reported the use of trypsin in the preparation of subcultures of LLC-MK_2-NCTC subline 3206 of monkey-kidney cells cultivated in a chemically defined medium. Recent unreported work in this laboratory shows that in subculturing L cells the obviously harmful effect of trypsin can be suppressed by addition of soybean trypsin inhibitor. The present studies have established that subcultures of L and LLC-MK_2 cells can be prepared enzymatically with Aspergillin-O in the absence of such additives as serum or soybean trypsin inhibitor. Comparison of the effect of trypsin and Aspergillin-O on cell lines cultivated in the presence of serum showed that cells become detached individually, with trypsin, and as small islets with Aspergillin-O. Because little is known about the exact mode of action of Aspergillin-O or trypsin on tissue cells, their activity in releasing cells from glass cannot therefore be explained. If tissue cells incorporate ovomucoids or other enzyme inhibitors from media containing serum, it is possible that these substances might provide a mild protective action against the hydrolyzing activity of Aspergillin-O but not of trypsin. Or, Aspergillin-O may be more specific in its action for substances binding cells to glass.

Experiments are in progress with various cell lines treated with Aspergillin-O to determine whether their sensitivities to a variety of viruses have been altered.

Summary. Aspergillin-O, an enzyme isolated from a submerged fermentation of *Aspergillus oryzae,* has been used to advantage: (a) to prepare subcultures of cell lines adapted to continuous growth in unsupplemented chemically defined medium (CMRL-1066) and (b) to obtain luxuriant primary cultures from fresh tissues.

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Investigations on Extraction of Rat Plasma Phospholipids.* (28574)

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Two of the most commonly used solvent mixtures for extraction of lipids from blood and other animal tissues are ethanol:ether (3:1 v/v)(1) and chloroform:methanol (2:1 v/v)(2).

In lipid work carried out in this laboratory over a number of years, alcohol and ether have been used extensively as primary extractants of lipids from rat tissues. With the advent of methods of separating lipid classes on silicic acid columns by elution with increasing amounts of ethyl ether in petroleum ether (3,4,5), it became convenient to evaporate the primary extract and reextract the lipid into petroleum ether for storage or for application to the column. Although 99% or more of the plasma cholesterol, triglycerides, or free

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fatty acids extracted into alcohol:ether could be recovered in petroleum ether, only 40-70% of alcohol:ether-soluble phosphorus was extracted(6,7). The same procedures extracted 95-99% of the alcohol:ether-soluble phosphorus from liver(6), red blood cells(7), adrenal, spleen, kidney, and heart muscle.‡

Other investigators have reported losses of 20-50% of the alcohol:ether-soluble phosphorus when the primary extract was re-extracted into petroleum ether. Such losses were attributed to: 1) nonlipid phosphorus in the original extract(8,9); 2) decomposition of phospholipid by heat or oxidation(10); and 3) insolubility of lecithin and sphingomyelin in petroleum ether(11). However, Man(10) and Ellis and Maynard(12) demonstrated that 95-100% of the alcohol:ether-soluble phosphorus could be extracted into petroleum ether if the primary extract were evaporated under mild conditions of reduced pressure and low temperatures.

Our own attempts to improve the plasma lipid phosphorus recovery by equally gentle methods still resulted in only variable and poor recoveries of the alcohol:ether-soluble phosphorus. We therefore undertook the present study to determine the nature of this petroleum ether-insoluble phosphorus and to see whether it represented degraded phospholipid or a specific phospholipid, such as sphingomyelin, which would not readily extract into petroleum ether.

Method. A pooled sample of blood was obtained from mature female rats maintained on laboratory stock diet. Each animal was anesthetized with an injection of 6% pentobarbital sodium (0.1 ml/100 g body weight). Blood was withdrawn by cardiac puncture with syringes which had been rinsed with 1% (w/v) heparin in 0.9% saline. The samples were centrifuged and the plasma was removed and pooled. Several aliquots of 4-5 ml each were taken for extraction. This quantity of plasma was chosen because it represented the amount routinely obtained from a single rat. To each aliquot was added 0.1 ml of 0.1% hydroquinone in 95% ethanol as an antioxidant.

‡ Unpublished data.

Two methods were used for the primary extraction of the plasma samples. One set of plasmas was extracted with 95% ethanol: diethyl ether in a manner similar to the procedure previously reported(13), except that the first extraction with 95% ethanol remained at room temperature overnight. The other set was extracted with chloroform: methanol (1:1, v/v)(14). All solvents used in extractions or chromatography were freshly redistilled.

In both instances the primary extracting solvents were removed in a rotary evaporator at reduced pressure, with temperatures maintained below 50°C. The material was then extracted 4 times with 10-ml portions of boiling petroleum ether, then 4 more times at room temperature. The residue in the flask was finally extracted with chloroform to pick up any phosphorus insoluble in the petroleum ether. Pooled petroleum ether-soluble extracts and pooled petroleum ether-insoluble residues in chloroform were then fractionated into separate phospholipid classes, on silicic acid columns(14).

Phosphorus was determined(15) on the petroleum ether-soluble and -insoluble material in order to estimate what proportion of the phosphorus was soluble or insoluble by each extraction method. Phosphorus was also determined on the individual phospholipids eluted from the silicic acid column.

Fatty acid composition of each phospholipid fraction was determined by gas-liquid chromatography as previously described(13). Methyl heptadecanoate was added as an internal standard after phospholipid fractionation in order to check the recovery of the fatty acids(16).

Results and discussion. Little difference was noted between the total amounts of phosphorus extracted initially with ethanol:ether or with choloroform:methanol (Table I). However, it was evident that, whereas primary extraction with chloroform:methanol allowed complete recovery of the phosphorus in petroleum ether, primary extraction with ethanol:ether caused a loss of 25.7% of the originally soluble phosphorus. The phosphorus insoluble in petroleum ether after alcohol:ether extraction could, however, still be

TABLE I. Amount of Phosphorus in μgP/ml Plasma Extracted from Rat Plasma and Recovered in Petroleum Ether by 2 Extraction Methods.

	95% ethanol: ether	Chloroform: methanol
P extracted initially	51.4	54.8
P extractable into PE*	38.2	56.3
P insoluble in PE (but soluble in chloroform)	11.2	0.6
% P recovered	96.1	104

* PE = petroleum ether.

extracted into chloroform.

In an effort to establish whether the petroleum ether-insoluble phosphorus was indeed phospholipid, it, along with the petroleum ether-soluble and chloroform:methanol-extracted samples, was separated into cephalin, lecithin, sphingomyelin, and lysolecithin fractions. The results of this fractionation are shown in Table II. The percentages of phosphorus recovered in the phospholipid fractions from the petroleum ether-insoluble material were almost identical with those obtained from the chloroform:methanol-extracted plasmas. The higher percentage of cephalin phosphorus in the petroleum ether-soluble phospholipid was thought to be due to elution of lecithins with the fraction. Since paper chromatography of the cephalin fraction failed to reveal choline-containing material, however, the cause of the high phosphorus in this fraction is unknown.

The petroleum ether-insoluble phosphorus therefore appeared to be a mixture of intact phospholipids similar to those extracted with chloroform:methanol and those soluble in petroleum ether. Had the phosphorus-containing material in this insoluble fraction consisted mostly of degraded phospholipids, the major portion of it would have remained on the column or would have been eluted with the last eluate. The small amount of phosphorus present in the neutral lipid fraction appeared not to be phospholipid, because when it was chromatographed by the method of Lis *et al.*(5), which employed an ether: petroleum ether eluant, 93% of the phosphorus came off in the triglyceride fraction and none in the phospholipid fraction.

TABLE II. Per Cent of Total Lipid Phosphorus Eluted in Each Phospholipid Fraction.

Fraction	Ethanol:ether extracted PE-* soluble	Ethanol:ether extracted PE-insoluble	Chloroform: methanol extracted PE-soluble
Neutral	1.5	8.0	5.1
Cephalin	11.6	4.8	5.5
Lecithin	61.8	64.0	66.3
Sphingomyelin	10.2	10.4	11.6
Lysolecithin	14.9	12.8	11.4
Total % phosphorus recovered from column	88.8	104	89.2

* PE = petroleum ether.

Fatty acid compositions of each of the phospholipid classes separated from petroleum ether-soluble, petroleum ether-insoluble, and chloroform:methanol extracts are shown in Table III. Although the percentages of linoleic and arachidonic acids appeared to be lower in the cephalin and sphingomyelin fractions of the petroleum ether-insoluble phospholipids than in the petroleum ether-soluble phospholipids, the differences were not thought large enough to affect the petroleum ether solubility of the two fractions. The sphingomyelin and lysolecithin fractions would be expected to be the most insoluble in petroleum ether. However, as indicated in Tables II and III, the percentages of phosphorus and fatty acid compositions in these fractions from the petroleum ether-insoluble residue were almost identical with the values for the same phospholipids recovered from the petroleum ether-soluble or chloroform:methanol extracts.

The results of these studies showed, therefore, that the petroleum ether-insoluble phosphorus extracted from rat plasma by alcohol: ether consisted principally of intact cephalin, lecithin, sphingomyelin, and lysolecithin, and that it had a similar fatty acid composition and occurred in similar proportions to those of the phospholipids soluble in petroleum ether or in chloroform:methanol. There was no evidence that the petroleum ether insolubility of the phospholipid was due to extensive degradation or resulted from the insolubility of a specific class of phospholipid. However, extraction of rat plasma with alcohol:ether may have caused some alteration of the phospholipids, perhaps from peroxides in the diethyl ether. Such an explanation does

TABLE III. Fatty Acid Composition of Plasma Phospholipids.*

Phospholipid fraction	Treatment	Myristic	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	Arachidonic
Cephalin	Alcohol : ether							
	PE†-sol	.4	10.6	.6	31.8	4.5	8.8	40.5
	PE-insol	1.6	17.1	3.3	57.7	7.2	5.5	31.4
	Chloroform: methanol	.9	9.5	1.8	33.4	5.4	7.8	39.8
Lecithin	Alcohol : ether							
	PE-sol	.7	19.6	1.4	29.8	5.8	13.8	27.8
	PE-insol	.5	18.3	1.2	26.8	5.2	12.9	26.9
	Chloroform: methanol	.5	19.2	1.1	29.9	6.2	15.2	26.9
Sphingomyelin	Alcohol : ether							
	PE-sol	3.1	13.2	2.1	29.4	5.8	13.0	32.6
	PE-insol	5.4	16.4	3.0	27.3	6.4	12.5	26.4
	Chloroform: methanol	2.6	19.6	4.4	26.4	8.3	14.7	20.3
Lysolecithin	Alcohol : ether							
	PE-sol	.5	31.5	1.5	35.3	4.9	12.6	12.6
	PE-insol	1.3	33.0	1.3	33.2	5.3	12.4	12.6
	Chloroform: methanol	.5	32.9	1.7	31.8	7.0	15.0	10.4

* Expressed as per cent of total phospholipid fatty acids in fraction. Some minor components are not shown here.

† PE = petroleum ether.

not appear too likely, however, since the same extraction procedures used on liver(6) and red blood cells(7) allowed 95% of the extracted lipid phosphorus to be recovered by petroleum ether. Also any effect on phospholipid solubility produced by alcohol:ether is reversible, since chloroform extraction of the petroleum ether-insoluble residue gives almost quantitative recovery of the phospholipids.

From these results it would appear, therefore, that if extraction of rat plasma phospholipid into petroleum ether is desired for further analyses, primary extraction with chloroform:methanol would be recommended over Bloor's alcohol:ether extraction. The reasons for the difference in the petroleum ether solubility of the phospholipids extracted by the two methods remain unknown.

Summary. The poor recovery of phospholipid when rat plasma was extracted into petroleum ether after primary extraction with ethanol:ether was shown to be a function of the extraction method, since complete recovery of the phosphorus was obtained after initial extraction with chloroform:methanol. When the petroleum ether-soluble and -insoluble phosphorus fractions obtained from the ethanol:ether extraction were fractionated into separate phospholipid classes, the phosphorus recovered and the fatty acid composition of the phospholipids were similar. Thus the petroleum ether-insoluble phosphorus was intact phospholipid and was not a specific phospholipid, such as sphingomyelin or lysolecithin.

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Antifertility Activity of an Oxidized Polyphenolic Acid from *Lithospermum ruderale.** (28575)

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Since Train *et al.*(1) reported that squaws of the Shoshone tribe used a cold water extract of the roots of *L. ruderale* (lithosperm) to reduce fertility, considerable work has been published dealing with the biological effects of various extracts of lithosperm. Cranston(2) and Zahl(3) investigated the inhibitory effect of the plant on the estrous cycle of mice while Plunkett *et al.*(4,5) studied its effects on the cycling of rats. It was demonstrated that dried roots of the plant impaired development of the gonads and accessory sex organs of the immature male rat and that injection of root extracts was at least 10 times more effective than oral administration. It was also shown that similar effects could be produced in the mature animal. Noble *et al*(6), Graham *et al*(7,8), and Noble *et al*(9) conducted experiments showing *in vitro* inactivation of gonadotropin (Pregnant Mare Serum) by lithosperm extracts. Zeller *et al*(10) studied the antiovulatory action of lithosperm in hens. Breneman *et al*(11) demonstrated the inactivating effect of its extracts *in vitro* and *in vivo* on pituitary gonadotropins using the cockerel. Breneman and Carmack(12) showed that the action of glucagon was inhibited in the chicken by *L. ruderale*. Using another species of plant, *(L. officinale)*, Kemper *et al* (13) demonstrated that its extracts could reduce blood sugar levels in the rabbit. Also, the incidence of mammary tumors in mice has been reduced by lithosperm(14).

Because *L. ruderale* roots contain a very active polyphenol oxidase which rapidly oxidizes the principal polyphenol present in the roots during water extraction, a logical step was to determine if the "active principle" could possibly be an oxidized form of the main polyphenol, which we have named "lithospermic acid" (LA). The effects reported here have been produced through use of this oxidized, polymerized acid which was isolated from the roots. A similar oxidized polymer was also prepared by isolation of the pure LA and subsequent controlled enzymatic oxidation.

Materials and methods. Isolation of lithospermic acid. The dried, ground roots (100 g) were vigorously stirred for 15 minutes with 500 ml of water at 25°C maintained at a pH of 2.0 with dilute HCl to prevent enzymatic oxidation. Following filtration, the infusion was extracted in a separatory funnel using 6 x 100 ml of peroxide-free diethyl ether. The ether extracts were combined, dried over anhydrous sodium sulfate and the volume reduced by distillation. The syrupy residue was dried *in vacuo*. The crude LA (1.4 – 2.0% of the dry roots) was purified by counter current distribution during 32 transfers using diethyl ether and water. The yield of chromatographically pure product was about 50% of the crude LA. LA is a polyphenolic acid having two 1, 2, 4 tri-substituted benzene rings each containing o-dihydroxy groupings and an olefinic grouping conjugated with one of the rings. Its chemical nature has been reported(15).

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