References and Notes

1. G. N. Woode and J. C. Bridger, *J. Med. Microbiol.* **11**, 441 (1978).
2. L. J. Saif, E. H. Buhl, K. W. Thiel, R. F. Cross, J. A. House, *J. Clin. Microbiol.* **12**, 105 (1980).
3. C. R. Madeley, *J. Infect. Dis.* **139**, 519 (1979).
4. J. E. Kaplan *et al.*, *ibid.* **146**, 190 (1982).
5. H. B. Greenberg, R. G. Wyatt, A. R. Halica, R. H. Yolken, R. Black, A. Z. Kapikian, R. B. Chanoch, *Perspect. Virol.* **11**, 163 (1981).
6. S. Chiba, Y. Sakuma, R. Kogasaka, M. Akihara, H. Terashima, K. Horino, T. Nakao, *J. Infect. Dis.* **142**, 247 (1980).
7. W. D. Cubitt, D. A. McSwiggon, W. Moore, *J. Clin. Pathol.* **32**, 786 (1979).
8. J. H. Gillespie and F. W. Scott, *Adv. Vet. Sci. Comp. Med.* **17**, 163 (1973).
9. S. H. Madin, in *Diseases of Swine*, H. W. Dunne and A. D. Lemans, Eds. (Iowa State Univ. Press, Iowa, ed. 4, 1975), pp. 286–307.
10. A. W. Smith, D. E. Skilling, R. J. Brown, *Am. J. Vet. Res.* **41**, 1845 (1980).
11. H. B. Gelberg and R. M. Lewis, *Vet. Pathol.* **19**, 424 (1982).
12. A. W. Smith, D. E. Skilling, A. B. Latham, *Am. J. Vet. Res.* **42**, 693 (1981).
13. R. Dulbecco and M. Vogt, *J. Exp. Med.* **99**, 167 (1953).
14. G. C. Revozzo and C. N. Burke, *A Manual of Basic Virological Techniques* (Prentice-Hall, Englewood Cliffs, N.J., 1973), pp. 150–151.
15. A. J. Howatson and G. F. Whitemore, *Virology* **16**, 466 (1973).
16. C. Wallis and J. L. Mulnick, *ibid.*, p. 504.
17. Y. C. Zee and A. J. Hackett, *Arch. Gesamte Virusforsch.* **20**, 473 (1967).
18. A. W. Smith, T. G. Akers, S. H. Madin, N. A. Vedros, *Nature (London)* **244**, 108 (1973).
19. A. E. Ritchie and A. L. Fernelius, *Arch. Gesamte Virusforsch.* **28**, 369 (1969).
20. There are San Miguel sea lion viruses types 1 to 12, one feline calicivirus serotype, six probable new calicivirus serotypes isolated from dolphins, minks, primates, walruses, snakes, and calves, and 13 serotypes of vesicular exanthem of swine virus. Of these 13 types, one has been lost. Typing serum but not antigen for the remaining 12 types was furnished to us by Plum Island Animal Disease Center.
21. A. W. Smith, C. M. Prato, H. L. Bray, *J. Wildl. Dis.* **12**, 326 (1976).
22. A. W. Smith, D. E. Skilling, A. E. Ritchie, *Am. J. Vet. Res.* **39**, 287 (1978).
23. G. G. Long, J. F. Everman, J. R. Gorham, *Can. J. Comp. Med.* **44**, 412 (1980).
24. A. W. Smith, D. E. Skilling, A. H. Dardiri, A. B. Latham, *Science* **209**, 940 (1980).
25. A. W. Smith, S. H. Madin, N. A. Vedros, R. A. Bankowski, *Am. J. Vet. Res.* **38**, 101 (1977).
26. A. W. Smith, C. M. Prato, D. E. Skilling, *ibid.* **39**, 287 (1978).

27. Supported by the Oregon State University School of Veterinary Medicine, Corvallis, and the Zoological Society of San Diego, San Diego, Calif. 92112.

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Sulfation Preceding Deiodination of Iodothyronines in Rat Hepatocytes

Abstract. *In man and animals iodothyronines are metabolized by deiodination and conjugation with glucuronic acid or sulfate. Until now these processes have been regarded as independent reactions. However, in the present study a close interaction of these pathways was observed in the hepatic metabolism of 3,3′-diiodothyronine and 3,3′,5-triiodothyronine. Studies with rat hepatocytes and liver microsomes indicated that sulfation of the phenolic hydroxyl group facilitates the deiodination of these compounds.*

The thyroid gland secretes mainly thyroxine (3,3′,5,5′-tetraiodothyronine, T_4), which is the precursor of the active form of thyroid hormone, 3,3′,5-triiodothyronine (T_3). Some 80 percent of the total T_3 production originates from outer ring deiodination of T_4 in peripheral tissues, especially the liver. Inner ring deiodination of T_4 yields 3,3′,5′-triiodothyronine (reverse T_3, rT_3), a biologically inactive compound. Both T_3 and rT_3 are further deiodinated to 3,3′-diiodothyronine (3,3′-T_2). A second important metabolic pathway for iodothyronines is conjugation with either glucuronic acid or sulfate. These principal pathways, deiodination and conjugation, have usually been regarded as functionally distinct processes. Here we present evidence for a close association of these processes in the hepatic metabolism of iodothyronines.

The metabolism of 3,3′-T_2 and T_3 was studied with monolayers of rat hepatocytes, prepared as described previously (*1*). Whereas hepatic T_3 metabolism is rather complex, 3,3′-T_2 metabolism is easier to follow as it is subject only to outer ring deiodination and sulfation (*2*). Initially, therefore, we focused on the metabolism of 3,3′-T_2 by incubating 3,[3′-^{125}I]T_2 with hepatocytes in monolayers. The medium was analyzed by chromatography on Sephadex LH-20. A good separation was obtained between $^{125}I^-$, produced by outer ring deiodination, and 3,3′-T_2 sulfate (T_2S) and unprocessed 3,3′-T_2. At substrate concentrations of 10 n*M* 3,3′-T_2 or less, iodide was the principal product observed (> 90 percent). At higher 3,3′-T_2 concentrations or during coincubation with 6-propyl-2-thiouracil (PTU), an inhibitor of deiodination, increasing amounts of T_2S accumulated in the medium at the expense of I^- formation (*2*). The rate of outer ring deiodination of 3,3′-T_2 in rat liver cells is similar to that of rT_3 (*3*). This is a surprising observation because 3,3′-T_2, in comparison to rT_3, is a poor substrate for outer ring deiodination by microsomal deiodinase activity (*4*). We, therefore, suspected that in intact hepatocytes deiodination of 3,3′-T_2 is preceded, and in effect accelerated, by sulfate conjugation. If indeed sulfation precedes deiodination of 3,3′-T_2, inhibition of the

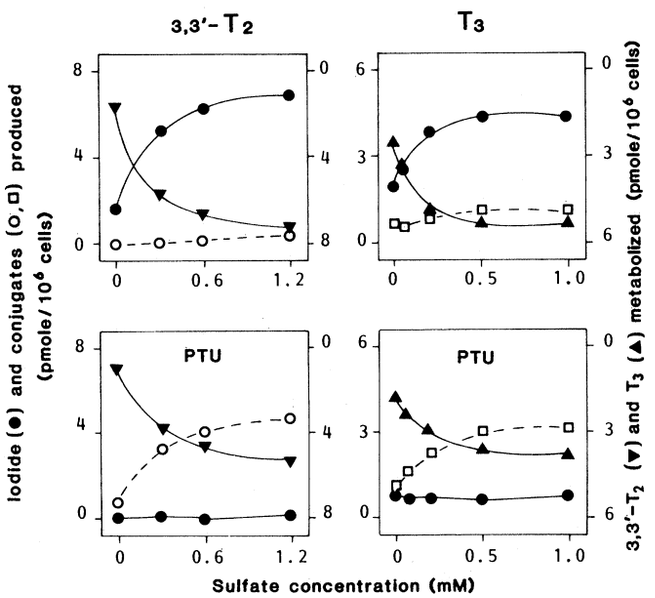

Fig. 1. Sulfate dependence of outer ring deiodination of 3,3′-T_2 and T_3 by monolayers of isolated rat hepatocytes. Approximately 2×10^6 hepatocytes were incubated for 60 minutes with sulfate- and protein-free Dulbecco's balanced salt solution to reduce the cellular sulfate content (*13*). Hereafter, incubations were performed with 4 ml of Dulbecco's medium containing 10 n*M* unlabeled plus 2 μCi of ^{125}I-labeled 3,3′-T_2 or T_3 and increasing sulfate concentrations. Identical incubations were conducted in the presence of 100 μ*M* PTU (lower panels) to inhibit deiodination. The 3,3′-T_2 incubations contained 0.5 percent bovine serum albumin. After 30 minutes for 3,3′-T_2 and 180 minutes for T_3, 100-μl samples were taken from the medium. After protein precipitation with ethanol, the supernatants were evaporated. The residues were dissolved in 0.1*N* HCl and chromatographed by subsequent 0.1*N* HCl and 0.1*N* NaOH elution on small (0.75 ml) Sephadex LH-20 columns. By this method a good separation was obtained between iodide and the conjugated and free iodothyronines, respectively. From the radioactivity in the various fractions, before and after incubation, the respective production and clearance rates were calculated. The results are expressed as picomoles produced or metabolized by 10^6 cells. The conjugates of 3,3′-T_2 and T_3 were identified by hydrolysis with sulfatase or β-glucuronidase (Sigma). Whereas 3,3′-T_2 was exclusively sulfated, T_3 was also glucuronidated. In the absence of PTU, T_3 glucuronide was the major conjugate. In the presence of PTU, the sulfate-dependent increment of T_3 conjugates represents accumulation of T_3S. Symbols: ●, iodide; ▼, 3,3′-T_2; ○, T_2S; ▲, T_3; □, T_3 conjugates.

former process will inevitably result in a reduction of deiodination.

Sulfation in hepatocytes may be influenced by (i) reduction of the SO_4^{2-} concentration in the medium and (ii) inhibition by compounds such as salicylamide, 2,6-dichloro-4-nitrophenol (DCNP), or pentachlorophenol (PCP) (*5*). We tested both possibilities. Figure 1 shows that I^- production in the absence of PTU and T_2S formation in the presence of this inhibitor are remarkably similar functions of the SO_4^{2-} concentration in the medium. The disappearance of 3,3′-T_2, with or without PTU, also depends on medium SO_4^{2-}. Addition of 10 μ*M* salicylamide, 100 μ*M* DCNP, or 100 μ*M* PCP reduced outer ring deiodination by 50, 48, and 70 percent, respectively. In the presence of PTU, T_2S formation was inhibited by these compounds to similar extents, that is, 60, 57, and 80 percent, respectively. These reductions were identical to the decrease of 3,3′-T_2 clearance.

After incubation of hepatocytes with [3′-^{125}I]T_3, metabolites were separated on Sephadex LH-20 into three peaks, that is, I^-, T_3 conjugates, and unreacted T_3 (Fig. 1). The conjugate peak contained both T_3 sulfate (T_3S) and T_3 glucuronide as revealed by enzymatic hydrolysis. Glucuronidation was not affected by PTU or SO_4^{2-}. At low SO_4^{2-} levels little T_3S formation was observed. The increase of the conjugate peak with increasing SO_4^{2-}, especially in the presence of PTU, reflects the accumulation of T_3S. A similar SO_4^{2-} dependence of T_3 deiodination was observed in the absence of PTU.

Deiodination of T_3 without PTU and T_3S formation with PTU were inhibited to similar extents by 25 μ*M* salicylamide, 10 μ*M* DCNP, and 1 μ*M* PCP, that is, 80, 85, and 75 percent, respectively. The greater effectiveness of DCNP and PCP in this case is explained by the use of albumin-free medium. No effect of these compounds on glucuronidation was noted.

The second part of this study consisted of the determination of the enzymatic characteristics of outer ring deiodination of 3,3′-T_2, T_2S, and rT_3 by rat liver microsomes. This subcellular fraction is the principal site of deiodinase activity in rat liver (*4*). Outer ring deiodination of the three ^{125}I-labeled substrates was estimated from the iodide production in incubations with microsomes. Figure 2 shows the rate of deiodination as a function of the substrate concentration. In accordance with earlier work (*4*) rT_3, with a Michaelis constant (K_M) of 0.1 μ*M*, is the best substrate for outer ring deiodination. The K_M of T_2S (0.3 μ*M*) is close to that of rT_3, making it a much better substrate for microsomal deiodinase than 3,3′-T_2 (K_M 9 μ*M*). Several compounds were tested as potential inhibitors of the deiodination of 0.01 μ*M* rT_3 or T_2S. A close correlation (r = .99, $P < .01$) was observed between the degrees of inhibition of both reactions by these compounds at 1 μ*M* (*6*). This observation is compatible with deiodination of rT_3 and T_2S by a single enzyme.

Our observations demonstrate that although 3,3′-T_2 and T_3 are poor substrates for deiodination by the microsomal fraction of rat liver, this process readily takes place in rat hepatocytes. Three lines of evidence support the view that sulfation of 3,3′-T_2 and T_3 in hepatocytes yields conjugates which are highly prone to deiodination. First, significant amounts of the sulfate conjugates accumulate only if deiodination is blocked with PTU. Second, deiodination varies with the sulfotransferase activity of the cells, either by restriction of SO_4^{2-} in the medium or by the addition of inhibitors. Of these, salicylamide is the most specific competitive inhibitor of phenolsulfotransferases (*7*). The metabolic inhibitors PCP and DCNP may, in addition, interfere with the synthesis of adenosine 3′-phosphate-5′-phosphosulfate (*7*), while PCP may also inhibit deiodination directly (*6*). The similar influence of the above conditions on I^- production and on sulfate conjugation in the presence of PTU strongly suggests that sulfation is the rate-limiting step preceding deiodination. Third, T_2S and T_3S are preferred substrates for microsomal deiodinase activity. This is illustrated by the low K_M of T_2S (0.3 μ*M*) in comparison with 3,3′-T_2 (9 μ*M*). For T_3S we observed an enhanced inner ring deiodination by rat liver microsomes [K_M 4.6 μ*M*, maximum velocity (V_{max}) 1050 pmole per minute per milligram of protein] compared with T_3 (K_M 10.7 μ*M*, V_{max} 33 pmole/min-mg protein). The T_2S generated is then rapidly deiodinated in the outer ring (*8*).

It is not surprising that 3,3′-T_2 and T_3 are sulfated in rat hepatocytes. They both are substrates for rat liver cytosolic sulfotransferases (*9*). However, the preferential deiodination of iodothyronine sulfates casts a new light on the processes involved with the peripheral metabolism of thyroid hormone. It is interesting that monkey hepatoma cells showed no reduction of T_3 inner ring deiodination when sulfation was inhibited (*10*). The very different characteristics of inner ring deiodination in hepatoma homogenates (K_M 0.034 μ*M*, V_{max} 223 fmole/min-mg protein) and the lack of inhibition by PTU (*10*) resemble the PTU-insensitive deiodination of T_3 in rat cere-

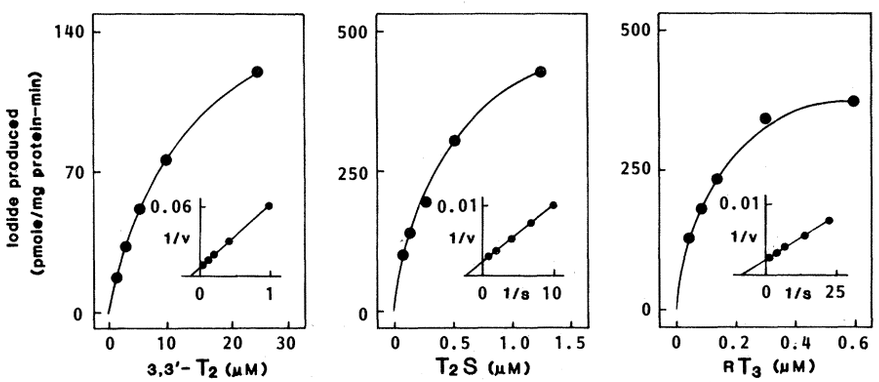

Fig. 2. Linear and double reciprocal (inset) plots of the rate of outer ring deiodination of 3,3′-T_2, T_2S, and rT_3 by rat liver microsomes as a function of substrate concentration. Deiodination was estimated according to the method of Leonard and Rosenberg (*14*) by measuring the production of $^{125}I^-$ in 200-μl reaction mixtures. The mixtures contained diluted rat liver microsomes and adequate concentrations of outer ring ^{125}I-labeled 3,3′-T_2, T_2S, or rT_3 in buffer (0.15*M* sodium phosphate (*p*H 7.2), 3 m*M* EDTA, and 5 m*M* dithiothreitol, the cofactor for deiodination). Microsomal protein concentrations were 8.4 μg/ml for rT_3 and T_2S and 336 μg/ml for 3,3′-T_2. After incubation for 10 minutes (rT_3 and T_2S) or 30 minutes (3,3′-T_2) at 37°C the reaction was stopped with 50 μl of human serum containing 5 m*M* PTU. Protein-bound substrate was precipitated by 350 μl of 10 percent trichloroacetic acid. Iodide was separated from other products in the supernatant by ion-exchange chromatography (Dowex 50W-X_2). Deiodination rates were calculated from the increase of radioactivity in the iodide fractions and expressed as picomoles per minute per milligram of protein (U). The K_M and V_{max} values are given as mean ± standard deviation: 3,3′-T_2, K_M 8.9 ± 3.9 μ*M*, V_{max} 188 ± 94 U (N = 4); T_2S, K_M 0.34 ± 0.07 μ*M*, V_{max} 353 ± 137 U (N = 3); rT_3, K_M 0.10 ± 0.02 μ*M*, V_{max} 445 ± 88 U (N = 4). T_2S was prepared by biosynthesis: 1 μ*M* 3,3′-T_2, 10 μCi of 3,[3′-^{125}I]T_2, and 100 μ*M* PTU were incubated in 4 ml of culture medium with rat hepatocytes at 37°C. After 2 hours approximately 80 percent of both labeled and unlabeled 3,3′-T_2 was sulfated. T_2S was purified by Sephadex LH-20 chromatography, and its yield was calculated from the recovery of added radioactivity.

bral cortex (K_M 0.021 μM, V_{max} 320 fmole/min-mg protein) (*11*). For this type of deiodinase sulfation does not appear to enhance deiodination. It remains to be established to what extent sulfation determines the hepatic deiodination and clearance of iodothyronines in vivo. Our results support the concept that sulfation is not merely a means to facilitate biliary and urinary excretion of hydrophobic aglycons (*12*).

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References and Notes

1. E. P. Krenning, R. Docter, H. F. Bernard, T. J. Visser, G. Hennemann, *FEBS Lett.* **91**, 113 (1978).
2. M. H. Otten, J. Blom, M. van Loon, T. J. Visser, *Ann. Endocrinol.* **43**, 52A (1982).
3. K. Sato and J. Robbins, *J. Clin. Invest.* **68**, 475 (1981).
4. T. J. Visser, D. Fekkes, R. Docter, G. Hennemann, *Biochem. J.* **179**, 489 (1979).
5. P. Moldeus, B. Andersson, V. Gergely, *Drug Metab. Dispos.* 7, 416 (1979); B. Andersson, M. Berggren, P. Moldeus, *ibid.* **6**, 611 (1978); G. J. Mulder and E. Scholtens, *Biochem. J.* **165**, 553 (1977); J. H. N. Meerman, A. B. D. Van Doorn, G. J. Mulder, *Cancer Res.* **40**, 3772 (1980).
6. The respective percentages of inhibition of outer ring deiodination of rT_3 and T_2S (0.01 μM) as induced by the following compounds (1.0 μM) were, respectively, T_4, 34 and 22 percent; T_3, 10 and 3 percent; rT_3, 90 and 86 percent; diiodotyrosine, 4 and 0 percent; PTU (10 μM), 80 and 90 percent; PTU (100 μM), 98 and 100 percent; iopanoic acid, 39 and 29 percent; salicylamide, 2 and 0 percent; dichloronitrophenol, 6 and 2 percent; pentachlorophenol, 68 and 70 percent. Linear regression yielded: $r = .99$, $P < .01$.
7. G. J. Mulder, in *Sulfation of Drugs and Related Compounds*, G. J. Mulder, Ed. (CRC Press, Boca Raton, Fla., 1981), p. 131.
8. T. J. Visser, J. A. Mol, M. H. Otten, *Endocrinology* **112**, 1547 (1983).
9. R. D. Sekura, K. Sato, H. J. Cahnmann, J. Robbins, W. B. Jakoby, *ibid.* **108**, 454 (1981).
10. K. Sato and J. Robbins, *J. Biol. Chem.* **255**, 7347 (1980); K. Sorimachi and J. Robbins, *Biochim. Biophys. Acta* **583**, 443 (1979).
11. M. M. Kaplan, T. J. Visser, K. A. Yaskoski, J. L. Leonard, *Endocrinology* **112**, 35 (1983).
12. G. M. Powell and A. H. Olavesen, in *Sulfation of Drugs and Related Compounds*, G. J. Mulder, Ed. (CRC Press, Boca Raton, Florida, 1981), p. 187.
13. K. Sato and J. Robbins, *Endocrinology* **109**, 844 (1981).
14. J. L. Leonard and I. N. Rosenberg, *ibid.* **107**, 1376 (1980).
15. Supported by grant 13-34-110 from the Division for Health Research TNO. We thank G. Hennemann, R. Docter, and E. P. Krenning for advice; H. F. Bernard, J. Blom, and M. A. C. van Loon for technical assistance; and Y. J. van Dodewaard for secretarial assistance.

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A Gene Controlling Condensation of Heterochromatin in *Drosophila melanogaster*

Abstract. *A temperature-sensitive lethal mutant of* Drosophila melanogaster *was used to identify an essential cell cycle function that is necessary for the mitotic condensation of heterochromatic but not of euchromatic portions of the genome. This mutant is an allele at a locus (*mus-101*) identified earlier by the use of mutagen-sensitive mutants. The data suggest that the mutagen-sensitive and repair-defective phenotypes of viable* mus-101 *mutants result from a disruption in chromosome organization.*

The DNA of eukaryotic cells is condensed some 7000-fold into metaphase chromosomes with such exquisite precision that each cell cycle reproduces chromosomes of constant size, arm ratio, and features of longitudinal differentiation such as primary and secondary constrictions, differential condensation of euchromatin and heterochromatin, and chromosome banding. Although the early steps of DNA packaging (initial coiling into 100-Å filaments and folding into 250-Å fibers) are relatively well defined, the subsequent steps in chromatin condensation are much less well understood (*1*). One approach to deciphering the processes of chromosome packaging is the analysis of mutants affecting chromosome condensation. Mutants in genes necessary for the occurrence of many steps in the cell cycle have been known in yeast and in mammalian tissue culture cells for some time [for reviews see (*2*)]. More recently, we have identified mutants defective in a variety of processes necessary for normal mitotic chromosome behavior in *Drosophila melanogaster* (*3–8*). In this report, we describe mutants at one of these loci whose wild-type product is necessary for the mitotic condensation of heterochromatin but not euchromatin.

One of our methods for identifying mutants in genes specifying essential mitotic functions has been to isolate temperature-sensitive (ts) lethal mutants and then screen them under semirestrictive conditions for those that increase the frequency of chromosome instability (*2, 3*). From a collection of 168 X-linked ts lethal mutants, we recovered 15 that affect mitotic chromosome behavior. Details of the isolation and preliminary characterization of all 15 mutants will be given elsewhere (*7*).

One of the X-linked ts lethal mutants is *mus-101*^{ts1}; adults homozygous or hemizygous for this mutation do not survive at temperatures above 22°C. Chromosome instability, detected both genetically and cytologically, is also temperature-sensitive [see (*7*) and below]. Conventional mapping placed *mus-101*^{ts1} at 41.6 on the X chromosome, and deficiency mapping showed that both the phenotypes of ts lethality and increased chromosome instability are uncovered by the deficiency *Df(1)HA92* (12A6,7 to 12D3) (*4*). These findings provide strong evidence that lethality and chromosomal effects result from the same mutation.

The mutagen-sensitive locus *mus-101*, previously identified by use of two homozygous viable mutants exhibiting hypersensitivity to killing by mutagens (*9*) and a defect in postreplication repair (*10*), is also located in the region uncovered by *Df(1)HA92* (*4, 11*). Complementation tests based on the phenotypes of methyl methanesulfonate sensitivity, female sterility, and mitotic chromosome instability all showed *mus-101*^{ts1} to be an allele at the *mus-101* locus (*7*).

To characterize the effect of *mus-101*^{ts1} cytologically, we shifted third-instar larvae reared at 18°C to the restrictive temperature of 29°C for various periods of time and made metaphase chromosome preparations by squashing ganglia in acetic orcein (*12*). When *mus-101*^{ts1} cells are kept at 18°C, they are cytologically normal and are indistinguishable from wild-type control cells. However, after 2 hours at 29°C there is a dramatic effect on the heterochromatic Y chromosome (Fig. 1 and Table 1). In more than two-thirds of metaphase preparations, the Y chromosome appears elongated and, in many cases, it exhibits one or more stretched areas of variable extension (Fig. 1, b to d versus a). After longer treatments, nearly all metaphases exhibit a clear undercondensation of the Y chromosome. After 2 hours at 29°C, a few cells with undercondensed autosomal centric heterochromatin are also observed. In many cases, thin threads of chromatin connecting the two autosomal arms are visible (Fig. 1, e and f), whereas in others the undercondensation of heterochromatin is so drastic that it mimics a chromosome break (Fig. 1d). In most affected cells exposed for 2 or 4 hours to the restrictive temperature, only one autosome (Fig. 1d) or a pair of autosomes (Fig. 1e) are affected; however, at subsequent times, cells showing undercondensation of the heterochromatin of all chromosomes are also observed (Fig. 1, f to i). The proportion of cells with a complete failure of heterochromatin condensation increases with the treatment time and at 12 and 24 hours is about one-third of the affected cells.