As for 5-pyridoxic acid, a 100-mg sample of 4-pyridoxic acid was dissolved in 1 l of buffer solution and placed in an oven at 37°. However, the buffer solution was placed in the oven for two days instead of three. Only 4-pyridoxic acid could be isolated from the Dowex-1-formate column indicating that no lactonization had occurred. When 4-pyridoxic acid was dissolved in 0.1 M formic acid and kept at room temperature only traces of lactone could be detected (ultraviolet spectrum) after one week. An appreciable amount of the lactone of 4-pyridoxic acid was formed after one month of storage at room temperature.

The change of pH accompanying the growth of Pseudomonas sp. MA was not given[2] so the behavior of 4-pyridoxic acid at a pH lower than 5.8 was not determined. It is probable that the growth medium reached a lower pH with Pseudomonas sp. MA than with sp. IA due to more acidic oxidation products. However, it seems unlikely that any lactonization takes place in short periods of time as only traces of lactone could be detected when the acid was suspended in 0.1 M formic acid (pH 2.4) for one week. Whether the lactone of 5-pyridoxic acid is an intermediate in the oxidation of pyridoxine by Pseudomonas sp. IA, is presently being studied in our laboratory with the aid of the ^{14}C-labeled lactone of 5-pyridoxic acid (IV). The synthesis of the ^{14}C-labeled lactone as well as the synthesis of ^{14}C-labeled pyridoxine will be published elsewhere.

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On the formation of estroprotein

Liver catalysis in the binding of [^{14}C]estrogens to albumin has been the subject of several reports in the last decade[1–6]. In contrast, the experiments described herein demonstrate that the metabolism *in vitro* of estrogens by liver slices does not lead to significant enzymatic binding to protein.

Rat-liver slices (250 mg) were incubated with 11 μg ($4 \cdot 10^5$ counts/min) of [16-^{14}C]estrone in 0.1 M phosphate buffer (pH 7.4) for 4 h as previously described[6]. The incubation mixture from a typical experiment, on centrifugation (900 $\times$ *g* for 5 min at 0°), afforded a supernatant fraction containing 75% of the added radioactivity. The liver pellet was rinsed with normal saline, removing another 3% of the radioactivity, then extracted with acetone for 24 h in a Soxhlet apparatus, and

finally saponified with 10% NaOH in 50% ethanol. The acetone extract contained 22% of the original radioactivity; the saponified liver, 7%.

The supernatant fraction, when subjected to paper electrophoresis in barbital buffer (pH 8.8) gave the same distribution of radioactivity (Fig. 1) observed in our earlier study[6]. The bands of ^{14}C at 10, 30, and 40 cm are designated respectively as the "origin", "albumin area", and "second peak". Fig. 1 also shows that the acetone extract of the liver yields a similar electrophoretic pattern. This observation in conjunction with the reported anodic migration of steroid conjugates (0.1 M barbital buffer (pH 8.6)[7]) casts doubt on the characterization of the moving peaks as "estroprotein".

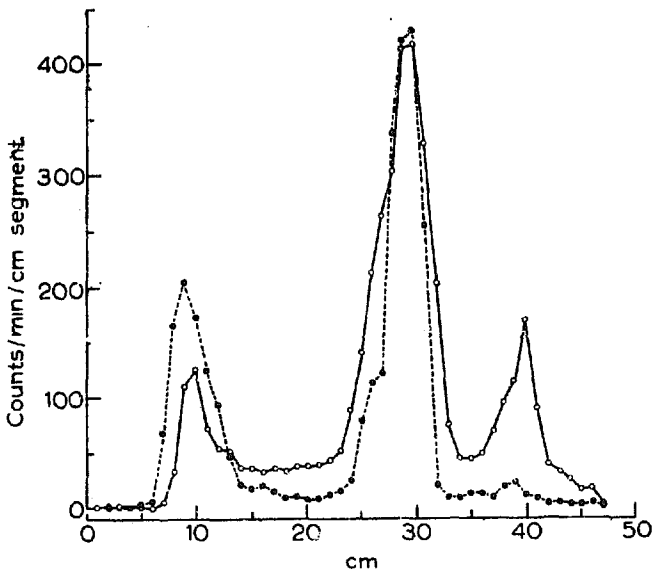

Fig. 1. The electrophoretic separation of estrogenic radioactivity following incubation of [16-^{14}C]-estrone in the presence of surviving rat-liver tissue in phosphate buffer (pH 7.4). O—O, electrophoretic pattern of supernatant fraction; ●---●, electrophoretic pattern of acetone extract of liver residue.

Elution of the radioactivity comprising the origin of the electrophoretic pattern with either 0.01 N NaOH or 0.9% NaCl followed by ether extraction of the acidified (pH 1) eluate led to a high recovery of estrogen(s) in this area (Table I). On the other hand, the majority of radioactivity in the albumin area and most of the radioactivity in the second peak remained in the aqueous phase following similar treatment. When the albumin area and second peak were eluted with 0.9% NaCl and then dialyzed

TABLE I

ETHER EXTRACTION OF THE THREE ELECTROPHORETIC PEAKS OF RADIOACTIVITY

Area	*Counts/min*			*Per cent removed*
	Initial	*Ether extract*	*Aqueous phase*	
Origin	3217	2219	501	69
Albumin	17360	5950*	9410	34
Second peak	5758	685	4273	12

* Analysis of this fraction established the presence of the same mixture of estrogens present in the origin.

for 24 h against the same saline solution, it was observed that the concentrations of radioactivity were equal on both sides of the membrane (Table II).

Analysis of the three electrophoretic bands of radioactivity has been accomplished through the use of gradient-elution partition chromatography[8] in conjunction with carrier steroids which were located by a KOBER colorimetric assay[9]. The non-conjugated steroids present in the origin of an electrophoretic pattern derived from a supernatant fraction proved to be a mixture of (28%) estrone (I), (15%) 2-hydroxyestrone* (II), and (30%) a presently unknown steroid (III) which were eluted from the column in the same order. The same mixture of non-conjugated steroids (34% of I, 8% of II, and 19% of III) was shown to be present in the origin of an electrophoretic strip of an acetone extract of residual liver.

TABLE II

DIALYSIS OF RADIOACTIVITY IN THE ALBUMIN AND SECOND ELECTROPHORETIC PEAKS

Conventional dialyses were performed by placing 2.5 ml of the saline eluate in a cellulose casing (Visking Corp., average radius of pores, 24 Å) and dialyzing against 20 ml of 0.9% NaCl at 4°. Experiments established that equilibration was secured after stirring the samples for 24 h.

	Albumin peak		*Second peak*	
	Inside bag	*Outside bag*	*Inside bag*	*Outside bag*
Volume (ml)	2.5	20	2.5	20
Initial counts/min/ml	670	—	222	—
Final counts/min/ml	67	64	27	23

The presence of estrogen glucuronide conjugates in the albumin area of paper strips obtained with supernatant fluid of residual liver is indicated by the following evidence. Acidified (pH 4.5) eluates of this area, which had been extracted previously at pH 1 with ether, were observed to liberate an additional 30% of ether-extractable radioactivity on treatment with beef β-glucuronidase (EC 3.2.1.31). The ether phase, on subsequent chromatography, yielded (15%) 2-methoxyestrone* (IV) followed by all of the steroids (4% of I, 8% of II, and 12% of III) detected previously at the origin. A similar observation was recorded for an eluate of the albumin region from an acetone extract of liver. Enzymatic hydrolysis yielded in this case 2% of I, 11% of II, 31% of III, and 7% of IV.

A second incubation with β-glucuronidase failed to release any additional ether-extractable radioactivity from an eluate of the albumin area. However, the same eluate, when subjected to hydrolysis with 3 N HCl for 2 h relinquished a substantial amount (30%) of ether-soluble radioactivity. Analysis of the extract, which, in all probability, represents estrogen sulfate conjugates, established the presence of II (14%). The remainder of radioactivity in this extract has not yet been identified though it is apparently of a more polar nature than the metabolites I, II, III, and IV.

β-Glucuronidase, 3 N HCl and 3 N KOH all proved ineffective in attempts to release the radioactivity from eluates of the second peak. Cleavage was effected, however, with alkaline phosphatase (EC 3.1.3.1) which yielded still another ether-soluble estrogen of unknown structure.

* The authors gratefully acknowledge the receipt of an authentic sample of this steroid from Dr. J. FISHMAN of the Sloan Kettering Institute for Cancer Research.

It is apparent from the present evidence that binding *in vitro* of estrogen to protein, *i.e.* the formation of estroprotein, is unlikely. Rather, the findings indicate the formation of glucuronide and sulfate conjugates of estrogen metabolites which, under the conditions of electrophoretic separation used, have mobilities coincidental with albumin. Non-conjugated estrogens constitute the origin of the electrophoretic pattern and an unknown phosphate conjugate is present in the second peak. The authors recognize that the small amount (7%) of radioactivity in the liver which was only released on saponification and, therefore, unavailable for study, may constitute protein-bound estrogen.

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