of known substances. Solvent: *iso*propanol-hydrochloric acid-water (97 : 25 : 28). Descending chromatography on Whatman No. 1 paper for 24 h.

Fraction	R_F	Marker	R_F
AMP	0.30		
Y	0.29	Adenine	0.28
ADP	0.28		
ATP_1	0.28		
ATP_2	0.29		
IMP	0.28	Hypo-xanthine	0.29
GMP	0.21		
GDP	0.21	Guanine	0.21
GTP	0.21		
		2′-UMP	
UMP	0.69	3′-	0.76
UDPX	0.69	5′-UMP	0.70
UTP	0.68		
		2′-CMP	
X	0.55	3′-	0.57
CTP	0.48	5′-CMP	0.50

As seen from the table, the nucleotides from *Lumbricus* muscle comprise derivatives of the five bases adenine, hypoxanthine, guanine, uracil and cytosine. Adenine and hypoxanthine have the same R_F-values but are easily distinguished by their different ultraviolet absorption curves. The fractions containing pyrimidine derivatives showed R_F-values corresponding to the 5′-monophosphates with the exception of fraction X. This fraction had the elution position of CMP and yields on paper chromatography after hydrolysis a spot with nearly the same R_F-value as 2′- and 3′-CMP. The ultraviolet spectrum, however, was different from that of the cytidylic acids, the absorption maximum in 0.1 N hydrochloric acid being at 270 mμ instead of around 280 mμ.

After the purine and pyrimidine components of the different nucleotides had been established, the degree of phosphorylation was determined by phosphorus analysis. The purity of the fractions was checked by paper chromatography before analysis. The propanol-ammonia-water system was used which separates the different phosphates of the same nucleoside well, and gave a further qualitative identification of the compounds. Table 4 gives the R_F-values of the different fractions in comparison with those of the authentic compounds.

With a few exceptions (fractions UDPX, X and Y) the R_F-values found are in good agreement with those expected from the elution positions in ion-exchange and the results of paper chromatography after hydrolysis.

The pure fractions were analysed for pentose, acid-labile and total phosphates. In those cases where paper chromatography indicated an impure fraction

Table 4. R_F-values of ion-exchange fractions from earthworm muscle, compared with the R_F-values of authentic substances. Solvent: propanol-ammonia-water (60 : 30 : 10). Descending chromatography on Whatman No. 1 paper (a 120 cm long strip) for 70 h.

Fraction	R_F	Marker	R_F	Fraction	R_F	Marker	R_F
AMP	0.30	AMP	0.30	GDP	0.13	—	—
ADP	0.21	ADP	0.21	GTP	0.11	GTP	0.12
ATP_1	0.17	ATP	0.17	UMP	0.23	5′-UMP	0.23
ATP_2	0.18			UDPX	0.30	—	—
Y	0.30			UTP	0.13	UTP	0.13
IMP	0.21	IMP	0.21	X	0.25	5′-CMP	0.23
GMP	0.15	GMP	0.14				

the analysis was made on the eluted spots after further purification by paper chromatography. Only purinebound pentose was determined quantitatively. The presence of pyrimidinebound pentose was detected by qualitative orcinol tests before and after treatment with bromine [16]. No quantitative data were, however, obtained. The results of the analyses are shown in Table 5.

The analytical data together with evidence both from ion-exchange and paper chromatography confirm the identifications made in Fig. 3. After hydrolysis the purine derivatives AMP, ADP, ATP, IMP, GMP, GDP and GTP gave the corresponding bases: adenine, hypoxanthine and guanine, respectively (Table 3). The state of phosphorylation was determined by paper chromatography and direct analysis (Tables 4 and 5). Furthermore, all the nucleotides were established as 5′-phosphates by periodate oxidation, which showed that the 2′- and 3′-positions of pentose were unsubstituted. On a molar base, every nucleotide consumed 0.95—0.99 moles of periodate.

The ATP-fraction appears in the ion-exchange chromatogram as a very asymmetric peak, which could indicate a heterogeneous fraction. For this reason the fraction was divided into two parts, ATP_1 and ATP_2, each of which were analysed separately. They gave, however, identical ultraviolet absorption curves, the same R_F-values on paper chromatography both before and after hydrolysis. In addition they gave the same values for acid-labile and acid-stable phosphorus, for pentose and on periodate oxidation. For these reasons both fractions were considered to be ATP.

The pyrimidine derivatives were identified as UMP, UTP and CTP as shown in Fig. 3 and Tables 3, 4 and 5. They are 5′-phosphates: on hydrolysis 5′-uridylic and 5′-cytidylic acid are formed and not the 2′- or 3′-derivatives as shown by paper chromatography.

Three fractions are not yet fully identified, namely those designed X, Y and UDPX. Fraction Y is in all probability 5'-adenosinediphosphoribose derived from DPN by degradation with alkali during elution. It contains two moles of phosphate and two moles of pentose per mole adenine and has a higher R_F-value than ADP on paper chromatography (Table 4).

The UDPX fraction was shown to be a derivative of 5′-UMP by the ultraviolet absorption curve and the R_F-value after hydrolysis which was identical

Table 5. Analysis of the fractions obtained by ion-exchange-chromatography of the norite-purified trichloroacetic acid extract from *Lumbricus* body wall musculature.

Fraction	labile P μg	total P μg	base μg	pentose μg	base : pentose: labile P: total P
AMP	0	3.7	13.1	15.3	1:1.02:0:1.20
UMP	0	3.6	11.6	—	1:—: 0:1.10
IMP	0	9.5	40.3	48.0	1:1.07:0:1.02
GMP	0.2	2.7	12.1	11.4	1:0.95:0.11:1.07
UDPX	7.2	14.6	23.3	—	1:—:0.99:2.01
Y	2.7	11.1	23.2	50.2	1:1.93:0.50:2.08
ADP	4.0	8.1	16.9	18.7	1:0.99:1.03:2.02
CTP	3.8	6.0	6.9	—	1:—:1.94:3.08
GDP	1.8	3.7	9.3	9.2	1:0.99:0.91:1.93
UTP	5.6	8.4	10.2	—	1:—:1.99:2.98
ATP_1	3.9	5.6	8.1	9.8	1:1.00:2.04:2.99
ATP_2	4.1	6.1	8.7	9.7	1:1.00:2.04:2.99
GTP	3.0	4.5	8.1	8.2	1:1.03:1.81:2.74

with that of 5′-UMP. Phosphorus analysis gave the uracil: acid-labile phosphate: total phosphate ratio (1:1:2) of UDP. The R_F-value of the unhydrolysed fraction in propanol-ammonia-water, however, was much higher than that of UDP indicating a derivative of UDP. On chromatography after hydrolysis in N hydrochloric acid at 100 °C for 10 min the fraction gave only one ultraviolet absorbing spot, corresponding to 5′-UMP and phosphorus spots corresponding to UMP and orthophosphate. Spraying with ninhydrin revealed a strongly positive spot which did not coincide with the ultraviolet absorbing one. The unhydrolysed fraction gave, however, only a very weak ninhydrin reaction. Spraying with carbohydrate reagents gave no definite results. Oxidation with periodate gave a consumption of 0.95 mole periodate per mole base indicating that the 2′- and 3′-positions of the ribose moiety of UDP were unsubstituted. Our results so far characterize the UDPX fraction as a 5′-UDP derivative which on hydrolysis liberates a strongly ninhydrinpositive component.

Fraction X gave one ultraviolet absorbing spot both before and after hydrolysis in N hydrochloric acid at 100 °C. The ultraviolet absorption curve

Table 6. The nucleotide content of earthworm muscle obtained by ion-exchange chromatography of crude and norite-purified trichloroacetic acid extracts.

Fraction	μmoles per 100 g		Fraction	μmoles per 100 g	
	purified extract	crude extract		purified extract	crude extract
AMP	20.6	20.6	IMP	2.5	3.9
ADP	53.2	67.5	UMP	2.5	2.4
ATP	108.0	102.0	UDPX	8.0	9.4
ADPR (Y)	4.4	1.0	UTP	3.6	3.5
GMP	2.8	4.2	CTP	2.5	1.5
GDP	3.4	2.8	DPN	—	4.0
GTP	8.0	8.5			

and the elution position in ion-exchange chromatography indicate the presence of a cytidine derivative. The R_F-value after hydrolysis was that of 2′- or 3′-CMP. Beside the ultraviolet absorbing spot several ninhydrinpositive components were revealed by paper chromatography of this fraction, the complexity of which hindered further identification with the small amounts hitherto available.

The same results were obtained on ion-exchange chromatography of the crude trichloroacetic acid extract as with the norite eluates. The amounts of the different nucleotides from the earthworm muscle obtained by ion-exchange chromatography of the two types of extracts are shown in Table 6. There are a few qualitative differences in the nucleotide fractions between these two types of extracts. DPN was not eluted from norite by our procedure unless degraded to adenosinediphosphoribose and was consequently not found in the norite-purified extract. From the crude extract, however, DPN was obtained in the first chromatographic fractions together with other phosphorus compounds. DPN was estimated as the cyanidecomplex according to Colowick, Kaplan and Ciotti [13]. The earthworm muscle contains 4 μmoles DPN per 100 g, calculated from the molar extinction coefficient (6×10^{-6}) for the DPN—CN complex at 325 mμ.

Fraction Y is almost lacking in the DPN containing crude extract but is present in the DPN free extract obtained after norite adsorption, in further support of the identification of fraction Y as ADP-ribose originating from DPN. 4 μmoles of fraction Y were obtained from 100 g muscle, in good agreement with the figures for the DPN content of the crude extract. Otherwise the same nucleotides are obtained in both types of extracts, as seen from Table 6.

The acid-soluble nucleotide content of the earthworm muscle shows the following general pattern: predominance of adenine nucleotides (nearly 90 %), equal amounts of guanine and uridine derivatives (about 5 % of each) and a small amount of cytidine compounds. The same pattern is shown by rabbit skeletal muscle although the absolute amount of nucleotides was about 3 times greater in the latter case.

The resemblance in nucleotide composition between these two muscles of widely different origin is not surprising since their functions are similar.

The earthworm muscle, however, can hardly be considered as true striated muscle and is — compared with vertebrate skeletal muscle — a slow working muscle, a fact which may explain the quantitative difference in the nucleotide contents.

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