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EARLY EFFECTS OF RETINOL AND RETINOIC ACID ON PROTEIN SYNTHESIS IN RETINOL DEFICIENT RAT TESTES

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SUMMARY: When an [^{35}S] labeled mixture of methionine and cysteine was injected intratesticularly into retinol-deficient rats, two hours later more than 980 cytosolic proteins were detected by computer aided two dimensional gel electrophoresis. Furthermore, two hours after oral refeeding retinyl acetate as the source of retinol to retinol deficient rats, synthesis of 286 proteins was inhibited and that of 101 proteins was activated. Refeeding with retinoic acid leads in two hours to even higher inhibition of protein synthesis and the labeling patterns of proteins are not identical when compared to retinol refed rats. The results indicate that retinol or retinoic acid quickly influence expression of many proteins and suggest that retinol action in the testes is not identical to that of retinoic acid.

Retinol plays an essential role in differentiation of the testes (1). Animals fed a diet lacking retinol show atrophy of the germinal epithelium, whereas Sertoli cells are still present and interstitial cells appear to be little affected (1). When dietary supply of retinol is restored, germinal cells eventually regenerate morphologically and functionally (2,3). Experiments with the whole animal (4) or with the teratocarcinoma cells in culture (5) indicate that retinol or its metabolite retinoic acid influence genomic expression. About forty specific genes were reported to be activated or repressed by retinol or retinoic acid (for review see 6). In the work reported here using the retinol-deficient rat, we describe some of the early events of retinol or retinoic acid action on protein synthesis. We used computer aided two dimensional electrophoresis of testicular cytosols to answer the following questions. Firstly, what are the effects of retinol deficiency on testicular protein synthesis? Secondly, how extensively is synthesis of proteins influenced by two hour refeeding of retinol or retinoic acid to deficient rat? Thirdly, does such short term refeeding of a retinol-deficient rat with retinol or retinoic acid influence synthesis of the same or different proteins?

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MATERIALS AND METHODS

Materials - Trans [^{35}S]-label, a mixture of [^{35}S]-labeled methionine and cysteine 70:20 (specific activity 1100 Ci/mmol), was purchased from ICN, Irvine, CA. Sprague-Dawley rats were obtained from Sasco Company, St. Louis, MO.

Intragastric Feeding and Labeling of Testicular Proteins - Animals - Rats (21 days old) were made retinol deficient as described previously (7). Serum retinol level of deficient rats was determined by fluorometric method (8) and ranged between 2-4 μg/100 ml, indicating that the rats were retinol deficient indeed.

Retinol-deficient animals were fed 0.2 ml of cottonseed oil alone or with 100 μg of retinyl acetate as the source of retinol or retinoic acid by intubation into the stomach (4). Chow fed rats were used also as retinol sufficient again after gastric intubation of 0.2 ml of cottonseed oil. Ten minutes later, the rats were lightly anesthetized with ether. Next, the right or left testicle was exposed by a small incision and injected with 0.9 mCi of Trans [^{35}S] label using a Hamilton syringe. The incision was stitched immediately after the injection and the rats were allowed to recover. After two hours, the animals were killed by decapitation, blood was collected for retinol determination, and the injected testicles were excised.

Preparation of Cytosols - Testes were homogenized in 10mM Tris HCl, 0.25 M sucrose, pH 8.5 buffer (1:6 w/v) with a Potter-Elvejhem homogenizer and the homogenate centrifuged at 105,000 x g for one hour to prepare cytosols.

Two Dimensional Gel Electrophoresis - Cytosols were treated according to the method of Garrels (9). Briefly, 400-500 μg of cytosolic proteins were diluted with SDS sample buffer (9.95 M urea, 0.3% SDS, 100 mM dithiothreitol and 4% NP-40) to give a protein concentration of 2 μg/μl, heated in boiling water for two min, then cooled on ice. 10 μl of DNA-RNase solution (1 mg DNase, and 0.5 mg RNase/ml per 200 μg of protein) were added next. Samples were then chilled on ice for two min with occasional vortexing and snap frozen in liquid nitrogen.

Two dimensional gel electrophoresis has been standardized, and up to 3000 labeled proteins can be separated and detected on a single autoradiograph (10, 11). The computer scanning system developed by Protein Data Base Inc. (Huntington Station, NY) can detect 1/10 of dpm. The samples were analyzed using gels containing pH 3-10 ampholines in first and a 15% acrylamide in second dimension. All samples were loaded at 100,000 dpm/gel. Dried, "Enhance" (New England Nuclear) treated gels were exposed for 10 days for autoradiography. Films were then scanned using a computer program which automatically detects and integrates the densities of spots. The molecular mass of proteins analyzed ranged between 10 kDa to 120 kDa. The computer analysis provided quantitative measurement of incorporation of radioactivity into proteins. The results of gels of cytosolic proteins from testes of retinol deficient rats were used for initial coordinates to match protein spots from testes of rats fed chow, refed retinyl acetate or retinoic acid.

RESULTS AND DISCUSSION

Protein Synthesis in the Testes of Retinol-Deficient Rats - Preliminary experiments have shown that two hours after intratesticular injection of 0.9 mCi of [^{35}S] label, sufficient radioactivity was incorporated into trichloroacetic acid precipitated proteins to perform two dimensional gel electrophoresis. In subsequent experiments, therefore, retinol-deficient rats were injected with this dose and cytosolic proteins were analyzed. Fig. 1 shows labeling patterns of testicular proteins in retinol deficient rats. Using computer analysis 983 protein spots were detected (Table 1).

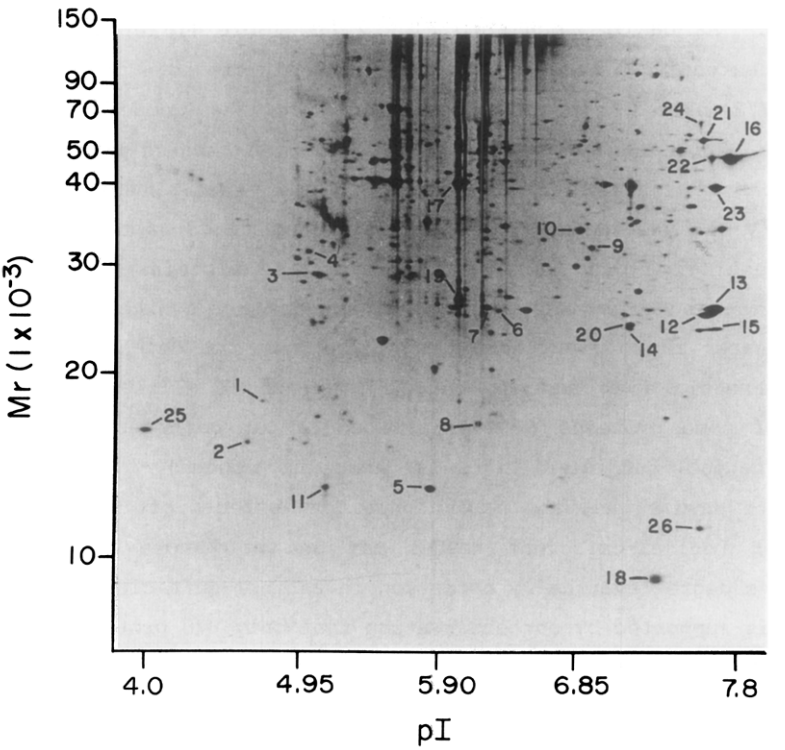

Figure 1. Fluorography of two dimensional gels of [^{35}S] labeled cytosolic proteins from retinol-deficient testes. Protein spot #1 was used as standard for relative quantitation of proteins. Some protein spots were assigned identification numbers. About 20 µg of cytosolic proteins (100,000 dpm/gel) were electrophoresed as described in MATERIALS AND METHODS.

Retinol or Retinoic Acid Repress and Activate Quickly Synthesis of Many Proteins - To study effects of retinol on synthesis of testicular cytosolic proteins, retinol-deficient rats were refed retinol for two hours and [^{35}S] labeled amino acids were injected intratesticularly as described in Materials

TABLE 1. Synthesis of Cytosolic Proteins in the Testes of Retinol-deficient Rats. Effect of Short Term Retinol and Retinoic Acid Refeeding

Animals	Number of Proteins Detected	Number of Proteins Synthesis of Which Was	
		Inhibited	Activated
Retinol-deficient	983	-	-
Retinol-deficient retinol refed[1]	772	286	101
Retinol-deficient retinoic acid refed[1]	698	364	111

[1] Retinol-deficient rats were refed 100 µg of retinyl acetate or retinoic acid intragastrically and killed two hours later.

and Methods. As can be seen from Fig. 2 and Table 1, retinol refeeding is followed by extensive alterations in the synthesis of these proteins. In this instance, 772 protein spots were detected by the computer scan. This represents a 22 per cent decrease in the number of labeled proteins, compared to the testes of retinol-deficient rats (983 proteins). When the change of radioactivity of individual protein spots was evaluated as the consequence of retinol refeeding, a drop in the labeling of 286 proteins was observed (see Table 1). The inhibition of some proteins which are synthesized in retinol-deficient testes is so pronounced that these were not detected after retinol refeeding (Protein # 15 and 16, see Fig. 1 and 2). Interestingly enough, synthesis of some proteins (# 14,15,16) could not be detected in testes of chow fed, retinol-sufficient animals (data not shown). It is therefore reasonable to assume that the early inhibitory effects of retinol on protein synthesis in retinol-deficient testes may be to restore the synthesis of proteins to a degree eventually occurring in retinol-sufficient animals. This contention is supported by our observation that only 678 proteins were labeled in testes of chow fed rats. The rapid decline in protein synthesis is in agreement with the earlier observation of decrease in testicular poly A-

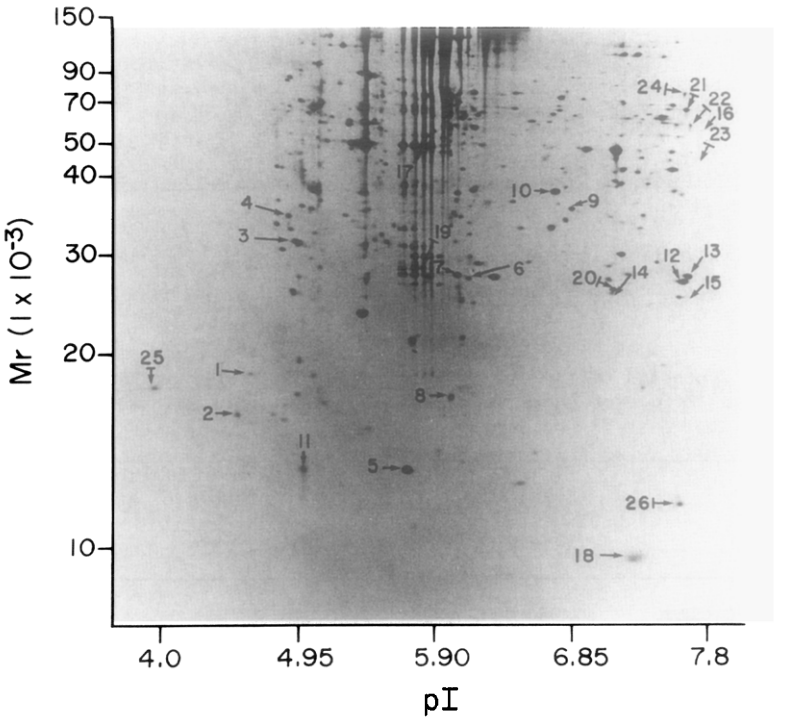

Figure 2. Fluorography of two dimensional gels of testicular cytosolic proteins after two hours of refeeding retinol-deficient rats with retinyl acetate. The horizontal arrows show spots, the intensity of which did not change relative to those detected in retinol-deficient testes. The upward and downward arrows point to the spots that have decreased or increased respectively relative to proteins from retinol-deficient testes. Conditions of electrophoresis as in Fig. 1.

containing RNA content one hour or two hours after oral administration of retinol to deficient animals (4).

Furthermore, as can be seen from Table 1, retinol refeeding causes not only the inhibition of synthesis of many proteins, but also concurrently to a lesser extent the activation of others (101 activated versus 286 inhibited). For instance, this effect can be seen by visual inspection of protein # 17 in Fig. 1 and 2. For quantitation, spot No. 1 in Fig. 1 showing 12 dpm was selected as the standard and assigned a value of 1.0. Using this calculation, the synthesis of protein # 17, increased 2.6-fold. On the other hand, the synthesis of some proteins was not affected by retinol refeeding (Fig. 1 and 2 proteins # 1 to 10).

Retinoic acid is an obligatory metabolite of retinol and has been shown to affect synthesis of few gene products (6). We have, therefore, refed retinol-deficient rats with retinoic acid and found, as shown in Table 1 and Fig. 3, that retinoic acid also quickly affects the synthesis of many proteins. Retinoic acid appears to be more effective than retinol by inhibiting the synthesis of 364 proteins. Similar to retinol action, retinoic acid refeeding activated synthesis of 111 proteins.

The data presented here indicate that synthesis of many cytosolic proteins is affected very quickly by retinol or retinoic acid. The total

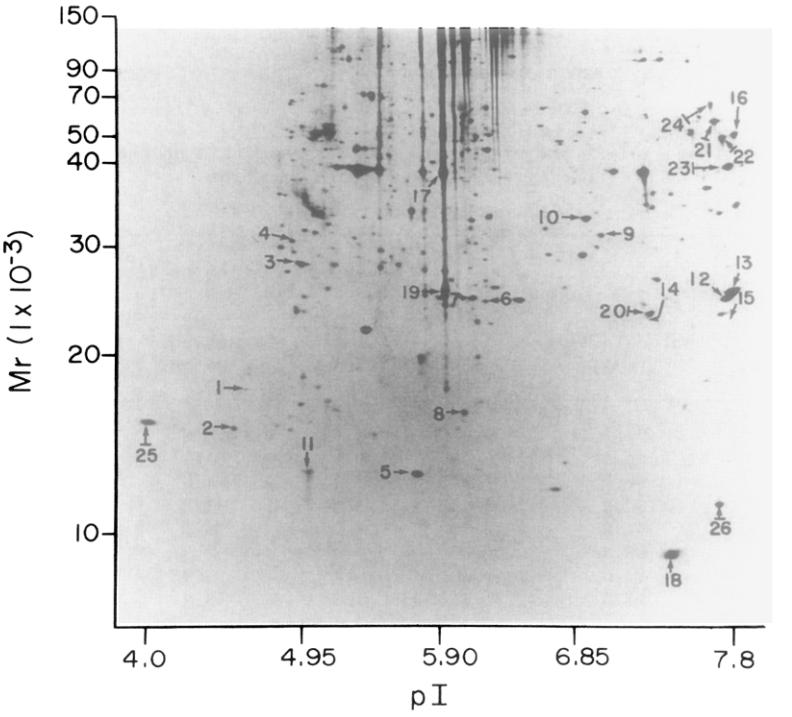

Figure 3. Fluorography of two dimensional gels of [^{35}S] labeled cytosolic proteins after two hours of refeeding retinol-deficient rats with retinoic acid. For details see legend to Fig. 2 and MATERIALS AND METHODS.

number of cellular proteins influenced by retinol or retinoic acid must be even higher as these compounds have been described to alter also cytoskeletal and membrane proteins (6). These results using whole animal are in apparent contrast to the studies with cells in culture where expression of most proteins with exception of transglutaminase (12) were described to occur in several hours (13-15,16). The reason for this difference in temporal response may be due to the use of higher specific radioactivity to label the proteins in our experiments as compared to previous reports, and probably also due to interplay among various cell types which takes place in the testes of the whole animal in contrast to the cells in culture. It is quite possible that retinol and retinoic acid effects may vary from cell to cell, tissue to tissue and may depend on intercellular communications.

Refeeding Effects of Retinol Are Not Identical to Those of Retinoic Acid - As the above described effects of refeeding retinyl acetate could be due to retinoic acid generated from retinol, we attempted to address the question, whether synthesis of testicular proteins is influenced by these compounds in an identical manner. Comparison of Fig. 2 and 3 allowed the summation in Table 2 that retinol and retinoic acid refeeding have different effects on labeling of cytosolic proteins. Previous studies from this laboratory have already shown that *in vitro* translation patterns of poly A-containing RNA were different in retinol and retinoic acid refed animals (4). Thus the results shown here confirm these observations. They also indicate that retinol and retinoic acid exert a common effect on the synthesis of some proteins which

TABLE 2. Differential Effect of Retinol and Retinoic Acid on the Synthesis of Some Cytosolic Testicular Proteins

Protein Spot[2]	Molecular mass (kDa)	pI	Relative Quantity of Incorporated Radioactivity[1]		
			Retinol Deficient	Retinol Refed[3]	Retinoic Acid[3] Fed
19	27.3	5.93	28.5	15.8	30.8
20	25.0	6.96	10.3	4.7	11.4
21	57.9	7.57	7.8	4.0	15.1
22	50.5	7.65	6.8	1.8	12.4
23	41.0	7.68	20.6	N.D.[4]	19.8
24	66.4	7.55	1.9	2.3	5.8
25	17.0	4.00	10.2	5.4	16.0
26	12.0	7.55	6.5	5.7	9.8

1- Relative quantity of radioactivity is based on protein spot # 1 showing 12 dpm (see Fig. 1) which was given an arbitrary value of one.
2- Protein numbering refers to their position in Fig. 1, 2 and 3.
3- Retinol deficient rats were refed 100 µg of retinyl acetate or retinoic acid intragastrically and killed two hours later.
4- N.D. non detected.

may be mediated by retinoic acid formed in situ. For instance, as shown in Figs. 2 and 3, synthesis of proteins # 11, 12, and 13 is suppressed and that of protein # 17 is activated by retinol and retinoic acid. Conversely, synthesis of three proteins was suppressed in retinol refed and activated in retinoic acid refed animals (see Table II proteins 21, 22, and 25). The synthesis of proteins 19, 20, and 23 also decreased in retinol refed rats but was unaffected in retinoic acid refed rats (Table 2). On the other hand, synthesis of proteins 24 and 26 increased in retinoic acid refed rats, but not in retinol refed animals. These results indicate that retinol and retinoic effects are common to a specific set of proteins and distinct on another set of proteins.

Although it appears that primary action of retinol and retinoic acid is on gene expression (6), it is not known whether the rapid changes in protein synthesis described here involve effects only on transcriptional mechanism. If this were the case, it remains to be elucidated whether the fast pleotropic effects of these compounds are due to a direct and simultaneous interaction with many transcriptional units, or alternatively whether an interaction with a single gene would trigger in turn rapid changes sequentially. If such sequential mechanism were operative indeed, it can be calculated from the results presented above (Table 1) that during first two hours after refeeding cascade-like alterations in synthesis of cytosolic proteins would proceed with a cadency of about 18 seconds per protein.

Finally, the results presented here suggest rather strongly that activation and repression of specific genes thus far reported (6), with the exception of the transglutaminase gene, represent a phenomenon which is a result of later effects of retinol and retinoic acid on cellular differentiation. Consequently, specific "early" genes suggested to be affected in this report remain to be characterized.

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REFERENCES

1. Wolbach, S.B. and Howe, R.R. (1925) J. Exp. Med. 43, 753-777.
2. Wolbach, S.B. and Howe, R.R. (1933) J. Exp. Med. 57, 511-526.
3. Thompson, J.N., Howell, J.M. and Pitt, G.A.J. (1964) Proc. R. Soc. Biol. 159.
4. Omori, M. and Chytil, F. (1982) J. Biol. Chem. 257, 14370-14374.
5. Strickland, S. and Mahdavi, V. (1978) Cell 15, 394-403.
6. Chytil, F. (1986) J. Am. Acad. Dermatol. 15, 741-747.
7. Lamb, A.J., Apivatanaporn, P. and Olson, J.A. (1974) J. Nutr. 104, 1140-1148.

8. Thompson J.N., Erdody, P. and Maxwell, W.B. (1973) Biochem. Med. 8, 403-414.
9. Garrels, J.I. (1979) J. Biol. Chem. 254, 7961-7977.
10. Young, D.A. (1984) Clin. Chem. 30, 2104-2108.
11. Levenson, R.M., Martin, E.V. and Young, D.A. (1986) Anal. Biochem. 15, 294-301.
12. Uhl, L. and Schindler, J. (1987) Exp. Cell Biol. 55, 28-33.
13. Paulin, D., Jakob, H., Jacob, F., Weber, K. and Osborn, M. (1982) Differentiation 22, 90-99.
14. Howe, C.C. and Solter, D. (1981) Dev. Biol. 84, 239-243.
15. Knowles, B.B., Pan S. Solter, D., Linnenbach, A., Croce, C.M. and Huebner, K. (1980) Nature (London) 288, 615-618.
16. Griep, A., Kendrick, N.C. and DeLuca, H.F. (1986) Arch. Biochem. Biophys. 249, 180-190.