Biochemical Pharmacology, Vol. 20, pp. 2959–2964. Pergamon Press, 1971. Printed in Great Britain

EFFECTS OF CLOMIPHENE ON VARIOUS BIOCHEMICAL REACTIONS IN THE MOUSE TESTIS *IN VITRO**

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(*Received* 12 *November* 1970; *accepted* 3 *March* 1971)

Abstract—Clomiphene (2×10^{-4} M) was found to inhibit mouse testis protein and RNA synthesis *in vitro*. The inhibition of protein synthesis was not principally the result of altered membrane transport since clomiphene had relatively little effect on the accumulation of α-aminoisobutyric acid-3-^{14}C in testis. The inhibitory effect of clomiphene on protein synthesis could be reversed by the addition of glucose. The drug was found to partially reduce the oxidation of D-Glucose-U-^{14}C to $^{14}CO_2$ and to significantly reduce protein labeling from D-Glucose-U-^{14}C.

CLOMIPHENE CITRATE (Clomid), 1-[(*p*-diethylaminoethoxy) phenyl]-1,2-diphenyl-2-chloroethylene, an analog of the nonsteroidal estrogen chlorotrianisene (TACE) has achieved recognition as a relatively effective inducer of ovulation in certain cases of female infertility.[1] Its principal function in these cases is believed to involve release of additional gonadotrophins from the pituitary. Apparently, clomiphene is also capable of eliciting gonadotrophin release in the male, as reported by Bardin *et al.*[2] The administration of clomiphene citrate to normal subjects, 100–200 mg/day for 6–9 days, was found by these investigators to result in increased plasma levels of both luteinizing hormone and testosterone. Clomiphene may, however, be able to act more directly at sites of action. Heller *et al.*[3] subsequently performed a study similar to that of Bardin *et al.*[2] except that an additional dose, 400 mg/day, was employed and the study was carried out for a longer duration of time. These authors reported that spermatogenesis was stimulated in volunteers receiving the lower doses but observed a reduction in sperm count with the high dose. It was suggested that this might represent a direct toxic effect of the drug on the testis at this dose level. Other investigators have also reported that clomiphene can interfere with spermatogenesis, in rodents such as the gerbil[4] and rat.[5]

Several studies have demonstrated the capacity of clomiphene to directly affect placental, ovarian, or testicular function. Smith *et al.*[6] have reported that clomiphene is capable of stimulating the aromatization of steroids by human placenta *in vitro*, while Hammerstein[7] has reported that clomiphene inhibits the incorporation of acetate-1-^{14}C into progesterone by slices of human corpora lutea. Previous data from this laboratory have demonstrated that clomiphene is capable of inhibiting rat

* This investigation was supported by General Research Support Grant RR-05684-02 from the General Research Support Branch, Division of Research Resources, National Institutes of Health.

testicular protein synthesis, *in vitro*.[8] The present study was designed in order to further investigate the possible mechanism for the inhibition by clomiphene of testicular protein synthesis.

EXPERIMENTAL

Materials. Mature male Swiss–Webster mice weighing 25–30 g were used in this study (Bio-Science Lab.). They were fed Purina Chow and water *ad libitum*. Clomiphene (supplied by Wm. S. Merrell Co.) was prepared by homogenizing in distilled water with a motor-driven teflon pestle (clearance 0·1–0·15 mm). The following radioactive precursors were employed in this study: L-lysine-U-^{14}C (sp. act. 312 mc/m-mole, Schwartz Bio-Research), Uridine-2-^{14}C (sp. act. 53·3 mc/m-mole, Schwartz Bio-Research), α-aminoisobutyric acid-3-^{14}C (AIB-^{14}C) (sp. act. 2·72 mc/m-mole, New England Nuclear) and D-Glucose-U-^{14}C (sp. act. 3·0 mc/m-mole, Amersham/Searle).

Incubation of the tissues. The mice were sacrificed by decapitation, the testis removed and decapsulated and each pair of organs (weighing 150–200 mg) were placed in flasks containing cold buffer. The main chamber of the Warburg flask contained 2·9 ml of Krebs–Ringer bicarbonate buffer at pH 7·0. The drug was added in a volume of 0·1 ml. The side-arm contained either $1{\cdot}3 \times 10^6$ disintegrations per min (dis./min) of L-lysine-U-^{14}C, $5{\cdot}29 \times 10^5$ dis./min of Uridine-2-^{14}C, $1{\cdot}03 \times 10^6$ dis./min AIB-^{14}C or $5{\cdot}8 \times 10^5$ dis./min of D-Glucose-U-^{14}C. The final concentration of L-lysine-U-^{14}C, Uridine-2-^{14}C, AIB-^{14}C and D-Glucose-U-^{14}C in the incubation medium was 5×10^{-7} M, $1{\cdot}2 \times 10^{-6}$ M, $2{\cdot}9 \times 10^{-5}$ M and $2{\cdot}7 \times 10^{-5}$ M respectively. The gas phase was 95% O_2–5% CO_2. An incubation temperature of 32° was employed.[9]

Incorporation of L-lysine-U-^{14}C into testicular protein. At the end of the incubation period, the reaction was terminated by the addition of 0·3 ml of 5 N perchloric acid (PCA). Proteins were isolated as described by Busch *et al.*[10] The dried protein pellets (10–15 mg) were dissolved in 1·0 ml of NCS solubilizer (Amersham/Searle) by heating at 60° for 10 min. Ten ml of toluene scintillation fluid containing 0·5% 2,5-diphenyloxazole (PPO) and 0·006% *p*-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP) was added to each sample. Radioactivity was determined in a Beckman LS-200B liquid scintillation spectrometer. Each sample was counted for either 10 min or to a 2 σ pre-set error of $\pm$0·5 per cent. Quenching was determined using external standardization. Efficiency ranged from 70 to 75 per cent.

Incorporation of Uridine-2-^{14}C into ribonucleic acid (RNA). Following incubation, the procedure for extraction of RNA, quantitation of RNA and determination of radioactivity was carried out as described elsewhere[11] with the following exception. Aquafluor (New England Nuclear) was used as the scintillation mixture for the aqueous samples.

AIB-^{14}C transport. AIB-^{14}C transport was measured by incubating testis tissue with AIB-^{14}C for various periods. Following incubation, the tissue was washed with buffer and blotted on absorbent paper. The entire tissue sample was then dissolved in 1·5 ml of Protosol (New England Nuclear) by heating at 55° for 20 hr. The solution was then assayed for radioactivity by the addition of 10 ml of the toluene scintillation mixture.

$^{14}CO_2$. The procedure for the collection of $^{14}CO_2$ evolved from D-Glucose-U-^{14}C, *in vitro*, was carried out as described previously[12] with the exception that NCS was used as the trapping agent.

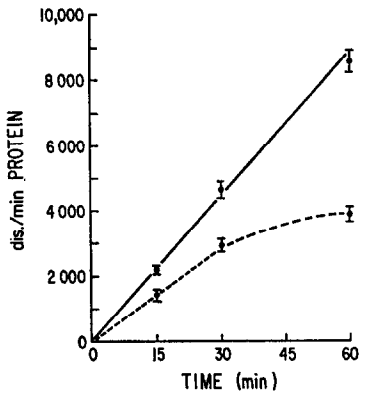

FIG. 1. Effect of clomiphene (2×10^{-4} M) on incorporation of L-lysine-U-^{14}C into testicular proteins of the mouse at various incubation times. Solid line represents control while dashed line clomiphene. Each point is the mean of three to six determinations. Vertical bars represent standard error.

RESULTS

Figure 1 presents the effect of clomiphene on the incorporation of L-lysine-U-^{14}C into testicular proteins of the mouse, *in vitro*, at various time points. Protein synthesis was inhibited by 35 per cent as early as 15 min of incubation ($P < 0{\cdot}05$). The degree of inhibition remained essentially the same (36 per cent) at 30 min and reached 54 per cent at 60 min.

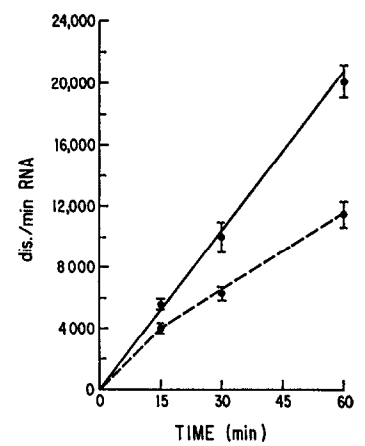

FIG. 2. Effect of clomiphene (2×10^{-4} M) on incorporation of Uridine-2-^{14}C into testicular RNA of the mouse at various incubation times. Solid line represents control while dashed line clomiphene. Each point is the mean of five to six determinations. Vertical bars represent standard error.

In order to determine if clomiphene might also be inhibiting RNA synthesis, the effect of clomiphene on the incorporation of Uridine-2-^{14}C into mouse testis RNA at the same time points was investigated (Fig. 2). RNA labeling was found to be decreased to a similar extent as protein synthesis. At 15 min, uptake of the tracer was diminished by 30 per cent, at 30 min 35 per cent and at 60 min 45 per cent.

TABLE 1. EFFECT OF CLOMIPHENE ON AIB-^{14}C ACCUMULATION *IN VITRO* IN MOUSE TESTIS*

Time (min)	Control† (dis./min/100 mg tissue)	Clomiphene† (dis./min/100 mg tissue)	P
15	8080 ± 410 (6)	7140 ± 240 (6)	> 0·05
30	9340 ± 290 (6)	8840 ± 870 (6)	> 0·05
60	11,090 ± 1230 (3)	8050 ± 630 (3)	> 0·05

* Testis, 150–200 mg, incubated in Krebs–Ringer bicarbonate buffer, pH 7·0, with AIB-^{14}C as described in text in an atmosphere of 95% O_2–5% CO_2 at 32°. Final concentration of clomiphene in incubation flask was 2×10^{-4} M.

† Each value is expressed as the mean ± S.E. Value in parentheses is number of determinations.

These results suggested that perhaps the inhibitory effect of clomiphene on both protein and RNA biosynthesis *in vitro* might be secondary to an action of the drug common to the two pathways. In view of the fact that estradiol-17β has been reported to affect the membrane transport of precursors in the uterus[13] and since clomiphene is capable of competitive antagonism of many estrogenic effects,[14] it seemed of interest to determine if clomiphene might be able to block membrane transport in the testis. Therefore, the effect of clomiphene on the accumulation of the non-utilizable amino acid AIB-^{14}C was studied. Table 1 indicates that clomiphene produced no statistically significant reduction ($P > 0{\cdot}05$) in the accumulation of AIB-^{14}C at any of the time points studied, although transport may be inhibited by as much as 27 per cent at 60 min. This suggests that the decrease in protein synthesis induced by clomiphene, *in vitro*, is not principally the result of reduced membrane transport of amino acid precursors.

It has been shown previously[15] that the protein biosynthetic process of the testis is extremely responsive to the addition of glucose. Therefore it was decided to investigate

TABLE 2. EFFECT OF GLUCOSE ON CLOMIPHENE INHIBITED PROTEIN SYNTHESIS IN MOUSE TESTIS, *IN VITRO**

Sample†	Specific activity‡ (dis./min/mg protein)	P
Control	8570 ± 300 (8)	
Clomiphene	4390 ± 380 (8)	
Glucose	13,650 ± 750 (6)	< 0·05§
Clomiphene and glucose	9790 ± 420 (5)	< 0·05‖

* Testis, 150–200 mg, incubated for 1 hr in Krebs–Ringer bicarbonate buffer, pH 7·0, in an atmosphere of 95% O_2–5% CO_2 at 32°.

† Final concentration of clomiphene and glucose in incubation flask 2×10^{-4} M and 9×10^{-3} M respectively.

‡ Each value is expressed as the mean ± S.E. Value in parentheses is number of determinations.

§ Compared to control.

‖ Compared to clomiphene.

what effect this hexose might have on clomiphene inhibited protein synthesis in the testis. Table 2 presents the results obtained. Glucose, alone, was found to stimulate mouse testicular protein synthesis by 59 per cent. In the presence of clomiphene, however, the stimulation was only 14 per cent. Since the stimulatory effect of glucose on testicular protein synthesis has been hypothesized to result from increased production of ATP,[16] it was decided to investigate whether clomiphene might be inhibiting glucose stimulated protein synthesis by interfering with its oxidation. Therefore, the effect of clomiphene on $^{14}CO_2$ production by the testis from D-Glucose-U-^{14}C was investigated. The results are presented in Table 3. Clomiphene was found to have no significant effect on the oxidation of glucose-U-^{14}C to $^{14}CO_2$ ($P > 0{\cdot}20$) although $^{14}CO_2$ production was reduced by 18 per cent. However, it was observed that protein labeling from radioactive glucose was nevertheless significantly decreased ($P < 0{\cdot}05$) to an extent similar to that obtained employing L-lysine-U-^{14}C as the precursor (41 per cent).

TABLE 3. EFFECT OF CLOMIPHENE ON $^{14}CO_2$ PRODUCTION AND PROTEIN LABELING FROM D-GLUCOSE-U-^{14}C BY MOUSE TESTIS, *IN VITRO**

Fraction	Control†	Clomiphene†	P
$^{14}CO_2$ (dis./min/100 mg tissue)	71,140 ± 3940 (6)	58,180 ± 3150 (6)	> 0·20
Protein (dis./min/mg protein)	795 ± 25 (9)	470 ± 35 (9)	< 0·05

* Testis, 150–200 mg, incubated for 1 hr in Krebs–Ringer bicarbonate, pH 7·0, in an atmosphere of 95% O_2–5% CO_2. Final concentration of clomiphene in incubation flask was 2×10^{-4} M.

† Each value is expressed as the mean ± S.E. Value in parentheses is number of determinations.

One additional possible mechanism was investigated. Perhaps clomiphene might be influencing ATP levels at a point beyond the catabolism of glucose. If this be the case then the level of endogenous ATP would be expected to be lower following incubation with clomiphene. There occurred no significant change in the level of ATP between control and drug-treated tissue (data not shown).

DISCUSSION

The data of the present investigation indicate that clomiphene is capable of blocking both protein and RNA synthesis in the mouse testis, *in vitro*. The mouse testis does not appear to be as sensitive as the rat testis to the addition of clomiphene in that a 10-fold greater concentration of the drug was required to inhibit protein synthesis by 50 per cent. Few reports appear in the literature regarding an effect of clomiphene on protein synthesis, although it has been observed that clomiphene is capable of blocking estrogen-induced protein synthesis in the rat uterus while having no effect on noninduced protein synthesis.[17] However, protein synthesis in that study was assayed on the basis of total uterine protein content, rather than employing radioisotope methodology which may be more sensitive than the former.

The fact that clomiphene's inhibition of RNA synthesis follows the same profile as its inhibition of protein synthesis suggests that clomiphene may be acting at a site

common to both pathways. It appears that a statistically significant effect on respiration can be ruled out although $^{14}CO_2$ production was decreased by 18 per cent in the presence of clomiphene. Interestingly, however, protein labeling from amino acids formed during the aerobic catabolism of glucose was still inhibited to approximately the same extent (41 per cent) as when labeled lysine was used as the precursor. These data suggest a site of action distal to the formation of alanine, glutamate and aspartate which have been shown to be the predominant amino acids formed by testis from D-Glucose-U-$^{14}C^{12}$ although inhibition of transamination cannot be ruled out.

In the present investigation, glucose was observed to stimulate the incorporation of L-lysine-U-^{14}C into mouse testicular protein by 59 per cent. The extent of the stimulatory effect of glucose on testicular protein synthesis is less than that which has been reported previously for the same concentration.[16] This may be explained, however, on the basis that in the previous study a different species was employed, namely the rat, and that testicular slices were used rather than teased tissue as in the present investigation. One would expect the latter variable to significantly influence the penetration rate of glucose into the tissue.

The results of the present study indicate that the inhibitory effect of clomiphene on protein synthesis in the testis is not related to lowered endogenous ATP levels. Incubation of testis tissue with the drug produced no significant change in the tissue level of this nucleotide.

The data of this investigation further demonstrate that the inhibitory effect of clomiphene on testicular protein synthesis, *in vitro*, may not be related to a membrane effect limiting the passage of amino acids. The drug was found to produce no significant effect on the accumulation of AIB-^{14}C in the testis although accumulation of AIB-^{14}C was decreased by 27 per cent at 60 min.

In conclusion, the results of this study suggest an intracellular site(s) of clomiphene action at a point probably independent of normal respiration and amino acid transport, although inhibition of transport and oxidation by clomiphene at 60 min add to 45 per cent.

Acknowledgement—The author would like to acknowledge the excellent technical assistance of Mrs. Freida Hwang.

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