Journal of Steroid Biochemistry, Vol. 10, pp. 299 to 304.
Pergamon Press Ltd. 1979. Printed in Great Britain

TESTOSTERONE-ACTIVATED RNA SYNTHESIS IN ISOLATED PROSTATE NUCLEI

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(*Received* 11 *July* 1978)

SUMMARY

RNA synthesis *in vitro* by prostate nuclei isolated from castrated rats injected with testosterone shows an increase two hours after the androgen administration. This enhanced RNA synthesis is the result of increased RNA initiation sites on chromatin as well as a stimulated endogenous RNA polymerase II activity. The RNA synthesized by the androgen-stimulated prostate nuclei contains poly(A)-rich RNA species. Sucrose gradient centrifugation analysis of the poly(A)-rich RNA indicates that it is heterogeneous and of high molecular weight.

INTRODUCTION

Administration of testosterone *in vivo* to castrated rats induces an early change in the prostate chromatin. Within 2 [1] or 4 [2] h after injection of testosterone into rats, the transcriptional template activity of chromatin and the euchromatin content of prostate are increased. In addition, there are increased RNA polymerase activities [3, 4] and conformational changes of chromosomal proteins [1]. The androgen-treated chromatin also binds more polylysine [1] and actinomycin D [3] than does the castrated control. In normal female mice injected with testosterone, elevated template activity of the kidney chromatin and increased RNA polymerase I and II activities have also been recorded within 2 h after the androgen treatment as compared with androgen-insensitive *tfm/y* mice [5]. These observations indicate that androgens induce an early structural alteration of the target cell chromatin, including augmented free DNA regions which are presumably available for additional RNA synthesis. If such is the case, the early androgen action should be manifested by increased RNA initiation sites on chromatin for enhanced primary transcriptional products. In the work reported here, we have investigated the early androgen-induced RNA synthesis by using the nuclei system of Marzluff *et al.* [6] which has been shown to transcribe several specific genes in nuclei from mouse myeloma [7, 8] and HeLa cells [9]. The effect of androgens on the early initiation sites on chromatin for transcription have been determined by incorporation of [γ-^{32}P]-ATP and [γ-^{32}P]-GTP into the transcribed RNA.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats, weighing 275–300 g and purchased from Blue Spruce Farm, Altamont, NY, were castrated by the scrotal route under ether anesthesia. The animals were each injected with 1.0 mg of testosterone in 0.1 ml sesame oil containing 10% ethanol via the tail vein 72 h after the operation. Control animals received only 0.1 ml of sesame oil containing 10% ethanol. The animals were killed by decapitation and the ventral prostates were dissected for use in the experiments.

Materials. The materials were obtained from the following sources: testosterone, non-radioactive adenosine-, guanosine-, cytidine-, and uridine-5′-triphosphate, Triton X-100, Sigma Chemical Co., St. Louis, MO; oligo(dT)-cellulose, P-L Biochemicals, Inc., Milwaukee, WI; [5-^{3}H]-uridine-5′-triphosphate, [γ-^{32}P]-adenosine-5′-triphosphate and [γ-^{32}P]-guanosine-5′-triphosphate, New England Nuclear, Boston, MA; α-amanitin, Boehringer Mannheim Biochemicals, Indianapolis, IN. All other reagents were of the highest quality purchased commercially.

RNA synthesis by isolated prostate nuclei. The preparation of nuclei from rat prostate using 0.1% Triton X-100 and the assay of RNA synthesis conducted at 25°C were performed as described by Marzluff *et al.* [6]. The salt concentration used in the isolated nuclei system for RNA synthesis was 0.15 M KCl [6]. After incubation for the time periods as indicated in the legends to figures, the reaction was terminated by the addition of cold 5% trichloroacetic acid and the acid-insoluble radioactivity was determined as described previously [10].

Isolation of in vitro synthesized RNA. The RNA synthesizing reaction mixture, after incubation for 15 min at 25°C, was mixed with 15 volumes of 0.5% sodium dodecyl sulfate containing 1.0 mM EDTA and 0.05 M sodium acetate, pH 6.0, by homogenizing in a loose-fitting Dounce homogenizer. The suspension was shaken at 55°C for 3 min with an equal volume of a 1:1 (v/v) mixture of redistilled phenol and chloroform containing 0.1% hydroxyquinoline [11, 12]. Following centrifugation, the aqueous phase was col-

lected, to which one-third volume of deionized formamide was added, and repeatedly extracted with phenol-chloroform at room temperature until no precipitate was visible at the interphase after centrifugation. The RNA was precipitated in the presence of 1 mg of carrier yeast RNA by the addition of 0.1 volume of 20% potassium acetate, pH 5.5, and 2 volumes of ethanol. The precipitated RNA was washed successively with 80, 90 and 100% ethanol, dried, dissolved, and treated with DNase I (20 μg/ml). The DNase-treated RNA was re-precipitated as above and dissolved in 2 ml of 0.5 M KCl containing 0.01 M Tris–HCl, pH 7.5 and 0.2 mM $MgCl_2$.

Isolation and sucrose gradient centrifugation of poly(A)-rich RNA. The RNA sample obtained above was applied to an oligo(dT)-cellulose column (0.9 × 2 cm) previously equilibrated with the same buffered 0.5 M KCl solution [12]. After washing the column thoroughly with 0.5 M KCl–0.01 M Tris–HCl, pH 7.5–0.2 mM $MgCl_2$, the poly(A)-rich RNA bound to the column was eluted with 0.01 M Tris–HCl, pH 7.5, and precipitated by 2 volumes of ethanol and 0.1 volume of 20% potassium acetate, pH 5.5. The RNA was dissolved in deionized formamide and dialyzed against 0.12 M NaCl–0.01 M EDTA–0.5% sodium dodecyl sulfate–0.01 M Tris–HCl, pH 7.5 (NEST) [12, 13]. The dialyzed RNA, in 0.2 ml volume, was layered on top of 5.0 ml of 5–20% sucrose gradients in NEST and centrifuged at 45,000 rev./min in a Spinco SW-50 rotor for 150 min, using *E. coli* 23S and 16S rRNA as markers. The gradients were fractionated in an ISCO gradient fractionator and the radioactivities were counted in 10 ml dioxane scintillation fluid and 0.5 ml NCS solubilizer (Amersham).

Determination of initiation of RNA transcribed from prostate chromatin. Chromatin was prepared from isolated prostate nuclei as described elsewhere [14]. RNA polymerase II was purified from rat liver nuclei [10] and used to transcribe RNA from chromatin. The *in vitro* RNA polymerase reaction mixture, in a final volume of 0.25 ml, contained the following: Tris–HCl, pH 7.9, 10 μmol; $MnCl_2$, 0.75 μmol; $MgCl_2$, 1.15 μmol; $(NH_4)_2SO_4$, 12.5 μmol; EDTA, 0.017 μmol; β-mercaptoethanol, 1.0 μmol; [γ-^{32}P]-ATP or [γ-^{32}P]-GTP, GTP (or ATP), CTP and [^{3}H]-UTP, 62.5 nmoles each; chromatin, equivalent to 10 μg DNA; and RNA polymerase. The reaction was carried out at 37°C for the time periods as indicated, and terminated by the addition of 1.5 ml of 0.01 M sodium pyrophosphate, pH 8.0, in 0.5% sodium dodecyl sulfate. The RNA was repeatedly extracted with phenol until there was no precipitate at the interphase after centrifugation. The RNA was then precipitated by 2 volumes of ethanol as described before. Carrier yeast RNA (1 mg) was added to facilitate the precipitation. The precipitated RNA was dissolved in 0.05 M Tris–HCl, pH 8.0, re-precipitated by 5% trichloroacetic acid, and counted as described elsewhere [10].

RESULTS

Effect of testosterone on RNA synthesis by prostate nuclei

Figures 1 and 2 show the RNA synthesizing system of Marzluff *et al.* [6] as applied to the prostate nuclei. The *in vitro* RNA synthesis was linear for the first 40 min, proportional to the concentration of nuclei.

The results of RNA synthesis carried out using nuclei isolated from prostate of rats 0.5, 1, 2, 6 and 12 h after injection with testosterone show two peaks of RNA synthesis occurring at 2 (CT_2) and 12 (CT_{12})

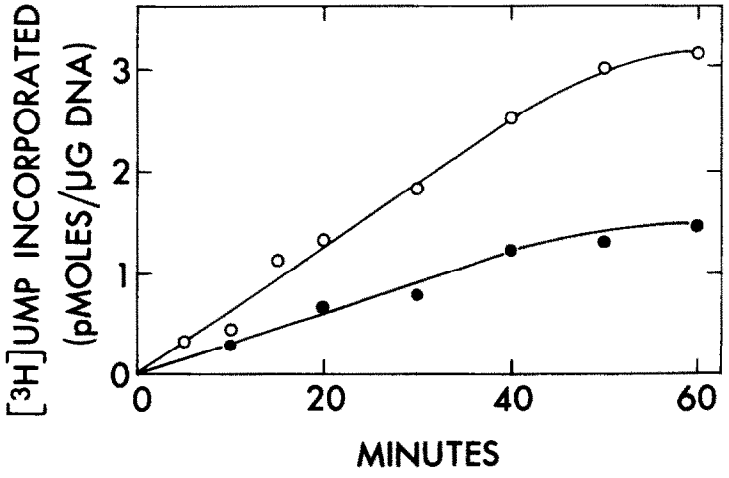

Fig. 1. RNA synthesis *in vitro* by rat prostate nuclei. The reaction mixture and assay, following those of Marzluff *et al.* [6], contained, in 0.25 ml: glycerol, 12.5%; $Mg(Ac)_2$, 5 mM; $MnCl_2$, 1 mM; Tris–HCl, pH 8.0, 25 mM; EDTA, 0.05 mM; dithiothreitol, 2.5 mM; KCl, 0.15 M: ATP, CTP and GTP, 0.4 mM each; [^{3}H]-UTP, 0.05 mM; and nuclei from castrated rat, 100 μg DNA. The reaction mixture was incubated at 25°C in the absence (○) or presence (●) of 1 μg α-amanitin per ml of reaction mixture, for the time periods as indicated. Processing and counting of acid-insoluble precipitate were as described under Methods.

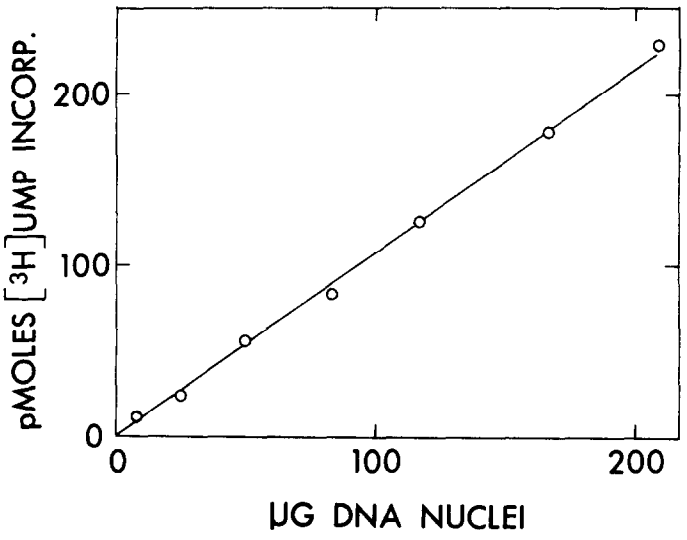

Fig. 2. RNA synthesis *in vitro* in relation to the concentration of nuclei. The reaction mixture and assay were as in Fig. 1, except that the incubation time was 20 min in the absence of α-amanitin throughout the experiment and the nuclei concentrations as indicated.

h post-testosterone administration (Fig. 3). RNA synthesis by CT_2 nuclei, however, was more active than those by other time preparations. This is consistent with the reports that CT_2 chromatin exhibits an enhanced RNA polymerase activity [3] and increased template activity in transcription and transcriptively active euchromatin [1]. These results suggest that the increased RNA synthesis by CT_2 nuclei is a result of the transcriptionally activated state of chromatin stimulated by androgens.

Analysis of the in vitro synthesized RNA

It has been shown that androgen administration results in increases in all classes of RNA, mostly ribosomal [15-17] and messenger [18, 19] RNA. Initial experiments of RNA synthesis by prostate nuclei from castrated rats indicated that α-amanitin, used at a concentration of 1 μg/ml of the reaction mixture, inhibited RNA synthesis by 55% (Fig. 1). The α-amanitin-insensitive RNA synthesis presumably represented ribosomal RNA and/or its precursor.

To determine the effect of testosterone on the synthesis of different classes of RNA, nuclei isolated from prostate of rats injected with testosterone for various hours were used for RNA synthesis in the presence of two different concentrations of α-amanitin: 1 μg and 160 μg per ml of reaction mixture, and in the absence of this toxin. This condition distinguishes the RNA synthesis catalyzed by each of the RNA polymerases I, II and III. The results in Fig. 4 show a pronounced RNA polymerase II-catalyzed RNA synthesis at 2 h after testosterone injection and a small but steadily increased RNA polymerase I-catalyzed RNA synthesis. The RNA synthesis by RNA polymerase III showed an increase at 0.5 h and, after declining, remained unchanged at a low level. The sum total RNA synthesis by the three RNA polymerases also indicate two major peaks at 2 h and 12 h after testosterone injection, showing 46% and 24% increases, respectively, over the zero-hour control. These values

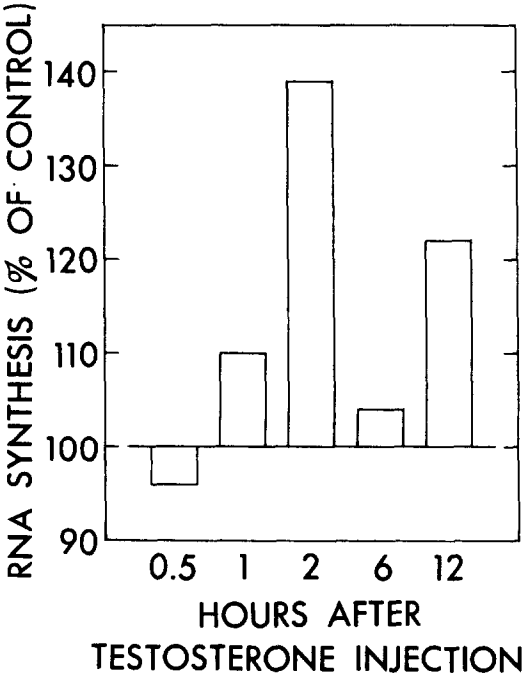

Fig. 3. Effect of testosterone administration on the RNA synthesis by isolated prostate nuclei. The experimental conditions were as in Fig. 1, except that the incubation was carried out for 20 min in the absence of α-amanitin. The prostate nuclei were isolated from castrated rat injected with testosterone (1 mg/0.1 ml sesame oil) for 0.5, 1, 2, 6 and 12 h as indicated. The RNA synthesis control (5,700 d.p.m.) by nuclei from rat injected with 0.1 ml sesame oil only is considered as 100%.

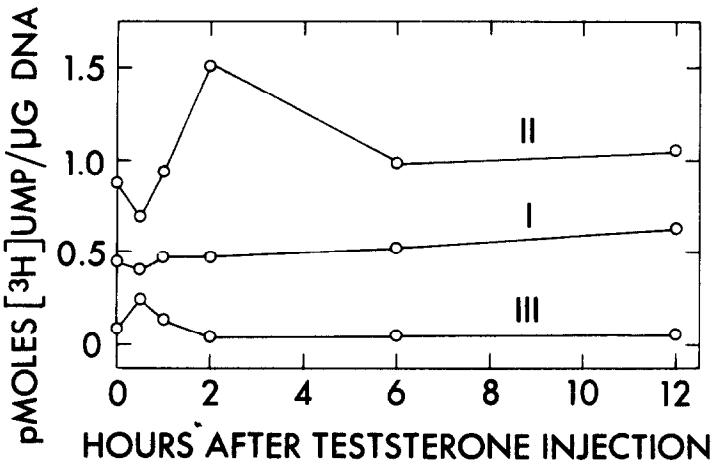

Fig. 4. Effect of testosterone on RNA synthesis by endogenous RNA polymerases I, II and III of isolated prostate nuclei. Each prostate nuclei preparation was divided into 3 equal portions and assayed as in Fig. 3 in the absence and presence of 1 µg or 160 µg α-amanitin per ml of the reaction mixture. The RNA synthesis in the presence of 160 µg α-amanitin/ml (a) represented the RNA polymerase I activity. The RNA synthesis in the presence of 1 µg α-amanitin/ml (b) represented RNA polymerases I + III activities which, when subtracted by (a), gave the RNA polymerase III activity. The differences in RNA synthesis in the absence of α-amanitin (c), which represented the total RNA polymerases activity, and (b) gave the RNA polymerase II activity. These values in RNA synthesis were expressed on the basis of the control RNA synthesis from rats injected with sesame oil only for various periods as the testosterone-treated animals. The zero hour controls were determined on nuclei from castrated rat injected with oil and killed immediately.

compare favorably with the 39% (2 h) and 22% (12 h) increases in total RNA synthesis shown previously in Fig. 3. Since the RNA synthesis were assayed within the linear range of incubation time and nuclei concentration, the data essentially indicate the various RNA polymerase activities and support an early increased RNA polymerase II activity stimulated by testosterone [3].

The increase in RNA polymerase II-catalyzed RNA synthesis by CT_2 nuclei strongly suggests that one of the early actions of androgens is a stimulated synthesis of heterogeneous nuclear RNA (hnRNA). Assuming that the androgen-stimulated hnRNA was polyadenylated, the poly(A)-rich RNA was isolated from the *in vitro* synthesized RNA by C and CT_2 prostate nuclei by oligo(dT)-cellulose chromatography and analyzed by sucrose gradient centrifugation. As shown in Fig. 5, the RNA synthesized by CT_2 nuclei contained more poly(A)-rich RNA than did C nuclei. The increased poly(A)-rich RNA stimulated by testosterone consisted mainly of RNA species of 20S-43S. Thus, the increased transcription of chromatin 2 h after testosterone administration [1] is supported, in part, by increased hnRNA synthesis.

Effect of testosterone on RNA initiation sites on chromatin

Since RNA polymerase activity is stimulated within 2 h following testosterone injection [3] and also by androgen-receptor complex *in vitro* [4], the enhanced hnRNA synthesis described above could be due to an enhanced RNA polymerase activity. Further, the increased high molecular weight RNA species synthesized by CT_2 nuclei could result from a stimulated elongation process of RNA. Moreover, although CT_2 chromatin contains more open DNA regions than does C chromatin [1], it is not known whether these augmented DNA sequences include additional RNA initiation sites. To resolve these questions, initiation of transcription of C and CT_2 chromatins by RNA polymerase II was analyzed by incorporation of [γ-^{32}P]-ATP and [γ-^{32}P]-GTP into RNA and compared with control C chromatin. As shown in Fig. 6, transcription of CT_2 chromatin showed increased incorporation of both [γ-^{32}P]-ATP and [γ-^{32}P]-GTP into RNA than the castrated control chromatin. Testosterone injection into castrated rat thus stimulated an early increased initiation of RNA synthesis.

DISCUSSION

Castration of postpubertal male rats results in several pronounced biochemical changes in the nucleus or chromatin of androgen-responsive cells. There is a marked decline in nonhistone chromosomal proteins and nucleoplasmic proteins [1, 4, 20] and in phosphorylation of nuclear proteins [21]. The lysine-rich histones, especially H1, is reduced [20]; and both nucleolar and nucleoplasmic RNA polymerases activities as well as RNA initiation sites of chromatin [3, 4, 22] decrease. Sustained testosterone replacement restores these deficiencies to

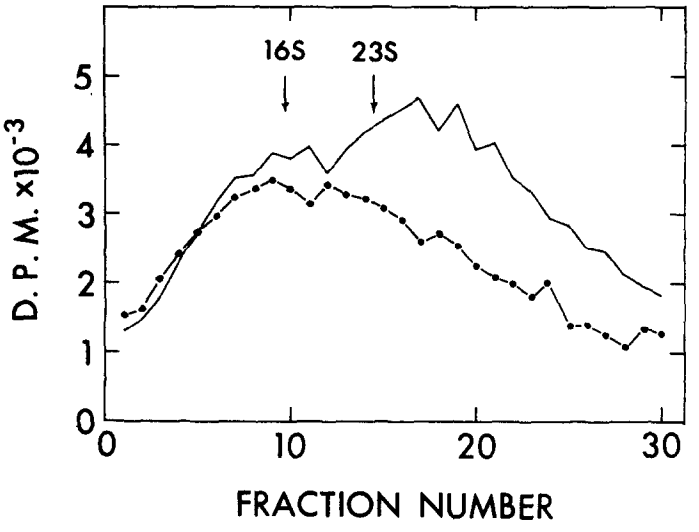

Fig. 5. Sucrose gradient centrifugation of poly(A)-rich RNA synthesized by prostate nuclei from castrated rats injected with testosterone for 2 h (——) and from castrated control (- - -). The isolation of *in vitro* RNA synthesized by prostate nuclei and of poly(A)-rich RNA are described under Methods.

near their normal levels. Androgen–receptor complex, the formation of which is correlated with androgenicity [23], has also been shown to stimulate RNA polymerase activity [4]. These androgenic effects are harmonious with the current concept of steroid-mediated regulation of gene expression [17], and are supported by the observations of early changes in chromatin structure [1, 3] and increased transcription [1, 2, 15–19, 24–26]. A direct correlation of the activated state of chromatin with transcriptional activity during the early stage of androgen action requires that androgen treatment results in new RNA initiation sites on chromatin for increased primary transcription products. The present data provide such evidence.

Enhanced testosterone-stimulated poly(A)-rich RNA synthesis in rat prostate at 2 h and sustained to 8 h after administration of the androgen has been

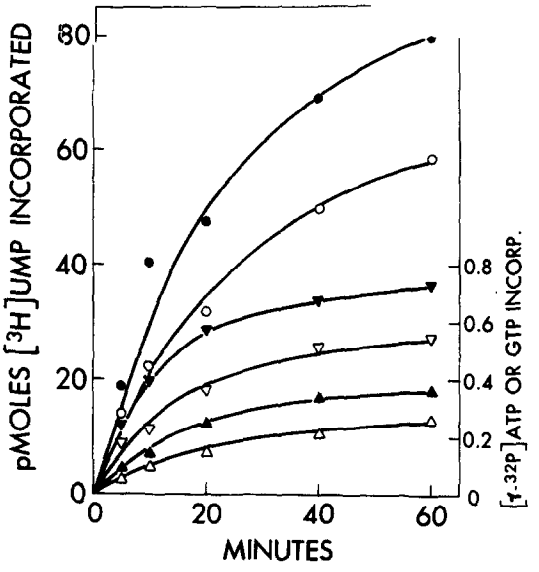

Fig. 6. Initiation and transcription of RNA from prostate chromatin of castrated rats (open symbols) and castrated rats injected with testosterone for 2 h (filled symbols) by rat liver RNA polymerase II. The reaction mixture is described under Methods, including C or CT_2 chromatins equivalent to 100 μg DNA, [^{3}H]UTP, ○ and ●, (64 d.p.m./pmol) and either [γ-^{32}P]-ATP, △ and ▲, (2934 d.p.m./pmol) or [γ-^{32}P]-GTP, ▽ and ▼ (2986 d.p.m./pmol). The reaction was carried out at 37°C for the duration as indicated. Processing and counting of RNA products were as described under Methods.

previously reported by Mainwaring *et al.* [18]. The synthesis of the prostate poly(A)-rich RNA is tissue- and steroid-specific and, when isolated from polyribosomes, appears as a 6-15S fraction that exhibits characteristics of mRNA. The androgen-stimulated poly(A)-rich RNA undoubtedly contains the primary transcriptional product which, after processing, is converted into the 6-15S RNA, both being a part of the early event in androgen action.

Davies *et al.* [22] reported that injections of testosterone to castrated rat increase steadily initiation sites on prostate chromatin and also the RNA chain length. The chromatin apparently undergoes a series of alterations after androgen treatment. The steady increases in the synthesis of poly(A)-rich RNA in prostate during the 8 h period after testosterone injection probably represent the net result of androgen-mediated differential transcription, involving the altered chromatin, increased RNA polymerase activity and perhaps also synthesis of factors involved in transcriptional control. These results and the present data, together, indicate that an early androgen action is an altered nucleus or chromatin resulting in new initiation of primary transcription product. The number and classes of gene copies being transcribed during the early stage of androgen effect, the possible regulation in RNA processing, and the mode of action by which androgens alter the target cell chromatin are necessary knowledge in our understanding of the mechanism of androgen action.

Acknowledgement—This work was supported by an N.I.H. research grant (HD-09443) from the United States Public Health Service.

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