Effect of DDS on o-diphenoloxidase of *Mycobacterium leprae:* Absorbance 480 nm ($\times 10^{-3}$)

Inhibitor	Intact bacilli			Disrupted bacilli		
	Bacilli + DOPA	Bacilli + DOPA + inhibitor	% inhibition	Bacilli + DOPA	Bacilli + DOPA + inhibitor	% inhibition
DDS suspension in water	58	52	11	50	36	28
DDS solution in ethanol	60	54	10	50	38	24
DDS suspension + polylysine	60	0	100	50	0	100
DDS solution + polylysine	60	0	100	50	0	100
Ethanol	70	72	-	50	51	-
Polylysine	64	65	-	50	50	-

due to the bacilli or the reagents. Each experiment was done at least 3 times; values given are for representative experiments, since no significant variations were observed between results of different experiments. Heating the bacilli at 100 °C for 3 min inactivated the enzyme.

Results and discussion. The results are presented in the table. DDS in suspension or solution showed very little inhibitory effect on the oxidation of DOPA by intact *M. leprae;* in the disrupted bacilli, the inhibition was greater, but it was still less than 30%. However, when mixed with polylysine, DDS produced 100% inhibition of the enzyme in both intact and disrupted bacilli. o-Diphenoloxidase is a copper protein. The sulfur atoms of DDS might bind the copper and inactivate the enzyme. We tested ethanol and polylysine separately for their effect on the oxidation of DOPA by *M. leprae.* Both reagents showed neither inhibition nor stimulation of the activity at the concentrations used.

The results reported here demonstrate that polylysine enables dapsone not only to pass through the bacterial cell membranes, but also to penetrate the enzyme molecules readily. Further studies have to be done with lower concentrations of DDS-polylysine combination to determine its effect on the growth of drug-resistant *M. leprae* in animals and to assess any toxicity it might have. It has been shown that polysine can interact with lipids[9]. The peptide, which remains stable over a wide range of conditions, can pass through the lipid bilayers of the cell membranes easily. This observation is consistent with our earlier data on the complete inhibition of o-diphenoloxidase of *M. leprae* by DDC, which contains lipid-soluble ethyl groups[4]. Rifampin which is bactericidal to the organism is a lipid-soluble drug. The leprosy bacilli have been shown to become resistant to both dapsone and rifampin[10-12]. Developing permeability barrier is one of the mechanisms by which bacteria become drug-resistant. In such cases, dapsone combined with polylysine or other similar compounds would be more effective than DDS alone. Rifampin-resistance in bacteria is mediated by substitution of one amino acid in the enzyme RNA polymerase. Since dapsone binds the copper moiety of o-diphenoloxidase and not the enzyme protein itself, mutations altering the protein structure may not affect its inhibitory action.

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Vitamin A antibodies: application to radioimmunoassay

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Summary. A radioimmunoassay for serum vitamin A is described which can detect as little as 1 ng of retinol. The statistical characteristics of this assay are presented and its use in a nutritional experiment is discussed.

Conrad and Wirtz[2] were able to produce antibodies to vitamin A by injecting into rabbits a retinoic-acid-albumin conjugate. The antiserum reacted equally well with retinal, retinol and retinoic acid and very poorly with retinyl palmitate, beta carotene and beta ionone. In this paper we describe a radioimmunoassay (RIA) for retinol in serum and plasma.

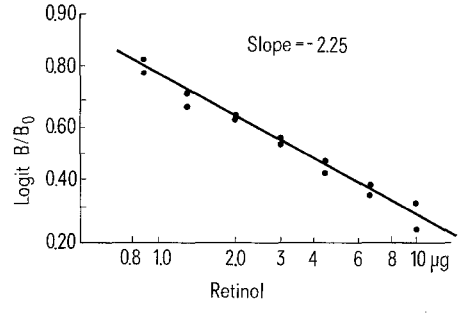
Materials and methods. Crystalline retinol was obtained from Eastman Organic chemicals. Tritiated-retinol and Aquasol-2 counting cocktail were obtained from New England Nuclear. Norit-A carbon came from Fisher Scientific Co. and dextran (clinical grade, 200,000-300,000) from Nutritional Biochemicals Corp. The vial of ^{3}H-retinol received from the supplier (0.25 mCi, 0.029 mg in 0.25 ml ethanol) was diluted with 62.5 ml absolute ethanol and stored under nitrogen at −20 °C. The buffer used in the radioimmunoassay was 0.05 M phosphate, 0.1 M NaCl, pH 6.8. Antiserums against vitamin A were produced in rabbits according to the method of Conrad and Wirtz[2]; the antiserum used in this work displayed an affinity constant of 2×10^8 l/M as determined by a Scatchard plot of RIA data. The antiserum was prepared for RIA by diluting 1/805 with buffer and adding 0.05 ml of diluted ^{3}H-retinol solution (see above) per 25 ml of diluted antiserum. The

dextran coated charcoal suspension consisted of 10 mg Norit A charcoal and 250 µg of dextran per ml of buffer.

Serum samples from fasted animals were prepared for analysis as follows[3]: 0.2 ml of serum was treated with 0.4 ml of absolute ethanol; the mixture was vortexed and centrifuged at 600×g for 10 min; 25 µl of the supernatant solution were delivered to a 12×75 mm disposable glass culture tube. To this was added 1.0 ml of the antibody-^{3}H-retinol mixture. The contents were mixed thoroughly and incubated in the dark at room temperature. After 1 h 0.5 ml of dextran coated charcoal suspension was added. The mixture was incubated an additional 15 min (in the dark) at room temperature and then centrifuged at 600×g for 10 min. The supernatant was decanted into 10 ml of counting fluid and counted in a scintillation counter. All of the samples subjected to the assay procedure were in 66.7% ethanol. Unknowns and standards were assayed in duplicate and controls were employed in triplicate. Table 1 describes the experimental controls and also the definitions used in manipulating the data. The separation of free ^{3}H-retinol from antibody bound ^{3}H-retinol was achieved through selective binding, by dextran coated charcoal, of the free ^{3}H-retinol; thus, the radioactive counts present in the supernatant fluid were supposedly those specifically associated with antibody. This assumption, however, was only an approximation since control Y (table 1) consistantly displayed a significant number of counts. The counts which did not, for reasons unknown, associate with dextran coated charcoal made up 10–20% of the total tritium label. Thus, the counts in the supernatant of each sample were elevated by this amount. To correct for this, a non-specific control (table 1) was subtracted from the counts of each sample. The level of the non-specific control varied with the preparation provided by the supplier and was usually within acceptable limits (10–15% of total counts) if the ^{3}H-retinol was used within 6 months of receipt. The concentrations of antibody and ^{3}H-retinol were adjusted so that the ratio of Bo to 'total ^{3}H-retinol' (table 1) fell in the range of 0.4–0.5. To construct the standard curve (or displacement curve) the logit the B/Bo was plotted versus the log of the standard retinol concentration; from the B/Bo calculated for each unknown its concentration could be read from the graph. These manipulations were carried out with the aid of logit-log graph paper[4]. The figure is an example of a standard curve. Each time we did an experiment, we included a sample of a standard serum pool as a check on the reliability of the assay.

Results and discussion. The statistical characteristics of the assay, and its application, were explored in 4 experiments (table 2). In experiment A, 10 replicate samples of serum (0.2 ml each) were treated with ethanol and the resulting extracts were assayed in duplicate for a total of 20 samples. For experiment B, a serum pool was first diluted 1/3.6 using 5% bovine serum albumin. 10 replicates were treated with ethanol and again assayed in duplicate. We were pleased to note that the SD did not increase sharply at the lower retinol concentration. In experiment C we examined the variation obtained by assaying the same serum pool on 10 separate days. The biological variation of serum retinol concentration from animal to animal was assessed in experiment D by assaying the serum of 12 male Wistar rats weighing between 255 and 295 g.

An experiment was carried out examining the recovery of retinol during the serum extraction procedure. Various quantities of retinol were added to serum samples which were then subjected to extraction and assay in duplicate; the quantity of the endogenous retinol was subtracted from the result obtained with each of the fortified serum samples. The results indicated that the ethanol treatment of serum allowed recovery and assay of 97% of the retinol present (data not shown).



Standard curve used in determination of serum retinol concentration.

Table 1.

	Standard or serum extract	66.7% ethanol	Antiserum and ^{3}H-retinol*	Buffer and ^{3}H-retinol*		Dextran coated charcoal	Buffer	
Sample	0.025 ml		1.0 ml		Incubate	0.5 ml		Continue
Control W		0.025 ml	1.0 ml		1 h room	0.5 ml		as described
Control X		0.025 ml	1.0 ml		temperature		0.5 ml	in text
Control Y		0.025 ml		1.0 ml		0.5 ml		
Control Z		0.025 ml		1.0 ml			0.5 ml	

Definitions: Non-specific control = Y/Z · X; B = sample minus non-specific control; Bo = W minus non-specific control; total ^{3}H-retinol = X minus non-specific control. * The ^{3}H-retinol concentration in these 2 mixtures is the same.

Table 2. Statistical properties of retinol radioimmunoassay

Experiment	N	Range B/Bo	Mean serum retinol concentration (µg/100 ml)	SD
A (replicate samples, same day)	20	0.52–0.59	46.0	3.4
B (replicate samples, same day)	19	0.80–0.89	12.1*	3.0
C (replicate samples, different days)	10	0.40–0.58	52.0	4.6
D (individual animals)	12	0.46–0.58	57.9	6.0

* This serum was diluted 1/3.6.

We found we could use this procedure to follow the decline of rat serum vitamin A during the onset of vitamin A deficiency. Male weanling rats placed on an A deficient diet experienced a fall in average serum retinol from 66 µg/100 ml to 12 µg/100 ml over a 52-day period. This method was useful for weanling rats since less than a ml of blood was needed for each assay.

The cross reactivity of the antiserum with various retinoids is relevant to the applicability of this RIA to retinol in fasting serum. The following facts indicate that the use of the antiserum for this purpose is appropriate: only a tiny fraction of the retinoids in the body are present as retinoic acid[5]; retinal is formed in the tissue where it is used[5]; retinyl esters and carotenes are prominant in serum only after eating[6], and in any case, react very poorly with the antibody[2].

The values obtained in this work for the retinol levels of normal rat serum extend from 46 to 57 µg/100 ml (table 2) and are consistant with published values[7,8]. Suthutuoravoot and Olson[9] have pointed out that normal serum vitamin A concentrations can vary widely and reflect not only vitamin A nutrition but also protein intake.

The RIA described here detects as little as 1 ng of retinol per assay tube. High performance liquid chromatography (HPLC) techniques provide the same order of sensitivity; they are reported to detect as little as 5–7 ng of vitamin A[10,11]. HPLC has the added virtue that it will separate the various retinoids as well as quantitate them. The RIA reported here has an advantage over HPLC for the routine measurement of serum retinol; sample preparation is simpler since it consists only of ethanol addition and centrifugation while the HPLC techniques require solvent extraction, and drying down or lyophilization[10,11]. Furthermore, the RIA technique can be applied in any laboratory to which a scintillation counter is available.

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A blocked mutant of *Claviceps purpurea* accumulating chanoclavine-I-aldehyde[1]

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Summary. An alkaloid-blocked mutant of *Claviceps purpurea* was isolated from a strain which produces ergotoxine alkaloids.

The mutant accumulates chanoclavine-I and the corresponding aldehyde. It lacks the ability to form tetracyclic ergolines.

Blocked mutants are particularly useful for studying the genetics of microorganisms producing secondary metabolites and the biosynthetic pathways of natural products. Furthermore, new compounds may be obtained by 'mutational biosynthesis'. This technique has been widely applied in the field of antibiotics[2–4].

Apparently alkaloid-blocked mutants have not yet been described. We wish to report our results with *Claviceps purpurea* (Fr.) Tul.

The parent strain *Claviceps purpurea* Pepty 695/S, in a sucrose-ammonium citrate medium[5] under submerged conditions, produces 1.2 g/l alkaloids. The alkaloid mixture is composed of ergotoxine (60%), ergometrine (20%), and clavine alkaloids including chanoclavine-I (20%).

During a screening program we selected single colonies and tested their ability to form alkaloids in a production medium[5]. Surprisingly 1 isolate, which showed no changes in morphology and pigmentation compared with the parent strain, did not synthesize any lysergic acid derivatives. The total alkaloid yield of this strain, designated Pepty 695/ch, amounted to 0.5 g/l. TCL (silica gel PF_{254} Merck) of a crude alkaloid extract revealed 2 spots designated A and B. R_f values of A and B in comparison to agroclavine and elymoclavine in different developing systems were as follows. In chloroform/methanol (80:20, v/v): A 0.08; elymoclavine 0.38; B 0.44; agroclavine 0.6. In methanol: A 0.12; B 0.27; agroclavine and elymoclavine 0.44. In chloroform/tert. butanol (3:1, v/v) 15% ammonia atmosphere: A 0.39; elymoclavine 0.48; agroclavine 0.88. 1 g of a crude alkaloid mixture obtained according to Erge et al.[5] and Maier et al.[6] was separated by repeated preparative TLC. Compound A was crystallized from acetone and B was obtained as an amorphous substance. The proportion of A: B was 4:1.

A was identified as chanoclavine-I (**IV**) and B as chanoclavine-I-aldehyde (**V**) on the basis of the following data.

A: m.p. 215 °C; $[\alpha]_{20}^{D} = -235°$ (c 1.0 in pyridine). $C_{16}H_{20}N_2O$[by HRMS, M^+ m/e 256,1586 (M^+ calc. m/e 256, 1576)]. Fragmentation: m/e 237; 223; 196; 183 ($C_{12}H_{11}N_2$, found 183, 0935, calc. 183.0922) 168, 167, 155, 154. PMR (100 MHz, CD_3OD, values are in ppm, δ scale): 1.85 (vinyl-CH_3, weak d); 2.42 (N-CH_3, s); 3.85–3.95 (C-10-H, m), 4,13 (CH_2O, br. s); 5.35 (vinyl-H, dq).

B: $C_{16}H_{18}N_2O$ [by HRMS, M^+ m/e 254,1414 (M^+ calc. m/e 254, 1419)]. Fragmentation: m/e 237 ($C_{16}H_{17}N_2$, found 237,1407, calc. 237, 1392), 235, 223, 211, 194 ($C_{14}H_{12}N$, found 194,1017, calc. 194,097), 183, 168, 167 155, 154. After treatment with D_2O a peak at m/e 256 was recorded. PMR (100 MHz, $CDCl_3$) 1.95 (vinyl-CH_3, weak d); 2.52 (N-CH_3, s); 4.33 (C-10-H, m) 8.0 (indol-NH, s), 9.4 (-CHO, s). IR