white would provide 50 g of L-threonine worth $3.50, 53 g of L-methionine worth $2.00, 17 g of L-tryptophan worth $1.50, and 55 g of L-lysine worth $0.60. (Values were rounded to nearest $0.1.) This represents an estimated total market value of $7.60 for only threonine, methionine, tryptophan, and lysine, which is twice the $3.80 estimated cost for the kilogram of dried duck egg white. The threonine alone almost covers the cost of the whole product.

This little exercise in price comparisons would seem to suggest that dried duck egg whites be considered as a currently economical source of the L-amino acids. Future price comparisons will depend on price trends of feed as compared to L-amino acid prices.

This discussion has centered on duck eggs as a source of economical amino acids. However, this is only a special case for an approach to nutrition that is not new but is sometimes overlooked in the confrontation between plant breeding and amino acid fortification as to which is the best answer to the world's protein problem. The data and discussion of this paper would suggest that an alternate approach to human protein nutrition be selection of appropriate supplementary foods that contain large amounts of the most needed essential amino acids. These might then be consumed as food additives (e.g. dried duck egg white) or as a separate food (e.g. whole duck egg).

It has been suggested that even the fortification of a vegetable protein with an animal protein can be practical if the right animal protein is selected. Even more economy might be achieved by production of vegetable proteins high in the most needed essential amino acids.

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In Vitro Biotransformations of 1-(*o*-Chlorophenyl)-1-(*p*-chlorophenyl)-2,2-dichloroethane (*o,p'*-DDD) and 1,1-Bis(*p*-chlorophenyl)-2,2-dichloroethane (*p,p'*-DDD) by Bovine Adrenal

In vitro biotransformations of ^{14}C-labeled 1-(*o*-chlorophenyl)-1-(*p*-chlorophenyl)-2,2-dichloroethane (*o,p'*-DDD) and 1,1-bis(*p*-chlorophenyl)-2,2-dichloroethane (*p,p'*-DDD) by bovine adrenal homogenates were studied. Homogenates were found to oxidize *o,p'*-DDD to *o,p'*-dichlorodiphenylacetic acid (*o,p'*-DDA), while *p,p'*-DDD was converted to bis(*p*-chlorophenyl)acetic acid (*p,p'*-DDA) and 1,1-bis(*p*-chlorophenyl)-2,2-dichloroethanol. Transformation products were identified by thin-layer chromatography and mass spectra of band extracts. The mitochondrial fraction was found to be the most active of the cortex subcellular fractions in transforming the substrates with some contribution by the soluble fraction. Transformation by the microsomal fraction was negligible. An NADPH generating system was necessary for these transformations, and they did not occur with boiled enzyme preparations.

The compound *o,p'*-DDD [1-(*o*-chlorophenyl)-1-(*p*-chlorophenyl)-2,2-dichloroethane] produces atrophy of the adrenal cortex, inhibition of ACTH stimulation of steroid production, and alteration of liver cortisol metabolism (Hart and Straw, 1971a). *o,p'*-DDD has Food and Drug Administration approval for human use in the treatment of Cushing's Syndrome, secondary to adrenal carcinoma (Verdon et al., 1960) or hyperfunction (Hellman et al., 1970).

The adrenal effects of *o,p'*-DDD occur in dogs and humans, but it is inactive in rats, mice, rabbits, and monkeys (Gaunt et al., 1965). Weber et al. (1958) reported that technical DDD caused adrenal atrophy in cattle. It has been reported that *p,p'*-DDD [1,1'-bis(*p*-chlorophenyl)-2,2-dichloroethane], the main component of technical DDD, is relatively inactive while the presence of *o,p'*-DDD is responsible for the adrenal effects of technical DDD (Nichols, 1961). Hart et al. (1973) found that equal doses of both isomers eventually produced cortex necrosis and inhibited adrenal steroid production in dogs, but *o,p'*-DDD was faster acting than *p,p'*-DDD.

A number of in vivo metabolites of *o,p'*-DDD in the human (Sinsheimer et al., 1972; Reif et al., 1974) and the rat (Reif and Sinsheimer, 1975) have been identified in these laboratories. Metabolism involved alkyl oxidation to *o,p'*-DDA [*o,p'*-dichlorodiphenylacetic acid] and aromatic hydroxylation in both species. Peterson and Robison (1964) identified a number of metabolites of *p,p'*-DDT [1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane] in the rat which could also be metabolites of *p,p'*-DDD. There was evidence of *p,p*-DDA [bis(*p*-chlorophenyl)acetic acid] formation, but aromatic hydroxylation of para,para'-substituted isomers has not been reported.

Since DDD concentrates in the adrenal gland (Moy, 1961) and the adrenal cortex is the target organ for the drug, the biotransformation of DDD isomers by adrenals is of interest. Of particular interest would be the possible para hydroxylation of *o,p'*-DDD by the adrenal cortex as the basis for the increased rate of activity of this isomer. The few reports of in vitro conversions of xenobiotics by adrenals have involved incubations with whole gland homogenates and would include transformations by both cortex and medulla. Jellinck et al. (1967) showed that rat

adrenal converted 7,12-dimethylbenzanthracene to 7-hydroxymethyl-12-methylbenzanthracene and a more polar compound. Sims (1970) indicated that 7-hydroxymethyl-12-methyl- and 12-hydroxymethyl-7-methylbenzanthracene were converted to phenols and dihydrols by rat adrenals. Other rat adrenal transformations reported are the conversion of methyl tetrahydrofurfuryl sulfoxide to methyl tetrahydrofurfuryl sulfone (Fujita and Ziro, 1967) and the conversion of digitoxigenin to 3-digitoxigenone (Talcott et al., 1973). Kupfer and Orrenius (1970) reported the demethylation of *p*-chloro-*N*-methylaniline and aminopyrine, and the ω-oxidation of laurate by guinea pig adrenal microsomes. Juchau et al. (1972) demonstrated the hydroxylation of 3,4-benzpyrene by human fetal adrenal gland.

The present study involves the in vitro metabolism of *o,p'*-DDD in its target tissue, the adrenal cortex. The adrenal cortex of cattle, a species in which *o,p'*-DDD shows adrenal effects, was chosen for study. Transformations with *p,p'*-DDD, the slower acting isomer, were also observed for comparison.

METHODS AND MATERIALS

Chemicals. Counsell and Willette (1966) synthesized *o,p'*-DDD and *p,p'*-DDD which were uniformly labeled with ^{14}C in the *p*-chlorophenyl moiety. The radiochemical purity of the *o,p'*-DDD was found to be 98.6% by reverse isotope dilution. Thin-layer chromatography (TLC) of *p,p'*-DDD using systems 2 and 4 (development three times) gave one radioactive band by liquid scintillation counting (lsc) of 0.5 cm sections of the plates. Unlabeled *o,p'*-DDD and *p,p'*-DDD were obtained from Aldrich Chemical Co. and were recrystallized three times from methanol before use. Reference standards *p,p'*-DDA and *p,p'*-DDE were obtained from Aldrich; Dr. R. E. Counsell supplied *o,p'*-DDMu and *p,p'*-DDMu (Haller et al., 1945). Glucose 6-phosphate, glucose-6-phosphate dehydrogenase, NADP, and disodium fumarate were obtained from Calbiochem. Literature methods (Cristol and Haller, 1945) were used to synthesize *o,p'*-DDA and its methyl ester.

FW-152 (Rohm and Haas), which was used as a source of 1,1-bis(*p*-chlorophenyl)-2,2-dichloroethanol, was purified by preparative layer chromatography using system 2. A band with R_f 0.26 to 0.46 was extracted with ether and recrystallized once with petroleum ether to give a compound melting at 108–109° (Pepper and Kulka, 1950) and mass spectrum (70 eV) *m/e* (rel intensity) 338 (0.16) M + 4, 336 (0.32) M + 2, 334 (0.25) M^+, 253 (63), 251 (100) M − $CHCl_2$, 237 (2.4), 235 (3.6), 165 (6.6), 139 (88), 141 (29).

Preparation of Homogenates. Bovine adrenal glands were obtained immediately after slaughter from a local meat packer. They were kept in ice enroute to the laboratory where homogenates were immediately prepared in a cold room at 10°. Whole homogenates were prepared as follows: glands were trimmed of fat, minced, and homogenized for 50 sec with a Waring Blendor in 0.1 *M* Tris buffer (pH 7.4) (10% w/v suspension). The homogenates were immediately frozen until used for incubations.

Adrenal cortices were separated from the medulla and outer capsule, minced, and homogenized for 50 sec with a Waring Blendor in 0.25 *M* sucrose–0.01 *M* phosphate buffer (pH 7.4) (10% w/v suspension). This cortex homogenate was then separated into a mitochondrial, a soluble, and a microsomal fraction by centrifugation following the procedure of Young et al. (1973).

Incubation and Extraction. Incubation mixtures contained the cofactors that have been used for in vitro adrenal steroid transformation studies (Hayano and Dorfman, 1962). Each mixture contained the following: (1) 2 ml of adrenal enzyme suspension, thawed immediately before use; (2) 2 ml of glucose 6-phosphate in water solution (5 μmol/ml); (3) 2 ml of NADP (1 μmol/ml), $MgCl_2$ (1 μmol/ml), and disodium fumarate (1 μmol/ml) in water; (4) 1 ml of a glucose-6-phosphate dehydrogenase in water solution (0.4 unit/ml); (5) 2 mg (0.006 μmol; 0.034 μCi) of ^{14}C-labeled *o,p'*-DDD or *p,p'*-DDD in 0.1 ml of absolute ethanol.

The mixtures were incubated at 37° for 2.5 hr. A procedure which gave nearly quantitative extraction of activity involved acidifying incubation mixtures with 10% HCl, extraction with 5 ml of ether, addition of 1 ml of formic acid to incubation mixtures, and further extraction with 3 × 5 ml of ether. The use of formic acid in extraction of DDT compounds from plasma was reported by Dale and Miles (1970).

Analysis. TLC plates (Brinkman 5 cm × 20 cm × 0.25 mm silica gel 60 F_{254} on glass) were developed to 15.5 cm. Detection of standard compounds was via fluorescent quenching and radioactivity was detected by liquid scintillation counting of 0.5-cm strips of silica gel scraped from the plates. A Beckman Model LS 200 scintillation spectrometer and a cocktail of 0.3% PPO (3,5-diphenyloxazole) in toluene were used. The following TLC systems were used for development: (1) methanol–chloroform–hexane (17:33:50); (2) methanol–chloroform–hexane (3:7:90); (3) benzene; (4) petroleum ether–hexane (10:90); and (5) formic acid–methanol–benzene (1:9:90).

A DuPont 21-490 single focusing instrument was used to obtain mass spectra. Solid probe determinations of residues from extracts of TLC bands were taken at intervals as the probe temperature was gradually increased from 50 to 250°.

TLC with system 2 and subsequent liquid scintillation counting were used to determine the percent transformation to in vitro metabolites. Identification of acidic metabolites was with the use of TLC systems 1, 2, and 5 and mass spectra of band extracts. System 2 origin band activity from *o,p'*-DDD incubations was also extracted with ether, methylated with diazomethane (Williams and Sweeley, 1964), and chromatographed with system 3 to further aid in its identification. System 4, development three times, was used to detect neutral metabolites such as *o,p'*-DDMu [1-(*o*-chlorophenyl)-1-(*p*-chlorophenyl)-2-chloroethylene], *p,p'*-DDMu [1,1-bis(*p*-chlorophenyl)-2-chloroethylene], and *p,p'*-DDE [1,1-bis(*p*-chlorophenyl)-2,2-dichloroethylene] in the presence of unchanged substrate.

RESULTS AND DISCUSSION

TLC R_f values for metabolites and reference compounds which cochromatograph are given in Table I.

Whole adrenal as well as cortex mitochondrial and soluble enzyme homogenates transformed *o,p'*-DDD to *o,p'*-DDA. Aromatic hydroxylation, either before or after side-chain oxidation to *o,p'*-DDA, was not detected. This is in contrast to our previous in vivo studies for *o,p*-DDD (Reif

Table I. TLC R_f Values[a]

Compound	System 1	2	3	4(3×)[b]	5
o,p'-DDD	0.68	0.47	0.57	0.32	0.65
o,p'-DDMu	0.71	0.52		0.52	0.67
o,p'-DDA	0.29	0	0		0.39
o,p'-DDA methyl ester	0.71	0.41	0.43		0.63
p,p'-DDD	0.69	0.45	0.60	0.29	0.67
p,p'-DDMu				0.59	
p,p'-DDE				0.65	
tert-Hydroxy *p,p'*-DDD		0.14	0.43		0.58
p,p'-DDA	0.28	0	0		0.40

[a] R_f values are an average of three determinations and are for metabolites and reference compounds which cochromatograph. [b] Plates were developed three times.

Table II. In Vitro Biotransformation of *o,p'*-DDD by Adrenal Cortex Subcellular Fractions

Subcellular fraction	% transformation[a] (av ± SD) Blank[b]	Transformation over blank[c]
Mitochondrial	0.51 ± 0.15	2.40 ± 0.47
Soluble	0.50 ± 0.14	1.33 ± 1.20
Microsomal	0.63 ± 0.28	−0.25 ± 0.16

[a] Percent transformation to *o,p'*-DDA was determined by TLC with system 2. [b] An average of five determinations of percent transformation was obtained with boiled enzyme incubations. [c] The values represent an average of five determinations after subtraction of the average blank value.

Table III. In Vitro Biotransformation of *p,p'*-DDD by Adrenal Cortex Subcellular Fractions

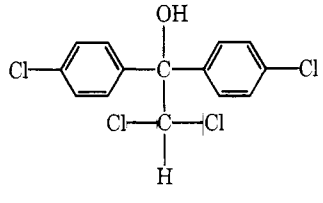
Subcellular fraction	% transformation[a] (av ± SD) Blank[b]	Transformation over blank[c]
Mitochondrial	0.47 ± 0.21	1.02 ± 0.17
Soluble	0.44 ± 0.15	0.45 ± 0.12
Microsomal	0.63 ± 0.08	0.03 ± 0.20

[a] Percent transformation to *p,p'*-DDA and the tertiary hydroxy-substituted *p,p'*-DDD was determined by TLC with system 2. [b] An average of five determinations of percent transformation was obtained with boiled enzyme incubations. [c] The values represent an average of five determinations after subtraction of the average blank values.

et al., 1974, Reif and Sinsheimer, 1975) and that of Feil et al. (1973) for *o,p*-DDT in rats.

TLC of methylated transformation activity with system 3 indicated only the methyl ester of *o,p'*-DDA. No transformation to *o,p'*-DDMu was detected by TLC with system 4. Results for *o,p'*-DDD incubations with cortex subcellular fractions are given in Table II. The activity was 88–96% extracted from the incubation mixtures. The mitochondrial fraction accounts for the majority of the transformation to *o,p'*-DDA. Incubations with one soluble enzyme preparation gave negligible transformation, while another resulted in considerable transformation. Thus, the standard deviation for the soluble enzyme preparation is high.

The results of *p,p'*-DDD incubations with cortex subcellular fractions are given in Table III. The activity was 83–99% extracted from incubation mixtures. The mitochondrial fractions show the highest percent transformation with some contribution by the soluble fraction. In addition to *p,p'*-DDA, tertiary hydroxylated *p,p'*-DDD



[1,1-bis(*p*-chlorophenyl)-2,2-dichloroethanol] was identified by TLC and mass spectra and accounted for 32–57% of the transformation radioactivity. Examination of incubation extracts with TLC using system 4 indicated the percent transformation of *p,p'*-DDD to *p,p'*-DDMu and/or *p,p'*-DDE was less than 0.2%.

The results of this study indicate that the adrenal cortex may play a role in the metabolism of xenobiotics. Transformations were not detected in the absence of a NADPH generating system nor in the presence of boiled homogenate controls. The observation that the mitochondrial fraction is most active in biotransformation of DDD species is of interest since it is the intramitochondrial steroid production which is inhibited by *o,p'*-DDD (Hart and Straw, 1971b).

The mode of biotransformation of DDD compounds by adrenal cortex appears to be side-chain oxidation rather than aromatic hydroxylation. Tertiary hydroxylation of *p,p'*-DDD has previously been reported in insects (Gatterdam et al., 1964), but no reports describing it as a metabolite in mammals were found. Alkyl hydroxylation of *o,p'*-DDD was not detected; ortho chlorination reportedly blocks such hydroxylation (Metcalf and Fukuto, 1968).

The biological activities and toxicities of the described in vitro metabolites are of interest due to their possible presence in the adrenal after DDD exposure. The tertiary hydroxy *p,p'*-DDD is active as a pesticide and synergist of insecticidal activity (Tahori et al., 1958). Larson et al. (1955) reported that it caused adrenal hypertrophy in dogs after chronic feeding; however, its source and purity for that study were not given. The compound *p,p'*-DDA has been classified as a relatively nontoxic metabolite (Smith et al., 1946). Bleiberg et al. (1962) reported that it inhibited choline acetylase while Johnson and Weiss (1967) found that it disrupted growth of KB and HeLa cell cultures. Little information concerning the toxicity of *o,p'*-DDA was found in the literature.

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New Volatile Constituents of Black Tea Aroma

The volatile components of black tea were isolated by extraction with supercritical CO_2 under pressure followed by atmospheric steam distillation and enrichment of steam volatiles on Porapak Q. The total black tea aroma fraction was separated into basic and neutral components. A total of 56 constituents, mainly pyridines, pyrazines, quinolines, thiazoles, aromatic amines, and carbonyls, have been identified for the first time in black tea aroma by using a combination of glass capillary gas chromatography and mass spectrometry. Identifications of the new compounds were accomplished by comparison of their mass spectra with authentic reference spectra measured on the same instrument and with mass spectral data given in the literature. GLC retention times were used to confirm identifications.

Many authors have carried out studies on volatile components of black tea, with the result that no single key aroma compound could be identified which shows a distinctly black tea like character. In recent years Japanese investigators have been studying the flavor of black tea (Ina and Sakato, 1968; Ina and Eto, 1972; Yamanishi et al., 1972, 1973a). The available information on the chemistry and biochemistry of black tea aroma formation was reviewed by Sanderson and Graham in 1973. Two recent publications by Cazenave et al. (1974) and Cazenave and Horman (1974) deal with studies on the composition of the aroma of black tea. Renold et al. (1974) identified 68 volatile constituents for the first time in a black tea aroma concentrate using the coupling technique of a mass spectrometer with a glass capillary column.

The present paper reports on the identification of hitherto unknown volatile black tea aroma ingredients, mainly nitrogen-containing constituents, which were separated by using a new mild isolation technique which is generally applicable in food odor research (Vitzthum et al., 1975).

MATERIALS AND METHODS

A commercial blend of black tea was extracted with supercritical CO_2 under pressure. The CO_2 system used in the authors' laboratory is shown schematically in Figure 1 and the CO_2 extraction procedure is briefly described. Dense gas phases, e.g. CO_2 above its critical conditions, are able to solve a great variety of substances. This solubility depends upon pressure and temperature of the compressed CO_2. In general, with increasing pressure solubility rises, too. On the other hand, with decreasing pressure a separation of the soluted substances from the gas phase occurs.

Vessel A contains air-dried black tea and a flow of compressed supercritical CO_2, by passing the tea, is charged with the aroma and flavor and a supercritical "solution" is formed. The pressures are of the magnitude of 100–300 bars. The gas flow is then fed into vessel B by expansion, and the separation mentioned above takes place at pressures in the range of 50–70 bars. The pure, now extract-free CO_2 is exhausted from B by the pump and compressed again in vessel A, etc. After careful removal of the remaining CO_2 a concentrate with a very intensive tea aroma is obtained in B.

Experimental details concerning operating parameters were previously described by HAG AG (1974b) and Vitzthum et al. (1975). The aroma concentrate was subjected to atmospheric steam distillation and fractionated into basic and neutral components essentially by the same procedure as described previously for coffee by Vitzthum and Werkhoff (1974a,b). Gas–liquid chromatographic analyses were performed on a Carlo Erba GI Model 450 gas chromatograph with an all-glass system using the direct injection technique on high-resolution glass capillary columns. The analytical chromatogram shown in Figure 2 was obtained with a 100 m × 0.30 mm i.d. glass capillary column coated with polypropylene glycol. The GC–MS equipment was similar to that described previously by Vitzthum and Werkhoff (1974a) except that a 200 m × 0.31 mm i.d. glass capillary column was coupled to the mass spectrometer.

N-Ethylacetamide and *N*-ethylpropionamide were prepared by reaction of aqueous ethylamine with acetyl chloride and propionyl chloride, respectively, according to the general procedure of D'Alelio and Reid (1937).

2,4-Dimethylthiazole was synthesized by the method of Schwarz (1945) by the reaction of thioacetamide and chloroacetone. 2,5-Dimethylthiazole and 2,4,5-trimethylthiazole were obtained as described by Kurkjy and Brown (1952). 2,5-Dimethyl-4-ethylthiazole was prepared by a modification of a method described by Takahashi and Hayami (1961). All other compounds identified in our laboratory were from reliable commercial sources.

RESULTS AND DISCUSSION

The compounds identified from the black tea aroma concentrate are listed in Table I. The chief advantage of the low-temperature CO_2 procedure (40°) consists in its rapidity and in the fact that it eliminates the risk of contaminating the aroma complex with solvents and their impurities