Table III. Amounts of *N*-Nitrosopyrrolidine Formed from Collagen When Heated at Various Temperatures and pH Values, and Dry for 2 hr[a-c]

Temp, °C	mg of *N*-nitrosopyrrolidine			
	pH 4.6[d]	pH 6.2[e]	pH 9.0[f]	Dry
120	N.D.	0.09	N.D.	N.D.
145	0.07	0.13	0.09	N.D.
170	0.38	0.86	1.02	N.D.
195	1.20	0.75	1.30	4.5

[a] All samples contained 0.300 g of collagen and 0.150 g of $NaNO_2$; buffered samples contained 1 ml of buffer. [b] pH was measured before heating. [c] Mean from two replicate experiments. Maximum variation of replicates from the mean was approximately ±30%. [d] Buffer prepared by adding 50 ml of 0.1 *M* potassium hydrogen phthalate to 8.7 ml of 0.1 *M* NaOH. [e] Buffer prepared by adding 50 ml of 0.1 *M* potassium dihydrogen phosphate to 25.9 ml of 0.1 *M* sodium hydroxide. [f] Buffer prepared by adding 50 ml of 0.05 *M* sodium bicarbonate to 10.7 ml of 0.1 *M* sodium hydroxide.

aqueous solution or dry under the conditions used. These results suggest that HP would not be a precursor of NPY. *N*-Nitroso-3-hydroxypyrrolidine may have been produced but it was not detected in this study.

Collagen samples were heated in buffer solutions at pH 4.6, 6.2, and 9.0 and dry using temperatures of 120, 145, 170, and 195° for 2 hr. Results are given in Table III, and three observations were noted: (1) only the pH 6.2 samples produced NPY at 120°, (2) small amounts of NPY were produced in all aqueous samples at 145°, and (3) in the temperature range 145–195° there was an increase in the amounts of NPY produced. The dry collagen samples did not produce NPY in the temperature range from 120 to 170° although it was formed at 195°. The results indicate that the pyrrolidine ring can be cleaved from a polypeptide chain and nitrosated in acidic, neutral, and alkaline buffers and dry at elevated temperatures. The identity of NPY in the heated samples was confirmed by comparing the mass spectrum obtained from the heated samples with a standard spectrum of NPY obtained under the same analytical conditions.

The results of this study suggest that collagen could be a precursor of NPY in cooked bacon. Caution must be observed in extrapolating the results of our work directly to cooked bacon because of the differences between the two systems. Nevertheless, PR makes up about 12% of the total amino acid residues in collagen. Raw bacon contains approximately 8% protein, and as much as 25% of the protein could be collagen (Price and Schweigert, 1971). The presence of nitrite, coupled with high cooking temperatures, could favor the formation of NPY during cooking.

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Analytical Studies of Cis–Trans Isomerization of Diethylstilbestrol Monomethyl Ether

Frank E. Gainer

Solutions of the monomethyl ether of diethylstilbestrol (MME) reach a cis–trans equilibrium mixture in Et_2O very rapidly. Chloroform solutions equilibrate less rapidly, in approximately 15 min, as determined by gas–liquid chromatography (glc) and nuclear magnetic resonance (nmr). The equilibrium mixture concentrations are about 27–30% cis and 70–73% trans. Silylation decreases the rate of equilibration in both solvents and alters the equilibrium mixture if MME is silylated before the addition of $CHCl_3$ at room temperature. Increasing temperature tends to increase the rate of equilibration of silylated MME sample solutions. Mass spectral data helped to confirm that the two peaks observed by glc were each due to MME.

The monomethyl ether of diethylstilbestrol (MME) was the subject of much investigation for several years immediately following the discovery of diethylstilbestrol (DES). Geschickter and Byrnes (1942) and Reid and Wilson (1942) reported the results of studies of its estrogenic activity. Gottlieb (1948) and Kelly and James (1952) reported general assay procedures for synthetic estrogens including MME. The synthesis of MME was reported by Dodds *et al.* (1939), Reid and Wilson (1942), Rubin *et al.* (1945), and Wilds and Biggerstaff (1945).

Analytical Development Department—Physical, Eli Lilly and Company, Indianapolis, Indiana 46206.

The synthesis of DES by Dodds *et al.* (1938), like many subsequent syntheses reported by Solmssen (1945), concludes with the demethylation of the dimethyl ether of DES (DME). Rubin *et al.* (1945) showed that MME can be formed under certain conditions during this demethylation step. The "British Pharmacopoeia" (1968) and The Pharmaceutical Society of Great Britain (1969) proposed thin-layer chromatography as a method of detection of MME in DES. More recently, Gainer and Chiasson (1974) reported the determination of MME by a gas–liquid chromatography (glc) assay.

The cis–trans isomerization of other stilbenes has been studied by Gegiou *et al.* (1968) and Saltiel and Megarity (1969). The cis–trans isomerization of DES has been investigated by Derkosch and Friedrich (1953), Rutherford

Table I. Glc Measurement of Cis-Trans Equilibration of Unsilylated MME in Chloroform

Sample 1			Sample 2			Sample 3			Sample 4		
Time, min	% cis	% trans	Time, min	% cis	% trans	Time, min	% cis	% trans	Time, min	% cis	% trans
15	28.8	71.1	0	20.4	79.6	0	19.4	80.6	4	18.9	81.0
30	29.3	70.5	9	25.6	74.4	16	27.2	72.8	14	27.0	71.6
60	28.3	71.6	25	27.7	72.3	24	27.9	72.1	26	29.8	70.2
120	28.3	71.3	60	26.9	72.8	32	27.3	72.5	38	23.8	76.2
			90	27.7	72.3	46	26.7	73.2	62	29.7	70.3
			120	27.1	72.6	68	27.4	72.5	80	29.6	70.5
			8 days	27.2	72.6	101	26.6	73.4			
						126	27.2	72.8			
						5 days	27.4	72.6			

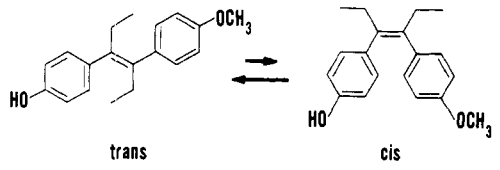

Figure 1. Cis-trans isomerization of MME.

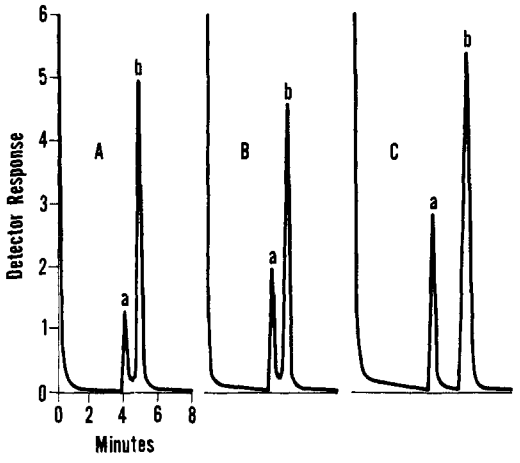

Figure 2. Typical glc chromatograms of silylated and unsilylated cis-trans isomers of MME: (A) unsilylated (not at equilibrium, 19.4% cis, 80.6% trans); (B) unsilylated (at equilibrium, 27.9% cis, 72.1% trans); (C) silylated (at equilibrium, 27.7% cis, 72.1% trans); (a) cis; (b) trans.

(1970), White and Ludwig (1971), and Winkler *et al.* (1971). Some mention of cis-trans isomers of DME has been made by Dodds *et al.* (1939) and Rubin *et al.* (1945), but little, if any, work has been published regarding cis-trans isomers of MME.

The purpose of this paper is to report some basic findings about cis-trans isomerization of MME without regard to type of isomerization. Such information is important, especially for use in interpreting analytical data from quantitative evaluations of MME.

EXPERIMENTAL SECTION

Apparatus. The gas chromatograph used was a Hewlett-Packard Model 402 equipped with a flame ionization detector; range 10; attenuation 128. A 4 ft × 0.25 in. i.d. glass column packed with 3% JXR on 80–100 mesh Gas Chrom Q (Applied Science Laboratories, Inc.) was used. Temperatures used were: column, 200°; injection port, 230°; detector, 230°. The flow rates of gases were: helium (carrier gas), 56 ml/min; hydrogen, 37 ml/min; oxygen, 300 ml/min. A Varian Associates Model A-60 nmr instrument was used.

Reagents and Samples. Solutions of MME, 1 mg/ml, in both $CHCl_3$ and Et_2O with and/or without 100 μl of Regisil, bis(trimethylsilyl)trifluoroacetamide (Regis Chemical Co., Chicago, Ill.), were prepared.

Table II. Nmr Measurement of Cis-Trans Equilibration of Unsilylated MME in Chloroform

Time, min	% cis	% trans
	Sample 1	
2	4.0	96.0
15	19.7	80.3
4 hr	23.0	77.0
20 hr	22.2	77.8
	Sample 2	
10	19.1	80.9
4 hr	22.4	77.6

Procedure. Two microliters of the unsilylated MME sample solutions was injected into the chromatograph immediately after dissolution and labeled as zero time. Additional 2-μl injections were made at various time intervals after zero time. The per cent that each peak (cis and trans) contributed to the total glc peak area was calculated electronically with the aid of an IBM 1800 computer connected to the gas chromatograph.

In order to observe the effect of silylation on cis-trans isomerization, Regisil was added to 1-mg MME samples contained in air-tight vials under two sets of conditions: before the addition of $CHCl_3$ and/or Et_2O, and immediately after the addition of $CHCl_3$ and/or Et_2O. These solutions were then chromatographed similar to the unsilylated sample solutions. Other silylated samples were heated for 15 min at 60° and then chromatographed.

A saturated solution of MME in $CDCl_3$ was prepared and a nuclear magnetic resonance (nmr) spectrum was obtained immediately at zero time with expanded horizontal scale in the 3.6–4.0 ppm region. Additional spectra were obtained on the same solution at various time intervals after zero time. The per cent of each isomer was calculated from measurements of integral height data.

RESULTS AND DISCUSSION

The isomerization of MME in solution to its cis-trans isomers was observed. No work was done to determine directly the extent of isomerization of MME in the solid phase. Isomerization of MME in two solvents, chloroform and diethyl ether, was studied. Results show that MME, in solutions of these two solvents, isomerizes to a relatively constant equilibrium mixture.

Figure 1 shows the proposed cis-trans isomerization of MME in solution with the trans isomer being favored as is the case with DES (White and Ludwig, 1971). All data support this conclusion. Data also support a higher percentage of the trans isomer in the solid state. The first gas chromatogram of MME immediately after dissolution (zero time) always shows a higher per cent trans than cis, and as isomerization proceeds, the per cent trans de-

Table III. Glc Measurements of Cis–Trans Equilibration of Silylated MME in Chloroform[a]

Time, min	% cis	% trans	
		Unsilylated	Silylated
	Sample 1		
0	19.9	37.9	42.1
11	23.0	27.3	49.7
28	23.8	17.8	58.5
60	24.6	7.3	68.1
90	25.0	3.1	71.9
5 days	24.7		75.9
	Sample 2		
0	25.3	45.0	29.6
5 days	27.4		72.6
	Sample 3		
0	29.5	55.9	14.6
6 hr	28.2	3.3	68.4
24 hr	27.8		72.2
	Sample 4		
0	29.6	56.3	13.9
24 hr	27.3		72.5
	Sample 5		
0	21.9	5.3	72.8
10	21.8	4.1	74.1
	Sample 6		
0	26.9	21.8	51.2
8	27.0	19.6	53.4

[a] Samples: 1, Regisil added before MME dissolution in chloroform; 2, 3, MME dissolved in chloroform immediately before addition of Regisil; 4, MME dissolved in chloroform 45 min before addition of Regisil; 5, same as 1 with 15 min of heating at 60° before chromatography; 6, same as 2 with 15 min of heating at 60° before chromatography.

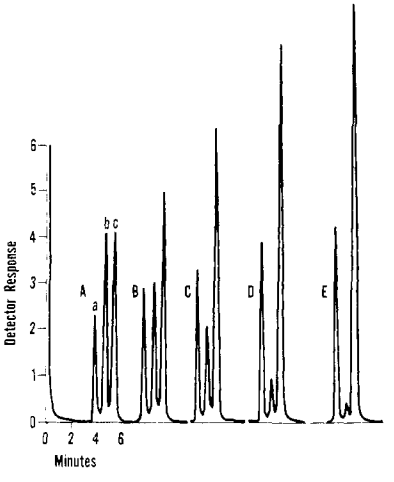

Figure 3. Typical glc chromatograms of silylated MME during cis–trans equilibration: (A) immediately after silylation; (B) 10.8 min after silylation; (C) 28 min after silylation; (D) 60 min after silylation; (E) 90 min after silylation; (a) silylated and/or unsilylated cis; (b) unsilylated trans; (c) silylated trans.

creases and the per cent cis increases until the constant equilibrium mixture is reached. Combined glc–mass spectrometry confirmed that peaks being measured were due to MME.

Isomerization in Chloroform Solution. Isomerization of unsilylated MME in chloroform solution proceeds to a 27–30% cis and 70–73% trans equilibrium mixture and is complete in approximately 15 min at room temperature. The equilibrium values are based on computed areas of the glc peaks corresponding to the cis–trans MME isomers. The cis–trans isomers are not completely resolved unless a silylating agent is used. Chromatograms of the unsilylated cis–trans isomers are seen in Figure 2. A noticeable difference is seen between the peak heights of the cis–trans isomers initially and 24 min later after equilibration is complete. Table I presents equilibration data obtained on different days and different chloroform solutions. The data demonstrate both the rate and extent of equilibration of unsilylated cis–trans MME. Zero time is used only to denote the initial injection immediately after dissolution of MME. Other times refer to the length of time the MME was in solution before the sample was injected. Once the cis–trans equilibrium mixture concentration has been reached, it remains constant as long as outside parameters are not introduced to change the equilibrium. The same equilibrium mixture concentration is reached even if the MME is extracted from DES in alkali solution; Gainer and Chiasson (1974).

Nmr data (Table II) support the glc data regarding the cis–trans isomerization of MME to an equilibrium mixture in chloroform solution. The chemical shift of the methoxy group in the cis isomer of MME is sufficiently different than that of the trans isomer to allow a semiquantitative measurement of the amount of each isomer present. Spectra were run in deuterated chloroform. The methoxy

Table IV. Glc Measurement of Cis–Trans Equilibration of Unsilylated MME in Diethyl Ether

Time, min	% cis	% trans
	Sample 1	
0		
8	28.5	71.5
16	28.3	71.5
24	28.1	71.9
32	28.0	72.1
43	28.5	71.4
60	28.0	72.0
	Sample 2	
0	29.4	70.6
10	29.7	70.3
20	30.1	69.9
30	29.8	70.2
50	30.3	69.7
60	30.4	69.6
18 hr	29.5	70.5

Table V. Glc Measurement of Cis–Trans Equilibration of Silylated MME in Diethyl Ether[a]

Time, min	% cis	% trans	
		Unsilylated	Silylated
	Sample 1		
0	28.8	11.7	59.2
8	28.9	9.3	61.6
18	26.5	7.9	65.1
26	28.6	5.8	65.4
34	28.6	4.4	66.8
50	28.5	2.3	68.9
70	28.6	1.2	70.1
100	27.5		72.4
	Sample 2		
0	1.0		98
	Sample 3		
0	0.9		98.5

[a] Samples: 1, MME dissolved in ether before addition of Regisil; 2, same as 1 with 15 min of heating at 60° before chromatography; 3, Regisil added before MME dissolution in ether followed by 15 min of heating at 60° before chromatography.

protons of the cis isomer appear at δ 3.67, while the methoxy protons of the trans isomer appear at δ 3.8. The integral of each peak was taken as a measure of the amount present when compared with the total integral of the two peaks.

The reaction of Regisil with MME does not stabilize the cis–trans isomers of MME in their respective forms. This response is unlike DES for which the isomers are effectively locked in their respective forms by the formation of trimethylsilyl derivatives prior to dilution with chloroform as reported by Rutherford (1970). Instead the silylation of MME with Regisil changes the rate of cis–trans equilibration as well as the per cent of each isomer.

The per cent of each isomer present in the mixture depends on whether the Regisil is allowed to come in contact with MME before or after dissolution of MME in chloroform. The equilibrium mixture concentrations remain the same as unsilylated MME if the MME is dissolved in chloroform before the addition of Regisil, but the per cent trans isomer increases if MME is dissolved in Regisil before the addition of chloroform. Regardless of the order of addition of Regisil, the rate of cis–trans equilibration decreases (Table III) due to the apparent slow silylation of MME. The chromatograms shown in Figure 3 support this statement.

The retention time of silylated *cis*-MME is the same as that of unsilylated *cis*-MME, but the retention time of *trans*-MME increases after silylation (Figures 2 and 3). The middle peak in chromatograms of silylated sample solutions is due to *trans*-MME and persists for several hours, decreasing as the peak for silylated *trans*-MME increases.

The application of heat to silylated sample solutions increases the rate of equilibration, although not dramatically (Table III). The addition of Regisil to a chloroform solution of MME already at cis–trans equilibrium offsets the equilibrium, and the equilibration process starts again just as with any other silylated sample (Table III).

Isomerization in Diethyl Ether Solution. Isomerization of MME in ether solution also proceeds to a 27–30% cis and 70–73% trans equilibrium mixture in accordance with equilibrium in chloroform solution. The equilibrium mixture concentrations were computed from glc chromatograms. Equilibrium is reached more rapidly in ether than in chloroform. Equilibration of unsilylated MME is complete almost immediately in ether (Table IV). The silylated MME samples reach equilibrium in less than 100 min (Table V). Data in Table V also show that heat has a greater influence on the cis–trans equilibration rate of MME in ether than in chloroform. The significant observation is the fact that the equilibrium concentration was pushed to nearly 100% trans by the use of Regisil and heat.

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