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An Improved Micro-Method for Avidin Assay

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A sensitive method for avidin assay was devised. Tritiated biotin is bound to avidin and this complex is then bound to bentonite. Radioactivity is converted into a gas form by combustion and counted in an automatic proportional counter with 55% efficiency and background of 3.7c.p.m. The sensitivity is 1–2ng of avidin.

Egg white and the oviducts of egg-laying vertebrates contain the protein avidin, which is induced by progesterone (Herz *et al.*, 1943). The determination of avidin is extremely simple compared with the complicated assays for other hormone-specific proteins. It can be assayed directly in the tissue homogenate without any purification procedure. Therefore the induction of avidin by progesterone is a good model to study the mechanism of action of steroid hormones. According to the present hypothesis steroid hormones induce transcription of specific mRNA. The translation of these specific mRNA species can be studied *in vitro* in cell-free systems, where only very small quantities of protein (avidin) can be synthesized (O'Malley *et al.*, 1972). In other words, the translation systems *in vitro* require sensitive assay methods for avidin because of the small amount of the protein produced. The present assay methods are not sensitive enough for this purpose (Wei, 1970; Korenman & O'Malley, 1970). Therefore we have devised a new improved method from the method of Korenman & O'Malley (1967).

Recently there has been developed a new low-background high-efficiency tritium counter for biological determinations (Parvinen *et al.*, 1973). The apparatus is a proportional counter with an automatic sample changer. The purpose of this study has been to investigate whether or not this gas counter can be used in the micro-determination of avidin.

All the reagents were made up in deionized water. (+)-[^{3}H]Biotin (specific radioactivity 114mCi/mmol) was synthesized by Roche Products Ltd., Basel, Switzerland (B.P. 628902; Robinson, 1966). In this method the imidazolidine ring was formed before the thiophane ring and tritium was added to the ring and side chain just before the protective benzoyl groups were removed. Bentonite was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Avidin standard (12 units/mg; 1 unit binds 1μg of D-biotin; Worthington Biochemical Corp., Freehold, N.J., U.S.A.) was made up in water. Tritiated biotin and bentonite were prepared in 0.2M-$(NH_4)_2CO_3$.

For the avidin assay 200μl of the sample and 50μl of [^{3}H]biotin (1nCi) were incubated at room temperature for 15min. Then 3mg of bentonite in 150μl of $(NH_4)_2CO_3$ was added to the incubation medium. The mixture was shaken for 5–10min and centrifuged for 5min at 2000g. The supernatant was removed and the bentonite was washed with 5×1.5ml of 0.2M-$(NH_4)_2CO_3$. After washing, the biotin–avidin–bentonite complex was transferred to counting ampoules with 0.15ml and 0.20ml of $(NH_4)_2CO_3$. The ampoules were centrifuged and $(NH_4)_2CO_3$ was removed carefully by suction. The biotin–avidin–bentonite complex in the ampoules was then dried under vacuum. Metallic zinc–Na_2CO_3 (4:1, w/w) (40mg) was added with a small dipper and the ampoules were closed and heated at 640°C for 1h. Radioactivity was counted in a proportional tube. The counting time was 100min. The efficiency of the counter was determined with n-[1,2-^{3}H]hexadecane (1.88μCi/g; The Radiochemical Centre, Amersham, Bucks., U.K.). The counting efficiency was, without bentonite, 51.9±2.4% (n = 15) (±S.D.), and with 3mg of bentonite, 55.3±4.3% (n = 11). This indicates that bentonite does not affect the efficiency. The background with pure methane was 1.2±0.1c.p.m. (n = 17). The method background was 3.7±0.9c.p.m. (n = 20) when 1nCi of [^{3}H]biotin was added to the bentonite solution and the bentonite was washed with 5×1.5ml of $(NH_4)_2CO_3$.

The avidin standard curve was derived from four independent determinations. Fig. 1 shows that the standard curve is linear, and the standard deviation is small. Avidin was assayed in oviduct homogenates obtained from diethylstilboestrol- and progesterone-pretreated immature chicks. The oviducts were homogenized at 0°C with a Teflon homogenizer in 10–40vol. of buffer (Korenman & O'Malley, 1967) The homogenate was centrifuged for 20min at 10000g and thereafter for 2h at 105000g. Avidin was assayed in the supernatant as described above. Fig. 2 shows that a plot of radioactivity against amount of supernatant was linear.

Our results show that the automatic gas counter can be used in the micro-determination of avidin.

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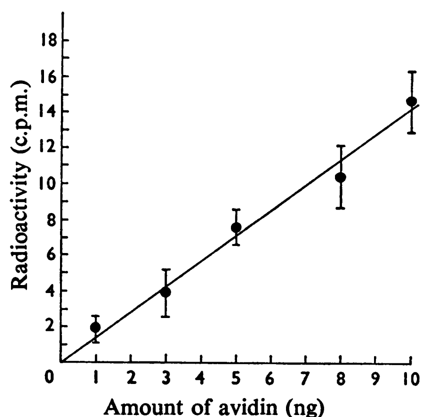

Fig. 1. *Avidin standard curve*

Every point was derived from four independent determinations. The curve was linear at least up to 100 ng of avidin. Standard deviations are given.

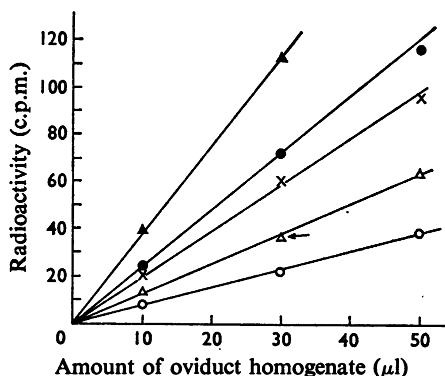

Fig. 2. *Determination of avidin in five different oviduct samples by using different amounts of tissue homogenate*

Every point is the mean of six independent determinations, except the point indicated by the arrow, which is the mean of 22 independent determinations of the same tissue homogenate. The value for this point is 36.3 ± 2.5 c.p.m.

The advantages of the gas counter over the liquid-scintillation counter are the absence of the quenching phenomenon, high and stable efficiency for tritium and very low background. When tritiated biotin is used, the sensitivity of the gas-counting method is 50–100 times higher than that of the liquid-scintillation counting method. Further, the efficiency of counting for tritiated biotin is variable in the liquid-scintillation counter, because the thick layer of bentonite in the bottom of the counting vial gives a variable quenching effect owing to the different sizes of the bentonite particles. This variation in the tritium-counting efficiency of the liquid-scintillation counter cannot be controlled by internal or external standardization. Therefore only [^{14}C]biotin is suitable for liquid-scintillation counting. However, the specific radioactivity of ^{14}C-labelled biotin cannot be increased significantly from the present available value (46 mCi/mmol; The Radiochemical Centre). The specific radioactivity of ^{3}H-labelled compounds can be improved up to 50 Ci/mmol, which is quite common today. If the highest possible sensitivity of the present method is calculated on the basis that the specific radioactivity of [^{3}H]biotin is 50 Ci/mmol, 20 pg of avidin can be detected. Thus this method can be even more sensitive than the laborious microbiological methods by which the highest sensitivity of 100 pg can be obtained (Launer & Fraenkel-Conrat, 1951). In the gas-counting system more than 3 mg of bentonite can be used for the avidin assay, which means that more tissue can be analysed. When 5 mg of bentonite was used, the avidin standard did not differ significantly from that in Fig. 1. If the ampoules are closed under vacuum, it is possible to use up to 10–15 mg of bentonite, with which amount the capacity of bentonite is enough for the determination of avidin obtained from oviducts weighing 60–80 mg. The present method is especially suitable for avidin assay in cell-free translation systems *in vitro*. In this kind of study the greatest problem is the determination of the small amount of newly synthesized avidin (Tuohimaa *et al.*, 1973) and thus a sensitive assay method is necessary.

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