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Induced Transcription-Dependent Synthesis of Mitochondrial Reduced Nicotinamide–Adenine Dinucleotide Dehydrogenase in *Drosophila*

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Salivary glands of *Drosophila hydei* recovering from an anaerobic treatment show a significant increase in apparent $V_{max.}$ of mitochondrial NADH dehydrogenase. This increase in $V_{max.}$ is based on an increase in enzyme molecules resulting from synthesis *de novo* in the cytoplasm, as indicated by the inhibition by cycloheximide of both the increase in apparent $V_{max.}$ and the increase in amino acid incorporation into enzyme fractions. The increase in enzyme activity is also inhibited by actinomycin D, which is in support of previous data indicating a causal relationship between transcription in puff 4-81B in the polytene chromosomes and an increase in apparent $V_{max.}$ of the enzyme. Gel electrophoresis of mitochondrial protein extracts revealed three protein fractions with NADH dehydrogenase activity. All three fractions showed increased activity as well as increased amino acid labelling in glands recovering from anaerobiosis compared with control glands. The data suggest that the increase in mitochondrial NADH dehydrogenase activity in salivary glands recovering from an anaerobic treatment depends on increased gene transcription.

A variety of treatments which interfere with the cellular respiratory metabolism results in the activation of a particular set of genes. In the polytene chromosomes this gene response is manifested by the occurrence of new chromosome puffs at loci which were inactive before the treatment (Ritossa, 1962; Berendes *et al.*, 1965; Ashburner, 1970; Leenders & Berendes, 1972).

In *Drosophila hydei*, the genome response after the release from anaerobiosis or incubation of salivary glands in media containing inhibitors of the terminal respiratory chain consists of the formation of four major puffs (Leenders & Berendes, 1972).

It has been previously shown that the appearance of these new puffs is followed by increases in apparent $V_{max.}$ of both mitochondrial NADH dehydrogenase (EC 1.6.99.3) and tyrosine aminotransferase (EC 2.6.1.5) (Leenders *et al.*, 1974; Koninkx *et al.*, 1975). No change in the apparent K_m of these enzymes was observed. Moreover, it was established that the increase in apparent $V_{max.}$ of the mitochondrial enzymes was dependent on synthesis *de novo* of RNA and cytoplasmic protein (Leenders & Beckers, 1972; Leenders *et al.*, 1974; Koninkx *et al.*, 1975).

The increase in NADH dehydrogenase activity could be correlated with the increase in activity of puff 4-81B, since treatment with antimycin A, which induces only three of the four puffs, did not lead to elevated NADH dehydrogenase activity. If the activity of locus 4-81B does indeed result in an increase in mRNA coding for NADH dehydrogenase this increase should be reflected in the synthesis of the enzyme *de novo*.

The aim of the present study was to demonstrate that synthesis *de novo* of this mitochondrial enzyme does indeed increase after puff induction.

Materials and Methods

Materials

Bovine serum albumin and actinomycin D were obtained from Calbiochem, Los Angeles, Calif., U.S.A.; 3-(4,5-dimethylthiazol-2-yl)-2,5-tetrazolium bromide was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.; cycloheximide was from Serva, Heidelberg, Germany; β-NADH (disodium salt) and chloramphenicol were from Boehringer G.m.b.H., Mannheim, Germany; acrylamide and bisacrylamide were from BDH Chemicals, Poole, Dorset, U.K.; [^{35}S]methionine (sp. radioactivity 325 Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K., and mixtures of ^{3}H-labelled amino acids (lot no. 787-172) and ^{14}C-labelled amino acids (lot. no. 828-021) were from New England Nuclear Corp., Boston, Mass., U.S.A. All other chemicals were of analytical grade from E. Merck A.G., Darmstadt, Germany.

In all experiments salivary glands of mid-third-instar larvae of *Drosophila hydei*, raised under standardized conditions in mass culture (Mitchell & Mitchell, 1964), were used. Salivary glands were isolated as described by Boyd *et al.* (1968).

Puff induction

Puffs at the chromosome loci 2-32A, 2-36A, 2-48BC and 4-81B were induced by incubating salivary

glands for 120min in Poels's (1972) medium flushed with N_2 under an N_2 atmosphere. The glands were allowed to recover for 75min by exposure to air. Glands used as controls were kept in the same medium (well aerated) for 195min. Before the glands were used for isolation of the mitochondria the chromosomal puffing pattern was analysed to ascertain that anaerobically treated glands had developed the specific puffs and that they were absent from the controls.

Preparation of mitochondria

After incubation, 150–250mg of glands was washed three times with 5ml of Mg–Ringer medium (see Leenders & Berendes, 1972) and homogenized in 3.5ml of a solution containing 0.154M-KCl, 1mM-EDTA and 1% (w/v) bovine serum albumin, pH7.2. The homogenate was centrifuged for 10min at 1000*g*, the pellet was resuspended in 1.5ml of the same solution and re-centrifuged for 10min at 1000*g*. The supernatants were combined and centrifuged for 10min at 6000*g*. After resuspension of the resulting mitochondrial pellet in 5ml of 0.154M-KCl–1mM-EDTA, pH7.2, and re-centrifugation for 10min at 6000*g*, the pellet was suspended in 0.35ml of the same solution and sonicated with a Branson sonifier (step 2; 3×10s). The sonicate was used for protein determination (Lowry *et al.* 1951; bovine serum albumin as standard) and for gel electrophoresis.

Gel electrophoresis and enzyme activity assay

Gel electrophoresis was performed as described by Davis (1964) and Ornstein (1964) on 10% (w/v) polyacrylamide gels for 3h. Bromophenol Blue was used as a marker. The current applied per gel was 2mA. The inner diameter of the Pyrex glass tube was 5mm, the outer diameter was 7mm; the length of the tube was 115mm. Three different buffers were used. The small-pore gel buffer was composed of 48ml of 1M-HCl, 36.6g of Tris and water added to 100ml, pH8.9. The large-pore gel buffer was composed of 48ml of 1M-HCl, 5.98g of Tris and water added to 100ml, pH6.7. The reservoir buffer was composed of 3.0g of Tris, 14.4g of glycine and water added to 1 litre, pH8.3. The protein quantity applied varied for different experiments between 50 and 150μg/gel. In all instances in which mitochondrial proteins from treated and non-treated glands were compared, equal protein quantities were applied to the gels.

NADH dehydrogenase activity was determined after gel electrophoresis of the mitochondrial protein preparations by incubation of the gels overnight at 0°C in 0.1M-Tris–HCl (pH7.2) containing 0.5mM-NADH and 0.5mM-oxidized 3-(4,5-dimethylthiazol-2-yl)-2,5-tetrazolium bromide. NADH dehydrogenase activity was detected on the basis of the formation of formazan [reduced 3-(4,5-dimethylthiazol-2-yl)-2,5-tetrazolium bromide] which has a blue–purple colour with a peak absorbance at 560nm (Sin & Leenders, 1975). The quantity of reduced 3-(4,5-dimethylthiazol-2-yl)-2,5-tetrazolium bromide formed was measured densitometrically at 560nm with a Zeiss PMQ II spectrophotometer, equipped with an integrating recorder. Control experiments revealed a linear relationship between the densitometrically established peak area of staining bands and the quantity of mitochondrial protein in the range from 20 to 150μg/gel.

After incubation of the gels in buffer with neither NADH nor oxidized 3-(4,5-dimethylthiazol-2-yl)-2,5-tetrazolium bromide, no stained bands were observed.

Incorporation of [^{35}S]methionine and ^{3}H- and ^{14}C-labelled amino acids

The incorporation of [^{35}S]methionine in salivary glands or in mitochondria of these glands was determined after incubation in 200μl of Poels's (1972) medium devoid of methionine sources other than the [^{35}S]methionine. After incubation, the glands were washed six times for 5min each with 5ml of ice-cold Mg–Ringer (pH7.2) containing 10mM-methionine (unlabelled).

To establish the time of maximal protein synthesis in glands recovering from an anaerobic treatment, protein was extracted by the procedure described by Tissières *et al.* (1974) and the radioactivity per μg of protein determined by liquid-scintillation counting in 10ml of scintillant containing 20ml of methoxyethanol (E. Merck A.G.), 5.5g of Permablend III (Packard Instrument Co., Downers Grove, Ill., U.S.A.) and 30ml of Soluene-100 (Packard Instrument Co.) per litre of toluene. Liquid-scintillation counting was performed with a Philips liquid-scintillation analyser PW 4510/01 which was equipped with a computer program for calculation of the number of d.p.m. from the number of c.p.m. and the external-standard ratio.

A comparison of the radioactivity incorporated by 30mg of glands incubated in 100μl of medium containing 10μCi of [^{35}S]methionine during 20min incubation periods beginning immediately after the release from anaerobiosis or 15min, 30min, 45min or 60min after the release from anaerobiosis revealed a higher rate of incorporation of radioactivity per μg of protein in the treated glands than in the control glands if the incorporation period begins at 45 or 60min after the release from anaerobiosis (Table 1). If the incorporation period of 20min begins at an earlier time, the incorporation per μg of protein in the

Table 1. *Rate of [^{35}S]methionine incorporation into total salivary-gland protein after various periods of recovery from anaerobiosis*

The treated salivary glands were recovering from a 2h (N_2) anaerobic treatment in a well-aerated medium at 25°C. The control salivary glands were kept in a well-aerated medium for the same period of time at 25°C. The salivary glands were pulse-labelled for 20min.

Time after release from anaerobiosis (min)	Radioactivity incorporated (d.p.m./μg of protein)		Treated glands/control glands
	Control glands	Treated glands	
0	5610	1611	0.29
15	5839	4305	0.74
30	5902	6012	1.02
45	6021	7230	1.20
60	5727	7322	1.28

treated glands is lower than in control glands (Table 1). On account of these data comparisons between pulse-labelled proteins of treated and non-treated glands or mitochondria prepared from them are always based on experiments in which the glands were pulse-labelled for a 30min period beginning at 45min after the release from anaerobiosis.

The patterns of newly synthesized pulse-labelled proteins were studied after electrophoresis on 10% (w/v) polyacrylamide gels which were either cut into 1mm slices and counted for radioactivity or radioautographed with Kodak medical X-ray films, RP/R14 (exposure time 5 days).

Double-labelling experiments were performed in which anaerobically treated glands were incubated for a 30min period beginning 45min after the release from anaerobiosis, in 75μl of medium containing 40μCi of a mixture of ^{3}H-labelled amino acids, and non-treated glands were incubated in 75μl of medium containing 15μCi of a mixture of ^{14}C-labelled amino acids. Equal amounts of the protein extracts from treated and non-treated glands were mixed and submitted to electrophoresis. The gels were cut into 1mm slices and the ^{3}H and ^{14}C labelling was determined by liquid-scintillation counting.

Inhibition experiments

To test whether or not the changes in the pattern of newly synthesized proteins after the release of salivary glands from anaerobiosis is dependent on synthesis *de novo* of RNA and protein, inhibition experiments were performed. RNA synthesis was inhibited during the recovery period by 20μg of actinomycin D/ml, cytoplasmic protein synthesis by 5μg of cycloheximide/ml and mitochondrial protein synthesis by 100μg of chloramphenicol/ml.

Results

Electrophoretic separation of proteins with NADH dehydrogenase activity

After electrophoresis of sonicates of mitochondria and incubation of the gels with NADH and oxidized 3-(4,5-dimethylthiazol-2-yl)-2,5-tetrazolium bromide overnight, three blue–purple bands [reduced 3-(4,5-dimethylthiazol-2-yl)-2,5-tetrazolium bromide] were detected. These bands, designated A, B and C, with relative mobilities of 0.157, 0.177 and 0.201 (Table 2) differed in E_{560} as measured by densitometry (Fig. 1). Measurements of the peak areas of densitograms of the three bands obtained from mitochondria of control glands revealed the proportion of A:B:C as 52.3:33.8:13.9.

Electrophoresis of sonicates of mitochondria isolated from glands 75min after the onset of recovery from an anaerobic treatment also revealed three bands. The R_F values of these bands as well as the relative absorbances were identical with those of the control glands (Table 2, Fig. 1). These findings demonstrate that the number and electrophoretic behaviour of protein fractions with NADH dehydrogenase activity are the same in mitochondria from control as in those from anaerobically treated glands.

However, the absolute values of the measured peak areas of all bands differed significantly when each band in the mitochondrial protein extract of treated glands was compared with the corresponding band of non-treated (control) glands (Table 2). In all protein fractions with NADH dehydrogenase activity, the activity was higher in mitochondria obtained from anaerobically treated glands than in control glands. This result agrees with previous data indicating an increase in the apparent $V_{max.}$ of the enzyme during the recovery of glands from anaerobiosis (Koninkx *et al.*, 1975).

Table 2. *Relative electrophoretic mobilities, relative absorbance values and increase in peak areas of mitochondrial protein fractions with NADH dehydrogenase activity*

The relative mobility of a protein fraction is defined as the ratio between the migration distance in polyacrylamide gels of the protein fraction and of the tracking dye Bromophenol Blue. The relative absorbance value is defined as the ratio between the peak areas of the individual fractions in densitograms made at 560nm and the total peak area of all fractions with NADH dehydrogenase activity in the same densitograms. The increase in peak areas of each mitochondrial protein fraction with NADH dehydrogenase activity from treated glands is expressed as percentage increase over the corresponding fractions in the controls. Each value is a mean of five experiments (±S.D.).

	Relative mobilities		Relative absorbance values		
	Untreated	Treated	Untreated	Treated	Increase in peak area
Band A	0.157±0.002	0.156±0.004	52.3±3.2	50.5±8.3	38.8±1.4
Band B	0.177±0.003	0.177±0.004	33.8±1.3	34.2±3.7	17.3±0.7
Band C	0.201±0.002	0.200±0.003	13.9±2.4	15.3±5.0	9.7±0.6

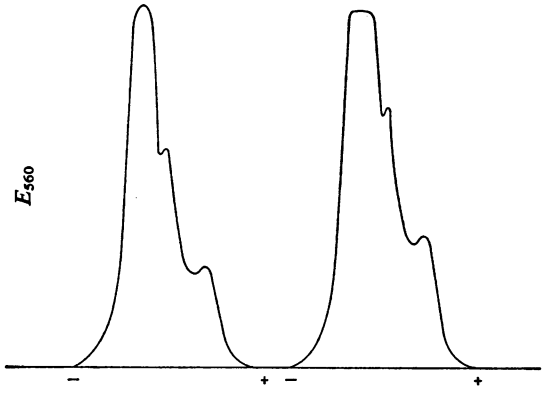

Fig. 1. *Densitometer tracings of polyacrylamide gels showing the protein fractions with NADH dehydrogenase activity*

The gels were measured at 560nm which is the peak absorbance of reduced 3-(4,5-dimethylthiazol-2-yl)-2,5-tetrazolium bromide. The densitometer tracing at the left is from gels of controls, the tracing at the right from gels of mitochondrial proteins from recovering glands.

To establish whether or not this increase in enzyme activity is based on an increase in enzyme molecules, both the rate of protein synthesis and the pattern of incorporation of labelled precursors in mitochondrial protein were determined.

Electrophoresis of [^{35}S]methionine-labelled mitochondrial proteins

The rate of protein synthesis is very low in glands which are just released from anaerobiosis (see Table 1 in the Materials and Methods section). At 50min after the onset of the recovery period the rate of protein synthesis is higher than in the control glands. The chromosome puffs attained their maximum size at

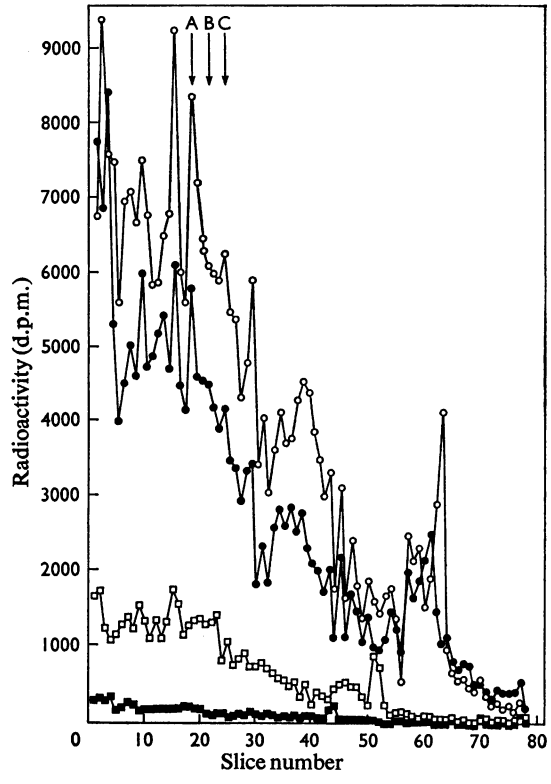

Fig. 2. *Radioactivity profiles of [^{35}S]methionine-pulse-labelled mitochondrial proteins isolated from salivary glands*

●, Non-treated glands; ○, glands after 75min recovery from anaerobiosis (identical with that of glands recovering in the presence of 100μg of chloramphenicol/ml); ■, glands after 75min recovery from anaerobiosis in the presence of 5μg of cycloheximide/ml; □, glands after 75min recovery from anaerobiosis in the presence of 20μg of actinomycin D/ml. The [^{35}S]methionine pulse was given from 45min until 75min after the onset of recovery. The inhibitors were present during the entire recovery period. Arrows indicate the position of protein fractions (A–C) with NADH dehydrogenase activity.

20min, whereas the greatest rate of increase in the apparent $V_{max.}$ of the NADH dehydrogenase occurs between 30 and 75min after the onset of the recovery period (Leenders & Beckers, 1972).

Fig. 2 shows the electrophoretic analysis of labelled mitochondrial proteins obtained after 30min labelling with [^{35}S]methionine beginning at 45min after the onset of recovery from anaerobiosis. This analysis demonstrated that, among others, the protein fractions with NADH dehydrogenase activity displayed a significantly higher incorporation in treated than in non-treated glands (arrows). As expected, no incorporation was seen in mitochondrial proteins of glands recovering from anaerobiosis in the presence of cycloheximide. Little incorporation was found when the glands recovered in medium with actinomycin D (Fig. 2). Chloramphenicol had no effect on the labelling of the protein fractions. These data suggest that the increase in label also depends on nuclear RNA synthesis *de novo*.

Double-labelling experiments

To exclude the possibility that the higher [^{35}S]-methionine incorporation in mitochondria of anaerobically treated glands merely reflects changes in amino acid pools, equal quantities of ^{14}C-labelled mitochondrial proteins from control glands and ^{3}H-labelled proteins from treated glands were mixed and run on polyacrylamide gels. After electrophoresis the ^{3}H/^{14}C ratio of each 1mm slice was determined. The ratios found for slices containing proteins with NADH dehydrogenase activity were significantly higher in mitochondrial extracts from treated than from non-treated glands (Fig. 3).

Inhibition experiments

Measurements of enzyme activity in gels of mitochondrial proteins extracted from glands recovering from anaerobiosis in the presence of cycloheximide revealed that the peak area of each of the three bands with enzyme activity is approximately the same as in extracts of control glands. This finding indicates that the increase in NADH dehydrogenase activity found after release from anaerobiosis is due to a net increase in enzyme molecules in the mitochondria. Since the activity of a mitochondrial enzyme synthesized in the cytoplasm can be increased in three different ways, i.e. decreased enzyme turnover in the mitochondria, increased uptake or increased synthesis *de novo* accompanied by increased uptake, the effect of cycloheximide on the apparent $V_{max.}$ of the enzyme was tested. An increase in enzyme activity owing to either a decreased turnover or an increase in uptake without an increase in synthesis *de novo* would be insensitive to cycloheximide inhibition of protein

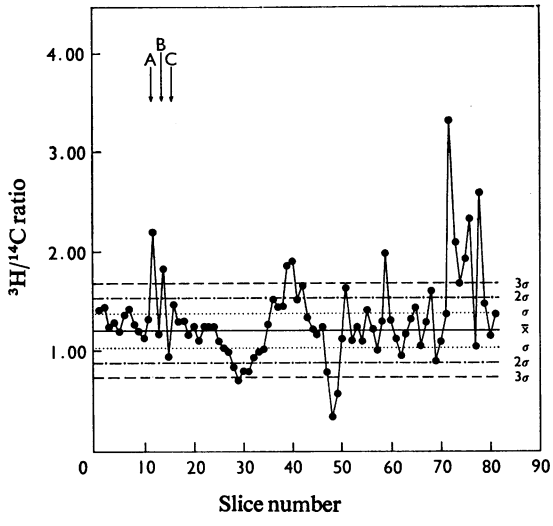

Fig. 3. *^{3}H/^{14}C ratios for individual fractions of gels in which a mixture of equal quantities of ^{3}H-labelled mitochondrial proteins from recovering glands and ^{14}C-labelled mitochondrial proteins from control glands were electrophoretically separated*

$\bar{x}$ is the mean of all ^{3}H/^{14}C ratios. σ is the standard deviation of these ^{3}H/^{14}C ratios which are indicated in the figure. Arrows show the positions of the fractions (A–C) with NADH dehydrogenase activity.

Table 3. *Apparent $V_{max.}$ of the mitochondrial NADH dehydrogenase*

The apparent $V_{max.}$ was measured in sonicates of mitochondria isolated from salivary glands (see the Materials and Methods section) after incubation of the control salivary glands in the absence (−) or presence (+) of cycloheximide (5μg/ml) and of salivary glands in the absence (−) or presence (+) of cycloheximide during the 75min recovery from anaerobiosis. The enzyme assay and the calculation of the apparent $V_{max.}$ were done as described by Leenders & Beckers (1972).

	Apparent $V_{max.}$ (μmol of NADH/min per mg of protein)
Control glands−cycloheximide	4.12
Control glands+cycloheximide	3.84
Glands recovering from anaerobiosis for 75min −cycloheximide	7.61
Glands recovering from anaerobiosis for 75min +cycloheximide	4.24

synthesis. Table 3 shows that the apparent $V_{max.}$ of mitochondrial NADH dehydrogenase in control glands incubated in medium without and with cycloheximide is slightly decreased when cycloheximide is present in the medium. The apparent $V_{max.}$ of the enzyme in mitochondria of glands recovering in medium without cycloheximide is significantly higher than in the controls (see Koninkx *et al.*, 1975), whereas this increase is almost completely absent from glands recovering in the presence of the protein-synthesis inhibitor. Thus the increase in apparent $V_{max.}$ of the enzyme during recovery from anaerobiosis depends on protein synthesis *de novo*.

Discussion

The present results demonstrate that the previously established increase in apparent $V_{max.}$ of the mitochondrial NADH dehydrogenase after experimental gene activation (Koninkx *et al.*, 1975) is correlated with an increased enzyme activity of three mitochondrial protein fractions as well as with an increased labelling of these proteins. The results of the inhibition experiments further suggest that the increase in apparent $V_{max.}$ depends on synthesis *de novo* of nuclear RNA and cytoplasmic protein rather than on changes in turnover rate or uptake of enzyme molecules without increased synthesis *de novo*. The apparent lack of labelled protein fractions in mitochondria of glands recovering in the presence of cycloheximide indicates that the synthesis of cycloheximide-insensitive mitochondrial proteins is very low or completely absent. As such, this finding is in accord with a previous suggestion that the increase in mitochondrial activity after recovery from anaerobiosis is not due to mitochondrial biogenesis.

The present data show a definite relationship between the induced activity of nuclear genes and increase in the activity of a mitochondrial enzyme which is known to occupy a key position in the terminal respiration (Hemmerich *et al.*, 1970).

It is tempting to suggest that one of the induced puffs, in particular puff 4-81B, is responsible for the production of mRNA molecules coding for the enzyme on the basis of the fact that this puff is not activated when the glands are treated with antimycin A and that under those conditions no increase in mitochondrial NADH dehydrogenase activity occurs.

An analysis of the pattern of newly synthesized cytoplasmic proteins in glands recovering from anaerobiosis revealed six strongly labelled polypeptide fractions in *Drosophila hydei* glands (Lewis *et al.*, 1975). Preliminary experiments indicated that the labelled mitochondrial protein fractions with NADH dehydrogenase activity separated by polyacrylamide-gel electrophoresis migrate in sodium dodecyl sulphate–polyacrylamide gels in the range of proteins with mol. wts. 67000–75000. In this region two or three heavily pulse-labelled protein fractions occur in cytoplasmic protein extracts of glands recovering from anaerobiosis. Since the NADH dehydrogenase isolated from rat heart mitochondria has approx. mol.wt. 300000 (King *et al.*, 1966), the mitochondrial protein migrating in the range 67000–75000 mol.wt. may be a subunit of the enzyme.

The relationship between the transcriptional activity of one chromosome locus (4-81B) and the increased synthesis of proteins with NADH dehydrogenase activity which are incorporated in the mitochondria supports Beermann's hypothesis (1952) that puffs are sites of mRNA synthesis.

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