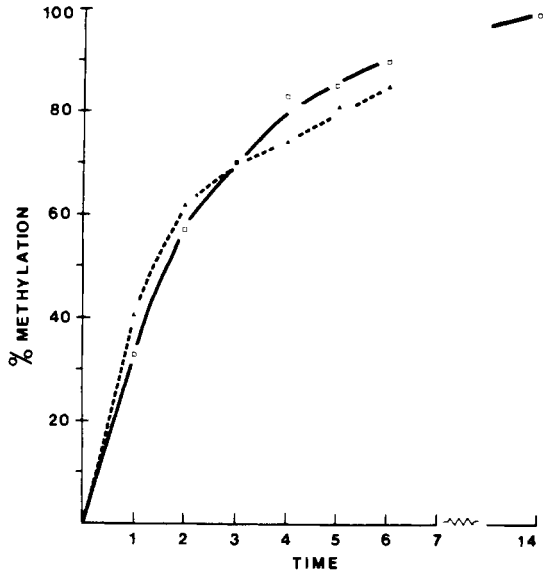

Figure 1. The percentage methylation of 2 mg of dichlorfop acid in 1 ml of methanol with time at 60 °C (▲- - -▲, abscissa in hours) and 25 °C (□- - -□, abscissa in days).

fragments with masses of 282 and 253, each containing 2 chlorine atoms, were also observed. The mass spectra of the parent dichlorfop-methyl and the remethylated acid were identical and showed a molecular ion weight of 340 (2 chlorine atoms) with ion fragments of masses of 282 and 253, both of which contained 2 chlorine atoms. No ion mass weights of 326 were observed. From these data the hydrolysis product was characterized as the acid (I, R = H).

From experiments in which a methanolic solution of dichlorfop acid had been used, it became apparent that with time there was a decrease in the acid concentration which was accompanied by formation of dichlorfop-methyl, the latter being identified by gas chromatographic and mass spectral analysis. This observation prompted a more detailed study of the fate of dichlorfop acid in methanolic solution.

The average results from duplicate experiments on the esterification of dichlorfop acid in methanol at two temperatures with time are summarized in Figure 1, from which it is seen that at 60 °C, methylation is rapid and 85% complete in 6 h. Reaction was complete within 24 h. At 25 °C, esterification is slower with approximately 30% occurring over a 24-h period. After 6 days at 25 °C, about 90% of the acid had undergone methylation, while methylation was complete within 14 days.

Thus, it was confirmed that dichlorfop acid in methanol can undergo complete methylation at room temperature. This is anomalous as the esterification of organic acids by alcohols at room temperature is generally insignificant and even at high temperatures does not normally go to completion but rather reaches an equilibrium. Horner et al. (1974) have reported that the herbicide 2,4-D (2,4-dichlorophenoxyacetic acid) on being heated under reflux with methanol (1.7 g in 10 ml) reached an equilibrium with the methyl ester when 52% esterification had occurred.

Separate experiments showed that the dichlorfop acid did not undergo any detectable esterification at room temperature in ethanol or 1-butanol over a 14-day period, indicating that the ethyl and *n*-butyl esters are either not formed under these conditions or are formed much more slowly than the methyl ester.

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Mercury in Chicken Eggs

Methylmercuric chloride (MeHgCl) and ethylmercuric chloride (EtHgCl) in approximately equivalent concentrations were isolated from the alkaline hydrolysates of chicken eggs purchased on the retail market. The mercury (Hg) from the two alkylmercuric compounds in 19 samples collected during 1973–1975 averaged 0.04 ppm. The range was 0.02 to 0.10 ppm. Comparative assays with an atomic absorption spectrophotometric method (AA) for six samples indicated that a large proportion of Hg was present as these two alkylmercuric compounds. Data are reported on the increase from 0.02 to 10.0 ppm of EtHg compounds, with no increase in MeHg compounds in eggs laid over a 32-day period by one hen fed approximately 1 mg of Hg per day as a grain coated with Ceresan M (ethylmercury *p*-toluenesulfonanilide).

The chemical form of mercury (Hg) determines its biological effect or toxicity. Elemental Hg is the least toxic, and alkylmercuric compounds are the most toxic. Other chemical forms of Hg in compounds with fungicidal properties, the inorganic Hg^{2+}, alkoxyalkylmercuric, and arylmercuric compounds, have toxicities between these two extremes. The low level of Hg in eggs has made it difficult to determine its chemical form. The 33 egg samples included in the Food and Drug Administration survey of the mercury content of food in 1970, 1971, and 1972 (Simpson, 1974) contained <0.002 to 0.005 ppm of Hg. Except in special circumstances, most of the Hg in foods other than

Table I. Alkylmercuric Compounds in Poultry Eggs (ppm)

Date sample collected	No. of samples	MeHg compds		EtHg compds		Total Hg as alkylmercuric compds	
		Range	Av	Range	Av	Range	Av
1973	5	<0.01-0.03	0.01	0.01-0.10	0.04	<0.02-0.10	0.05
1974	8	0.01-0.04	0.02	0.01-0.03	0.02	0.04-0.05	0.04
1975	6	0.01-0.02	0.01	0.02-0.03	0.02	0.02-0.05	0.03

fish is believed to be in the form of inorganic Hg^{2+} (Lu, 1974) or in the form of MeHg compounds derived, presumably, from residues in feeds containing fishmeal or treated cereal grains (*WHO Tech. Rep. Ser.*, 1972). The bulk of the Hg in fish flesh has been isolated as the methylmercuric ion ($MeHg^+$) and identified as methylmercuric chloride (MeHgCl) (Westoo, 1966; Johansson et al., 1970).

This communication summarizes some studies with eggs indicating the occurrence of ethylmercuric (EtHg) compounds and methylmercuric (MeHg) compounds. When MeHgCl is isolated from the alkaline hydrolysate of eggs by the method developed for the assay of MeHg compounds in fish (Schafer et al., 1975), a second compound with a retention time identical with EtHgCl is observed. The ratio of the two alkylmercuric chlorides is about 1:1. We reported the presence of a compound with a retention time similar to EtHgCl in the extracts from fish alkaline hydrolysates and suggested that it might account for the difference between the assays for total Hg and MeHg compounds in some fish samples. Information is reported here on (1) the concentration of Hg as the two alkylmercuric compounds in 19 samples of eggs from the retail market, (2) a comparison of assays for Hg by AA and as the sums of the GLC assays for MeHgCl and EtHgCl in six egg samples, and (3) the accumulation of EtHg compounds with no increase in MeHg compounds in hen eggs during the oral ingestion of a fungicide containing an EtHg functional group.

EXPERIMENTAL SECTION

Samples. Each sample, 12 eggs, was purchased in the local market: 5 samples in 1973, 8 in 1974, and 6 in 1975. They included eggs from the farm used for the in vivo study described below and those packed in cartons labeled with names of distributors located in Ohio, Minnesota, and Georgia.

Sample Preparation. Equal weights of water and whole eggs were stirred by hand until homogeneous. Care was taken to prevent denaturation. The homogenate was strained through three thicknesses of cheesecloth. The viscous albumin was removed by straining and discarded. That portion of the sample intended for assays by AA was collected in a glass-stoppered reagent bottle and stored at 5 °C until assayed. For the GLC assays, 18-g portions of the homogenate were collected in tared 150-ml beakers. 2-Propanol (15 ml) was added, and the beakers were covered with Parafilm and stored at −20 °C until assayed. Each sample for the in vivo study, 1 egg, was prepared for assay as described above.

Methodology for GLC Assays. The alkylmercuric compounds were extracted from alkaline hydrolysates and assayed using a modification of the procedure developed for the estimation of MeHg compounds in fish (Schafer et al., 1975) that increased the sensitivity from 0.5 to 0.05 ppm. The modification, an increase in the fractional volume of the initial benzene extract used for the remainder of the cleanup, made the method applicable for the assay of alkylmercuric compounds in eggs. The EtHgCl, purchased from Alfa Products, Ventron Co., was used as received. For recovery estimates, the samples were fortified before alkaline hydrolysis with 2 to 8 μl of benzene solutions of EtHgCl (0.50 to 1.00 mg/ml). Calibration standards were prepared to contain similar amounts of MeHgCl and EtHgCl, ranging from 0.02 to 0.2 ng/2 μl, in benzene. Peak heights were used for estimations of each compound.

Methodology for AA Assays. Digestion techniques of Munns and Holland (1971) and Hoover et al. (1971) were used to optimize conditions for the assay of 0.05-ppm levels of total Hg in the whole egg. Concentrated HCl solutions of pure EtHgCl were added before digestion for the recovery estimates.

In Vivo Study of the Increase of $EtHg^+$ in Eggs from Oral Ingestion of Grain Coated with Ceresan M. Ceresan M (product of DuPont de Nemours and Company) labeled 7.7% ethylmercury *p*-toluenesulfonanilide (3.2% total Hg as metallic) and 92.3% inert material was added as a water slurry to cracked corn to prepare a fungicide-treated corn containing 19.8 ppm of Hg (AA assay of final product). One chicken was given approximately 50 g of this treated corn (990 μg of Hg) per day, plus mash containing <0.001 ppm of Hg and water ad libitum for 32 consecutive days. The placement of the Hg-treated grain on top of the mash was sufficient inducement for its consumption. Individual eggs laid by the chicken were assayed for the alkylmercuric compounds and total Hg. The chicken was kept isolated on a chicken farm for this study. Eggs laid by chickens fed mash and water ad libitum were used as controls.

RESULTS AND DISCUSSION

During 1973–1975, 19 egg samples from the retail market were assayed for alkylmercuric compounds. The arithmetic averages of MeHg compounds, EtHg compounds, and total Hg as the sum of the two alkylmercuric compounds are given in Table I. Two of the five samples assayed in 1973 exceeded 0.05 ppm of Hg; one contained 0.07 ppm, and the other, 0.10 ppm. At the 0.1-ppm level, the precision and recovery estimates for EtHgCl added to eggs were similar to those for MeHgCl added to fish (see Schafer et al., 1975). The uncertainty for assays at the 0.02-ppm level was probably ±0.01 ppm. Reagent blanks prepared by substituting 18 g of water for 18 g of egg homogenate in step 1 of the isolation procedure contained no detectable compounds with retention times similar to EtHgCl and MeHgCl and provided evidence that these compounds were not contaminants in the reagents, including the $HgCl_2$ used in step 3. They were associated only with egg samples. When the alkaline hydrolysates of the egg samples were acidified in step 3 before the addition of $HgCl_2$, no peak with a retention time of EtHgCl was observed. A typical chromatogram of an extract from the alkaline hydrolysate of one sample from the retail market containing 0.02 ppm of MeHgCl and 0.04 ppm of EtHgCl (0.04 ppm of Hg) is shown in Figure 1. The overall average for the 3-year period was 0.04 ppm of Hg, or approximately 0.002 mg per egg. The suggested practical residue limit for Hg in foods other than fish is 0.05 ppm (Food and Agriculture Organization, 1968). The provisional tolerable weekly intake for man is 0.2 mg per person for MeHg (expressed as Hg) *WHO Tech. Rep. Ser.*,

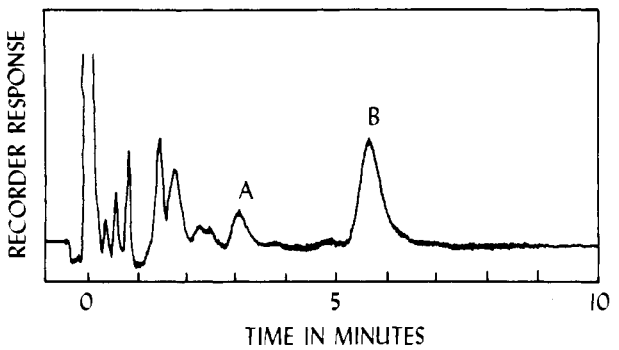

Figure 1. Typical chromatogram of an extract from the alkaline hydrolysate of a whole egg sample purchased on the retail market: (A) MeHgCl at 0.02-ppm level; (B) EtHgCl at 0.04-ppm level; injection volume, 2 μl; column, 3.5 mm i.d. × 210 cm glass packed with 5% phenyl diethanolaminesuccinate on 60/80 mesh Chromosorb W (HP); N_2 flow rate, 80 ml/min; column, 150 °C; injector, 205 °C, ^{63}Ni ECD, 270 °C.

Table II. Comparison of Assays for Total Mercury in Eggs (ppm)

Sample no.	GLC data: Hg as MeHgCl	GLC data: Hg as EtHgCl	GLC data: Total Hg from alkylmercuric compds	AA data, total Hg
1	0.02	0.03	0.05	0.06
2	0.01	0.01	0.02	0.03
3	0.01	0.02	0.03	0.04
4	0.01	0.02	0.03	0.03
5	0.01	0.03	0.04	0.02
6	0.01	0.03	0.04	0.03

1972), or about 100 times greater than the average concentration found in each egg.

The six samples collected in 1975 were used for the comparison of assays for total Hg by AA with the Hg from the GLC assays for the two alkylmercuric compounds. The data for each sample are shown in Table II. The recovery estimates for the AA method ranged from 96 to 103 on five replicates. For 16 replicates of egg sample no. 5, 8 g each, the arithmetic average was 0.021, and the standard deviation was 0.005. Egg sample no. 1 contained the highest concentration of Hg with both assay procedures. The average total Hg for the 6 egg samples was 0.03 ppm for each assay procedure. Thus, the comparative assays indicate that a large proportion of the Hg was present in these six egg samples as the two alkylmercuric compounds.

The oral consumption of 30 mg of Hg as a Ceresan-M coated grain over a 32-day period had no adverse effect on the appearance of the hen, feed consumption, or egg production. The chicken laid 3 eggs during the first 6 days and 7 eggs between days 7 and 32. One egg laid on the 18th day had a hole in one end and was not assayed. The amount of Hg in the individual eggs laid by the chicken is shown in Table III. All of the control eggs and the eggs laid within the first 6 days by the hen receiving Hg-treated grain contained <0.01 ppm of MeHgCl and 0.02 ppm of EtHgCl. There was no detectable increase in the MeHg compounds in eggs laid by the hen fed the treated grain. The first 3 min of the chromatograms from the assay of these eggs resembled Figure 1. The peak with a retention time for MeHgCl was smaller. Estimates of the concentration of EtHgCl in these samples were made from suitable dilutions of the final benzene extracts until they contained 0.02 to 0.2 ng of EtHgCl/2 μl. The concentration of EtHg compounds in the egg laid on the 7th day was 35 times greater than that in the control eggs, but it accounted for less than 1% of the Hg that had been consumed. On the 32nd day the concentration of EtHg compounds was 500 times greater than that in control eggs. The egg laid on the 32nd day contained twice as much total Hg (assay by AA) as that accounted for as the two alkylmercuric compounds. About 10% of the total Hg ingested by the hen in the 32-day period was found in all the eggs laid by the hen during this time period.

No information is available on the source of the alkylmercuric compounds reported here in eggs purchased on the retail market. The 8 control eggs collected from the farm during the in vivo study contained the lowest concentration of alkylmercuric compounds observed in eggs to date. There is no known basis at this time for attributing the levels of the alkylmercuric compounds in these control eggs to background contamination in the isolation procedure since the chromatograms from the reagent blanks (prepared as described above) were at the base line at the retention times characteristic of these two compounds. The similarity in the assay results for EtHgCl from the 8 control samples, an arithmetic average of 0.0232 and a standard deviation of 0.00129, indicates this could be a background contamination characteristic of eggs from this farm at this time. Tables I and II are uncorrected data. Insufficient evidence was available to justify the correction of these for the 82.8% recovery observed when eggs were fortified with EtHgCl at the 0.1-ppm level, or for the lowest concentration observed to date in eggs as discussed above.

Possible explanations for the higher concentration of Hg residues in eggs reported in this study (see Table I) as compared with those reported by Simpson et al. (1974) include the geographical location for the source of the eggs, procedures for sample preparation, and methodology. Both residue levels are well below acceptable tolerance levels. The discrepancy between the two levels has no bearing on the studies reported here on the chemical form of mercury in eggs laid by a chicken fed grain coated with an EtHg compound.

Table III. Effect of Oral Consumption of Ceresan-M Coated Grain on Concentration of EtHg Compounds in Eggs Laid by the Sample Chicken

No. of days chicken was on diet when egg was laid	Total Hg consumed, mg	GLC assays: Hg present as EtHg compounds, ppm	GLC assays: Total Hg per egg, mg	AA assays: Total Hg, ppm	AA assays: Total Hg per egg, mg
0-6	0-5.9	0.02	0.001[a]	0.01	0.0006
7	6.9	0.7	0.04		
9	9.9	2.0	0.12	2.5	0.15
12	11.9	4.9	0.29	6.4	0.30
15	14.8	7.5	0.45	10.0	0.60
21	20.8	6.3	0.38		
32	31.7	10.0	0.60	20.0	1.20

[a] The assay for Hg from MeHg compounds, < 0.01 ppm.

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An Automated Fluorometric Method for the Measurement of Tryptophan in Plasma

The fluorometric analysis of tryptophan described by Hess and Udenfriend has been automated to facilitate the estimation of tryptophan in plasma. Details of manifold construction and reagent composition are presented along with data concerning the reliability of the method.

A variety of procedures (Hier and Bergeim, 1946; Spies and Chambers, 1948; Scott, 1961; Wapnir and Bessman, 1965) are available for the determination of tryptophan in biological materials, but the fluorometric method of Hess and Udenfriend (1959) has been found particularly suitable for blood plasma (Young et al., 1971; Tontisirin et al., 1973; Lewis and Speer, 1974). The method is based on the conversion of tryptophan to the fluorophore, norharman, in a two-step reaction: (a) the cyclization of tryptophan to a tetrahydronorharman derivative and (b) the subsequent oxidation to norharman. Norharman is then determined fluorometrically by activation at 365 nm and measurement at 440 nm. Modifications of the original method have been published by Denckla and Dewey (1967) and Peters et al. (1969). The various procedures are sensitive and specific, but are not convenient for the analysis of large numbers of samples. The purpose of this paper is to describe an automated procedure, based on the original method by Hess and Udenfriend (1959), capable of analyzing 30 samples per hour. In adapting the method for autoanalysis, only two basic changes were made from the original method: the concentration of formaldehyde was reduced from 18 to 15%, and the concentration of peroxide was increased from 5 to 30%.

MATERIALS AND METHODS

Reagents used included: hydrogen peroxide, 30% w/v; formaldehyde, 15% w/v; sulfuric acid, 0.2 N; wash solution, 0.5 ml of Brij-35 solution (30%) per liter of water containing 3.2% w/v trichloroacetic acid (Cl_3CCOOH). Standard solutions were prepared daily by combining 0.5, 1.0, 1.5, 2.0, and 2.5 ml of a stock solution of 2 mg/100 ml tryptophan with 4 ml of 8% w/v Cl_3CCOOH and diluting to 10 ml. When samples are prepared as described in the next section, the values of the standards correspond to 0.5, 1.0, 1.5, 2.0, and 2.5 mg of tryptophan per 100 ml of plasma. Cl_3CCOOH is added to the standards and the wash solution to provide a background similar to the samples.

Sample Preparation. Blood is withdrawn with heparinized syringes and centrifuged immediately and the plasma separated. Samples are prepared by combining 1 ml of plasma with 4 ml of 4% w/v Cl_3CCOOH and centrifuging at 19 000*g* for 15 min. The supernatant liquid is decanted and analyzed without further dilution. The deproteinizing agent, sulfosalicylic acid, produced a high fluorescence that interfered with the analysis.

Automated Assay. The flow diagram for the automated method is shown in Figure 1. The system was assembled with the following components: one Sampler II, one Proportioning Pump I, and one 95 °C Heating Bath (Technicon Instruments Corp., Tarrytown, N.Y.), one Model III Fluorometer (G. K. Turner Associates, Palo Alto, Calif.) fitted with a Corning No. 7-60 excitation filter and a Wratten No. 3 emission filter and with sensitivity set at 3×, and one vom 5 Recorder (Bausch and Lomb Inc., Rochester, N.Y.). Pump tubing and glass fittings are Technicon designations.

Deproteinized plasma solution was aspirated at the rate of 30 samples per hour using a wash to sample ratio of 2 to 1. A water wash cup was placed behind the highest standard and samples were then run consecutively without alternating wash cups. The heating bath was equipped with two 12.2-m coils. Each sample remained in the heating bath for approximately 7.5 min on the first pass and 7.0 min on the second pass. The tubing between the fluorometer debubbler and flowcell was kept short to minimize the possibility of bubble formation in this region.

RESULTS AND DISCUSSION

The effect of varying hydrogen peroxide and formaldehyde concentrations on fluorescence of tryptophan standards was investigated (Table I). Linear response to the higher concentrations of tryptophan was obtained only if 30% hydrogen peroxide was used. The highest con-