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AFFINITY BINDING OF NON-HISTONE CHROMATIN PROTEINS TO THE X CHROMOSOME OF *DROSOPHILA* BY *IN SITU* CHROMATIN RECONSTITUTION AND ITS SIGNIFICANCE

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SUMMARY

Cytophotometric analysis of the *in situ* binding affinity of non-histone chromosomal protein (NHCP) to the polytenic X chromosome and autosome of *Drosophila melanogaster* has been carried out using Feulgen–Napthol Yellow S staining technique. The results reveal that the mean transformed absorbance ratio (male:female) with a 547 nm interference band filter for the two specific segments of the X chromosome is close to 0·5, while for a specific segment of an autosome it is close to 1·0, in the two sets of control; namely, the positive control (no treatment) and the negative control (treated with 1 M-urea+2 M-NaCl) as well as in the reconstituted chromosomal preparations, which received 1 M-urea+2 M-NaCl and the NHCP isolated from *D. melanogaster*. In contrast, the transformed absorbance ratios (male:female) with a 433 nm interference band filter yielded an interestingly different result. The ratios with a 433 nm filter for the X chromosome segments are significantly greater than 0·5 in all three sets of experiments. This finding by itself suggests that the NHCP binding affinity is dissimilar for the X chromosomes of male and female. When the 433 to 547 nm absorbance ratios were compared among the three sets, the data clearly revealed that in both positive control and NHCP reconstituted samples, the absorbance ratios (i.e. 433:547 nm) are significantly different between X chromosomes from males and those from females, while they are different between autosomes from males and females. The ratios are also not significantly different between male and female, either for the X chromosome or for the autosome in the negative control. These findings, therefore, suggest that there is a stronger binding affinity of NHCP for the male X chromosome of *Drosophila*, and reinstate the view that the X-chromosomal hyperactivity in male *Drosophila* is the consequence of a regulated organizational change in the DNA template.

INTRODUCTION

The X chromosome of the male *Drosophila* is nearly twice as active as the individual X chromosomes of the female (Mukherjee & Beermann, 1965). This has been well substantiated more recently (Lakhotia & Mukherjee, 1969; S. N. Chatterjee & Mukherjee, 1971; Maroni & Plaut, 1973; Mukherjee & Chatterjee, 1975, 1976; Lucchesi, 1974; R. N. Chatterjee & Mukherjee, 1981; Stewart & Merriam, 1980; Baker & Belote, 1983). The hyperactivity is considered to be the cytological counterpart of dosage compensation, a term given to the phenomenon of equalization of gene product of X-linked genes in the two sexes (Muller *et al.* 1931),

Key words: chromatin, X chromosome, *Drosophila*.

and initiated at the level of transcription (Kaiser *et al.* 1986; see also Dey & Lucchesi, 1983).

Lack of mosaic expression of X-coded genes in females heterozygous for coat colour mutations and expression of 6-phosphogluconate dehydrogenase allozymase, PGD^A (fast) and PGD^B (slow), showing two parental forms and an intermediate form in *trans*-heterozygotes, as resolved by polyacrylamide gel electrophoresis, convincingly rule out the operation of dosage compensation in *Drosophila* females by inactivation of one set of X-linked genes (Kazazian *et al.* 1965; see also Lucchesi, 1977).

In earlier reports it was proposed that the chromatin organization of the X chromosome in *Drosophila* males may be at a higher state of template activity than in females (Maroni & Plaut, 1973; Chatterjee & Mukherjee, 1981; Lakhotia & Mukherjee, 1970; Chatterjee *et al.* 1981). This proposition was substantiated by data from *in situ* transcription with *Escherichia coli* RNA polymerase (holoenzyme) as well as *Drosophila* RNA polymerase II with and without high salt (Chatterjee & Mukherjee, 1981; Chatterjee *et al.* 1981).

It is well established that chromosomal proteins, histones and non-histones, contribute considerably toward the chromatin organization and influence the template activity (Elgin & Weintraub, 1975). Since the X chromosome in *Drosophila* males is hyperactive and has higher template activity as well, we intended to determine whether such proteins discriminate differentially between the autosomes and the X chromosomes in the male and whether they bind, upon reconstitution *in situ*, differently with the two types of chromosomes. We have examined the possibility by *in situ* affinity binding and measuring the bound proteins cytophotometrically, by the Feulgen–Napthol Yellow S (FNYS) staining method for DNA and non-histone chromosomal proteins (NHCP) after Deitch (1966), using a non-scanning Cytophotometer (Leitz, MPV II). The results have yielded important information with regard to the role of X-chromosomal DNA–protein organization in setting up the template for hyperactivity of the male X chromosome.

MATERIALS AND METHODS

Materials and genetic strains

In these experiments we used polytene chromosomes from late third instar larval salivary glands of the wild-type Oregon R^+ strain of *Drosophila melanogaster* obtained from the *Drosophila* Stock Center, Pasadena, in 1966 and maintained in this laboratory since then. The strain has been and is reared in standard *Drosophila* medium, with Nipagin and propionic acid as anti-fungal preservative, at 23(±2)°C.

Cytological procedure for chromosomal preparation

Salivary glands from late third instar larvae of *D. melanogaster* were dissected out in *Drosophila* Ringer (pH 7·2), fixed in 7·5% formaldehyde in 0·01 M-phosphate buffer (pH 7·6) for 30 s, and squashed on 50% acetic acid. Coverslips were immediately removed by dry freezing. The slides were then passed through a formalin/ethanol series, upgrade and subsequently downgrade to water. This series of procedures was necessary to preserve the NHCP *in situ* (Deitch, 1966). Quick removal of acetic acid by dry freezing and subsequent washing in formalin/ethanol prevents removal of the proteins.

Isolation of non-histone chromosomal proteins

Adult flies from the wild-type male and female *D. melanogaster* were homogenized and nuclei were isolated (Elgin & Hood, 1973; Phillips & Forrest, 1973). Chromatin was pelleted from the nuclei (Chiu *et al.* 1975) and suspended in 5 M-urea in 50 mM-phosphate buffer (pH 7·6), stirred for 4 h using a magnetic stirrer, centrifuged at 15 000 rev. min^{-1} at 4°C for 0·5 h in a fixed-angle rotor and the supernatant was collected. The supernatant should contain 90–95 % of the NHCP (Chiu *et al.* 1975). The protein concentration was estimated according to the modified method of Lowry *et al.* (1975). The yield was on average 1·5 to 1·75 mg/10 g flies. Isolated proteins were dialysed overnight in a cold room (2–4°C) against 50 mM-phosphate buffer (pH 7·6).

Treatment and in situ *chromatin reconstitution*

Chromosomal preparation made by the procedure described were grouped into three batches: in one, slides were incubated in 2 M-NaCl/1 M-urea in 50 mM-phosphate buffer for 90 min and NHCP for 90 min; in the second batch, slides were incubated in 2 M-NaCl/1 M-urea in 50 mM-phosphate buffer (pH 7·6) for 90 min (negative control); the third batch of slides were given no treatment (positive control).

Staining procedure for cytophotometry

All preparations were hydrolysed in 1 M-HCl at 60°C for 10 min, and stained in Schiff's reagent (E. Merck, Germany) for about 90 min according to the Feulgen reaction for DNA (Stowell, 1945). Slides were then rinsed in 5 % acetic acid very briefly and counterstained in Napthol Yellow S (Fluka, England) for 60 min (Deitch, 1966). The specific segments of the chromosomes were then measured cytophotometrically as described below.

Cytophotometry

For quantitative estimation of the DNA of a specific part of a chromosome or of an entire chromosome per cell, the slides were stained by the Feulgen–Rossenbeck technique (Stowell, 1945). Transmittance measurements were recorded with a 547 nm interference band filter (Leitz). For measurements of NHCP, the slides were stained with Napthol Yellow S after Deitch (1966) and transmittance was recorded with 433 nm interference band filter in the Leitz MPV II attached to an Orthoplan Microscope (Leitz). All measurements were taken with a 100× oil immersion objective. The MPV II cytophotometer (Leitz) is a non-scanning non-integrating system.

Two specific segments of the X chromosome (1A–3C, referred to as X_b or distal, and 11A–14A, referred to as X_a or proximal) and a specific segment of the autosome 2L (21A–25F) from each nucleus were examined for quantification of DNA and NHCP. The specific segments of the chromosomes mentioned above can be easily identified in the squash preparations of polytene chromosomes by their banding patterns and puffs (see Beermann, 1964). Each specific segment of the chromosome(s) thus identified was outlined by the adjustable rectangular slit attached above the photocell. The area of illumination was adjusted by another rectangular slit inserted in the illumination path such that the area of illumination was only slightly larger than the measuring area. Each measurement was based on the 'one wave length/two areas' principle (Mariano-Garcia & Iorio, 1966).

The transformed absorbance was calculated by the following equation:

$$EA_1 = \log \frac{1}{T} \frac{(1-T_1)A_1}{1-T_1},$$

$$\text{where } T = \frac{I_s}{I_o} = \frac{\text{recorded value on the chromosome segment}}{\text{recorded value on the background}}.$$

Since I_o was adjusted to 100 %, the value of EA_1 becomes:

$$= \log \frac{100}{T} \frac{(1-T_1)A_1}{(1-T_1)},$$

where E = extinction, A_1 = area of the specific segment of the chromosome, A_i = area slightly greater than A_1, T_1 = transmittance for the area A_1, and T_i = transmittance for the area A_i.

A number of nuclei from each set was first examined at random with the specific interference band filters (547 nm and 433 nm), and the transmittance spectrum and the peak were monitored through a motorized variac attached to a recorder to ensure that the dye binding gives the peak at one and only one wavelength. The two segments of the X chromosome (namely X_a and X_b) and the autosomal segment (21A–25F) were examined and measured with both the filters.

The nuclei from each slide were so selected that the specific chromosomal regions to be measured were not embedded in or overlying any cytoplasmic materials. Furthermore, only the arms of the chromosomal segments concerned that were sufficiently free, i.e. without overlapping chromosomal material, were chosen for measurement.

Photomicrography

Photomicrographs were taken under a ×100 oil immersion objective in a Zeiss Differential Interference contrast microscope attached with a Wollaston Prism (Plan 100/1·25 oil no. 47591) in the nose-piece, using the same interference band filters (namely, 547 nm for Feulgen and 433 nm for NYS).

RESULTS AND DISCUSSION

Figs 1 and 2 present the staining affinity of the X chromosome relative to the autosome in the male (XY2A) and Figs 3 and 4 present those in the female (XX2A), as resolved by the interference band filters.

Table 1 presents the mean ratios of transformed absorbance in the male to that in the female with the 547 nm filter for the proximal (X_a or 11A to 14A) and distal (X_b or 1A to 3C) parts of the X chromosome and for a specific length of an autosome (21A–25F of the 2L arm), in untreated chromosomal preparations (as positive control), in high-salt/urea-extracted preparations (as negative control) and in high-salt/urea-extracted and NHCP-reconstituted chromosomal preparations. It is evident from the data that while the ratios are close to unity for the autosome, they are approximately 0·5 for the X chromosome segments (X_a and X_b). This is expected as 547 nm filter gives the absorbance for DNA in FNYS-stained chromosomes, and since the male has only one X and two sets of autosomes, while the female has two X chromosomes and two sets of autosomes.

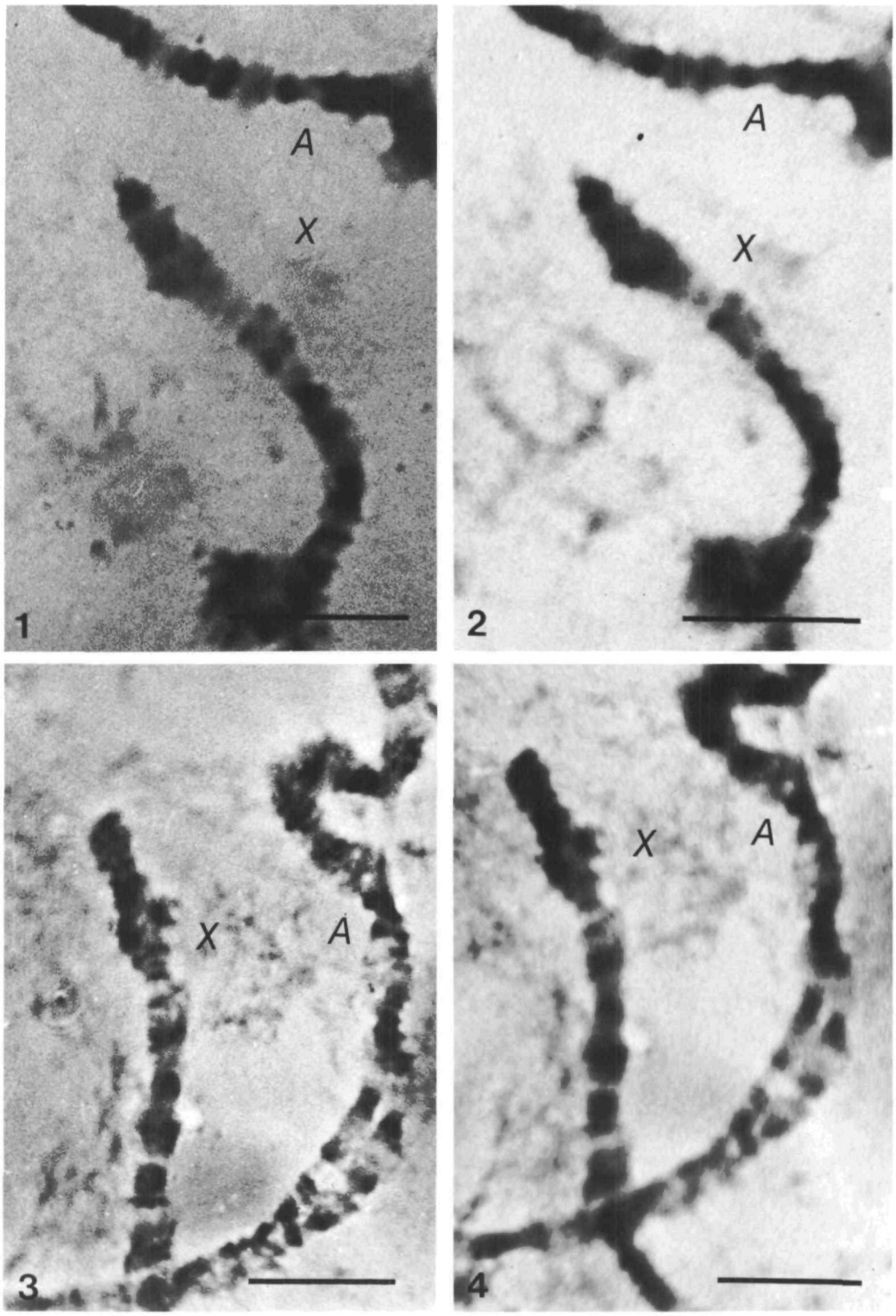
In contrast, the transformed absorbance ratios (male to female) with the 433 nm filter reveal interestingly different result (Table 2). While for the autosomes the mean ratios for the two controls and the NHCP-bound chromatin are not statistically

Fig. 1. Photomicrograph showing Feulgen–Napthol Yellow S-stained polytene chromosome segments from a larval salivary gland of *D. melanogaster* male after treatment with the extraction buffer alone (2 M-NaCl, 1 M-urea in 50 mM-phosphate, pH 7·6). Photograph taken with a 547 nm interference band filter. *X*, X chromosome; *A*, autosome. Bar, 10 μm.

Fig. 2. Photomicrograph of the same nucleus as in Fig. 1, but it is taken with a 433 nm interference band filter. Symbols as for Fig. 1.

Fig. 3. Photomicrograph showing Feulgen–Napthol Yellow S-stained polytene chromosome segments from a larval salivary gland of *D. melanogaster* female after treatment with the extraction buffer. Photograph taken with a 547 nm filter.

Fig. 4. Photograph of the same nucleus as in Fig. 3, but taken with a 433 nm filter.


A
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4

Table 1. *Transformed absorbance (male to female) ratio at 547 nm*

Experiment	No. of observed nuclei	Mean absorbance ratio ± S.E.		
		X_a	X_b	Autosome
1. Control	18	0·53 ± 0·01	0·51 ± 0·02	0·84 ± 0·02
2. Only buffer (2 M-NaCl/1 M-urea in 50 mM-phosphate buffer)	20	0·53 ± 0·01	0·55 ± 0·02	0·94 ± 0·02
3. Buffer+NHCP (2 M-NaCl/1 M-urea in 50 mM-phosphate buffer +NHCP (1 mg ml^{-1}))	20	0·54 ± 0·03	0·54 ± 0·03	0·90 ± 0·04

Table 2. *Transformed absorbance (male to female) ratio at 433 nm*

Experiment	No. of observed nuclei	Mean absorbance ratio ± S.E.		
		X_a	X_b	Autosome
1. Control	18	0·81 ± 0·05	0·79 ± 0·02	0·94 ± 0·06
2. Only buffer (2 M-NaCl/1 M-urea in 50 mM-phosphate buffer)	20	0·68 ± 0·06*	0·68 ± 0·04	0·81 ± 0·04
3. Buffer+NHCP (2 M-NaCl/1 M-urea in 50 mM-phosphate buffer +NHCP (1 mg ml^{-1}))	20	0·74 ± 0·03	0·72 ± 0·03	0·93 ± 0·07

*$P < 0·05$.

significantly different from each other, those for either segment of the X chromosome are significantly different ($P < 0·05$), between the positive control (no. 1) and urea/salt-extracted preparations (no. 2) and between urea/salt-extracted preparations (no. 2) and the NHCP-bound preparations (no. 3).

If the NHCP affinity were similar for the X chromosome of males and females, the absorbance ratio should have been close to 0·5. In contrast, the results in Table 2 reveal that the ratios are considerably greater than 0·5 in all three sets.

Since the affinity of NHCP to chromatin may be influenced by the amount of available DNA template, the 433:547 nm absorbance ratios were compared in all three sets of experiments. The results are presented in Table 3. In these cases the absorbance ratios (i.e. 433:547 nm) were compared in males and females separately. The data clearly reveal that in both positive control and NHCP-reconstituted samples, the transformed absorbance ratios are significantly different between male and female X chromosomes, while they are not significantly different for autosomes and for both X and autosomes for 2 M-NaCl/1 M-urea-extracted chromosome preparations.

These results lead us to one unavoidable proposition that the male X chromosome of *Drosophila* has a distinctly different organization that is more amenable to the

Table 3. *Transformed absorbance ratio 433:547 nm in male and female*

Experiment		No. of observed nuclei	Mean ratio 433:547 nm ± S.E.		
			X_a	X_b	Autosome
1. Control	Male	18	1·84 ± 0·09*	1·94 ± 0·10**	1·36 ± 0·06
	Female	18	1·33 ± 0·06	1·33 ± 0·04	1·26 ± 0·07
2. Only buffer (2 M-NaCl/1 M-urea in 50 mM-phosphate buffer)	Male	20	1·12 ± 0·03	1·13 ± 0·04	1·05 ± 0·04
	Female	20	1·03 ± 0·03	1·05 ± 0·05	1·15 ± 0·04
3. Buffer+NHCP (2 M-NaCl/1 M-urea in 50 mM-phosphate buffer +NHCP (1 mg ml^{-1}))	Male	20	1·75 ± 0·03***	1·73 ± 0·03***	1·23 ± 0·03
	Female	20	1·30 ± 0·03	1·31 ± 0·04	1·25 ± 0·04

*$P < 0·05$, **$P < 0·01$, ***$P < 0·001$.

RNA polymerase required for template activity and has a greater affinity for NHCP than the female X chromosomes (see Bautz Ekkehard & Dangli, 1983).

In order to ascertain whether the preferential binding of the NHCP with the male X chromosome is a reflection of non-specific binding, with more in some and much less in others, we analysed the NHCP removal (*in situ*) and reconstitution data for X chromosome and autosome on a per cell basis. The slopes calculated on the basis of the transformed absorbance data are presented in Fig. 5. The data indicate that in all experiments the transformed absorbance values for X_a and X_b segments are reasonably positively correlated with those for the autosome. The r values (linear regression) for X_a and X_b in the positive control, negative control and high salt/urea/NHCP-treated samples are 0·70 and 0·64, 0·51 and 0·56, and 0·34 and 0·36, respectively, in the male; and they are, 0·47 and 0·48, 0·53 and 0·54, and 0·62 and 0·83, respectively, in the female. It may be noted that only in the NHCP-reconstituted sample are the r values minimal. It is clear from the regression lines (Fig. 5) that the absorbance values in the NHCP-bound chromosomes from the male simulate the positive control with respect to the intercept and slope values, while those for the negative control male sample show drastically different slope and intercept values. This set of data suggests that while the NHCP binding follows first-order kinetics for the X chromosome in both male and female in relation to the autosome in the genomic complement, the low r values for the two segments of the chromosome in the NHCP-bound samples perhaps indicate an altered functional state of the X chromosome and autosomes following the *in situ* treatment.

Earlier, Rudkin (1969) showed that the X chromosome of the male contains about 11 % more histone-type proteins than that of the female. In this report we have used two large segments of the X chromosome and have shown that not only does the X chromosome of male *Drosophila* contain more NHCP but it may also preferentially bind more NHCP in reconstitution assays. The results corroborate the findings of Chatterjee *et al.* (1980), who by using scanning cytophotometry showed that the X chromosome of male *Drosophila* contains nearly twice as much NHCP as that of the female. Berendes (1968) demonstrated that there is an accumulation of NHCP at the point of initiation of puffing activity.

Recently, it has been shown that RNA polymerase binding precedes NHCP accumulation during gene activation (Bautz Ekkehard & Dangli, 1983). It is, therefore, reasonable to contend that NHCP binding and, or, accumulation is an indicator of gene activation. Perhaps such NHCP accumulation is required for the maintenance of the template activity and organization of the DNA. As a corollary to these conclusions and to those resulting from the present observations, we suggest that the hyperactivity of the male X chromosome is mediated through a predisposed modulation of the organization of the X chromosome in the male. Further work along this line in such strains as *mle* and *msl* (autosomal male specific lethals) and in a mutant that evokes a 4X activity in the male (Ghosh & Mukherjee, 1983; Mukherjee & Ghosh, 1986), may yield information with regard to the genetic contribution of the different regulatory genes involving hyperactivity (Belote & Lucchesi, 1980).

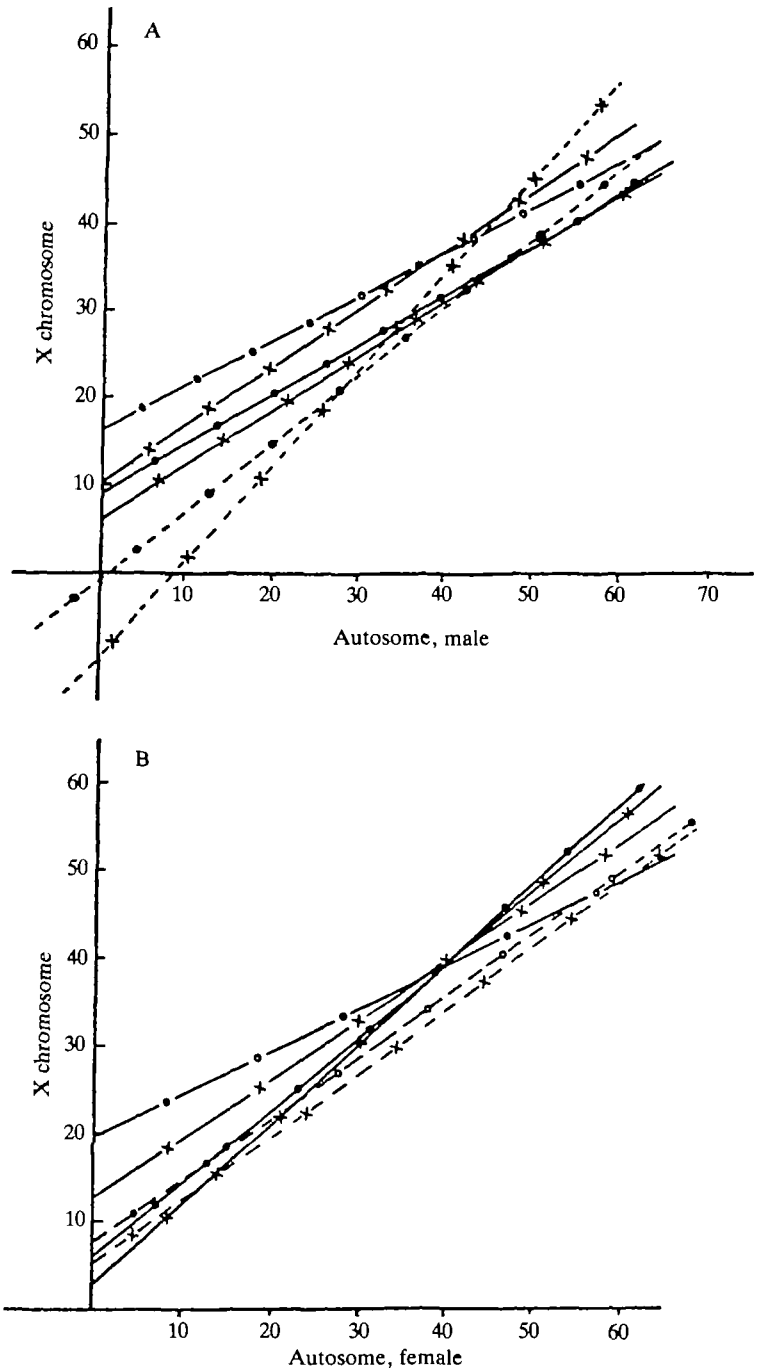

Fig. 5. Regression analysis of transformed absorbance at 433 nm of the X chromosome against the autosome in male (A) and female (B). (○——○) Xa, without treatment; (×——×) Xb, without treatment; (●——●) Xa, buffer, NHP; (×——×) Xb, buffer, NHP; (○– – –○) Xa, only buffer; (×– – –×) Xb, only buffer.

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