PCB Abbot 5 Proc. Society Experimental Biology & Medicine 121 1966 Accepted SEP 25 1979 RBS

and 1.8 mEq Ca^{++}/l accumulated K^+ when reimmersed in a solution containing 106 mEq Na^+, 10 mEq K^+ and 1.8 mEq Ca^{++}/l. When Na^+ was replaced by Li^+ in the second immersion fluid, K^+ accumulation was inhibited. Removal of Ca^{++} from the Li^+ Ringer used for the second immersion did not significantly increase the uptake of K^+ by the fibers. Muscles kept in Ca^{++} free Li^+ Ringer during both immersions had a significantly lower K^+ content than muscles immersed throughout in Li^+ Ringer containing 1.8 mM Ca^{++}. Increasing the Ca^{++} content of the fluid used for the second or for both immersions had no significant effect on the final K^+ content of muscles kept throughout the experiment in media containing normal concentrations of Na^+. It is concluded that the inhibition of K^+ uptake by frog stomach muscle observed when Na^+ is replaced by Li^+ in the second immersion medium does not arise from metabolic disturbances caused by an increased entry of Ca^{++} into the fibers.

1. Armstrong, W. McD., Am. J. Physiol., 1965, v208, 61.
2. Armstrong, W. McD., Fariduddin, K. M., 23rd Intern. Congr. of Physiol. Sciences (abst.), 1965, 88.
3. Judah, J. D., Ahmed, K., Biochem. Biophys. Acta, 1963, v71, 34.
4. ———, Biol. Revs., 1964, v39, 160.
5. Cosmos, E., Harris, E. J., J. Gen. Physiol., 1961, v44, 1121.
6. Skou, J. C., Physiol. Revs., 1965, v45, 596.
7. Burnstock, G., Straub, R. W., J. Physiol., 1958, v140, 156.
8. Adrian, R. H., ibid., 1961, v156, 623.

Received January 12, 1966. P.S.E.B.M., 1966, v121.

Chick Edema Factor: Some Tissue Distribution Data and Toxicologic Effects in the Rat and Chick.* (31029)

T. C. CAMPBELL† AND L. FRIEDMAN
Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge

The chick edema factor (CEF), responsible for a large number of deaths in the broiler industry in the fall of 1957, was traced to the unsaponifiable matter (unsap) of the fat used in the broiler rations. It since has been crystallized(1,2,3) and its structure proposed as that of a hexachlorohexahydrophenathrene(2). It is known that in the toxic fat, a mixture of related compounds can be found, some toxic and some relatively nontoxic.

To lay the groundwork for a study of the specific physiological effects of pure CEF compounds, a few short studies have been completed to learn the distribution of the toxic material in the body and which organs were primarily affected.

Experimental. Adult rats and day-old White-Rock chicks were used. Because the pure material was not available in sufficient quantity, we used, from the toxic fat, the unsap which represented 38% of the original toxic fat and was estimated to contain at least 10 ppm CEF. The unsap was force-fed because the animals' food intake was drastically curtailed when it was mixed in the diet. All animals were offered water and commercial feed *ad libitum*.

Table I shows the experimental plans and dosage levels employed for all 3 Trials. Feed consumption and fecal and urinary excretion were measured for the rats. Body weights were recorded in all experiments. All animals, upon sacrifice, were examined grossly for pathology and selected organs were weighed and frozen. Hydropericardial fluid (HPF) volume was measured in the chicks.

In addition to the examination for gross effects, the presence of CEF material in various tissue was determined. Adrenals, kidneys, and livers were assayed in the rats; livers only

* Supported by USPHS Grant EF 00305. Contribution 858 from Dept. of Nutrition and Food Science, Massachusetts Inst. of Technology.

† Present address: Dept. of Biochemistry and Nutrition, Virginia Polytechnic Inst., Blacksburg.

DSW 255066

STLCOPCB4060757

TABLE I. Experimental Plan of Trials.

Trial	Species	No. of animals	Group	Dosage (per kg) Unsap (ml/day)	CEF (estimated) (μg/day)
I (14 days)	Rat	2	High	2.0	18
		2	Low	1.0	9
II (6 days)	Rat	4	High	2.0	18
		4	Low	1.0	9
		4	Control*	0	0
III (6 days)	Chick	6	High	5.4	48
		6	Low	1.1	10
		6	Control*	0	0

* Control animals were not intubated.

have been analyzed in the chicks. To obtain a picture of the amount of material being absorbed and perhaps excreted, analyses of the feces and urine of rats and of the combined chick excreta were made.

The assay method for CEF is being reported in detail elsewhere.‡ In short, the sample is homogenized with water and saponified with alcoholic KOH. The unsaponifiable portion is extracted with petroleum ether and chromatographed first on an alumina column. The eluate obtained with 25% ethyl ether in petroleum ether, following prior elution with petroleum ether and 5% ethyl ether in petroleum ether, is concentrated and chromatographed on 500 μ thin layer silica gel plates with 3% ethyl ether in petroleum ether. The silica gel in the area of R_f 0.80-1.00 is removed and eluted with ethyl ether, the solvent removed, and the residue is redissolved in isooctane for gas chromatography. We used an F & M Model 400 gas chromatograph with an electron capture detector and a U-tube column, 3 ft × 6 mm (o.d.) × 4 mm (i.d.), packed with 1% SE-30 on ANAKROM ABS (Analabs). The operating conditions were: temperatures, column oven 180°C, EC detector 200°C, flash heater 240°C; gas flows, helium carrier gas, 60 cc/min; argon, 10% methane purge gas 180 cc/min. The limit of detection by this method is approximately 0.2 ppb, assuming a sample size of 5 g and a sensitivity of the EC detector of 5×10^{-11} g.

The so-called CEF components can be seen as a gas chromatographic pattern of peaks shown in Fig. 1. This pattern is that of a highly concentrated material isolated from the toxic unsap. It is similar to a material isolated by Yartzoff *et al* and kindly supplied to us by Firestone(3). We have numbered the peaks 1 through 8 as shown. Peak 4a was not seen in the Firestone preparation.§

Results and discussion. Table II shows the effects upon body weights, feed conversion, and feed consumption for the rats. The results for the vital organ weights and HPF volume are shown in Table III. There was no apparent gross pathology in either species with the exception of HPF and some ascites and subcutaneous edema in the chicks.

TABLE II. Gross Effects in Rats.

Trial	Group	% Body wt change	Feed intake depression* (%)	Dry matter dig.coeff. (%)
I (14 days)	High	—15	—	76
	Low	— 8.5	—	78
II (6 days)	High	— 6.6	—38	72
	Low	— 3.7	—29	75
	Control	— 1.0	—	77

* Depression below control animals.

While only the chicks develop hydropericardium, apparently both are equally sensitive to liver weight increase, as shown in Table III. In the case where each species was maintained on CEF for 6 days, (Trials II and III), the rats (9 μg/kg/day) showed an increase over controls of 21% while the chicks (10 μg/kg/day) showed an increase

‡ T. C. Campbell and L. Friedman, in press, J. AOAC, "Chemical Assay and Isolation of Chick Edema Factor."

§ The chromatogram for the Firestone preparation is presented elsewhere.

DSW 255067

STLCOPCB4060758

TABLE III. Organ Weights Expressed as % of Body Weights.

Trial	Group	Heart	Liver	Spleen	Kidney	Adrenals
II	High	.34	4.08‡	.20	.90	.21†
Rats	Low	.32	4.08‡	.20	.82	.18
	Control	.31	3.36	.19	.76	.14
					HPF (ml)*	
III	High	.86	3.85†	.61	.65†	
Chicks	Low	.70	3.15	.54	.12‡	
	Control	.78	2.73	.65	.04	

* HPF—hydropericardial fluid (ml).
† Significant statistically at 1% probability level.
‡ At 5% probability level (Hogben L test)(5).

of 15%. (The increase in liver weight in the chicks was not due to moisture or fat.)

Also, in Table III, neither heart nor spleen weights in either species are significantly affected. In rats, there appears to be a slight increase in kidney weights, though not statistically significant, and a highly significant increase of 50% in adrenal weights for animals on the high level. Whether this adrenal weight increase is simply a non-specific stress effect from intubation is not known, although it would seem that this cannot be entirely responsible, since the high level group showed an increase of nearly twice that of the low level group.

Some other observations which have been

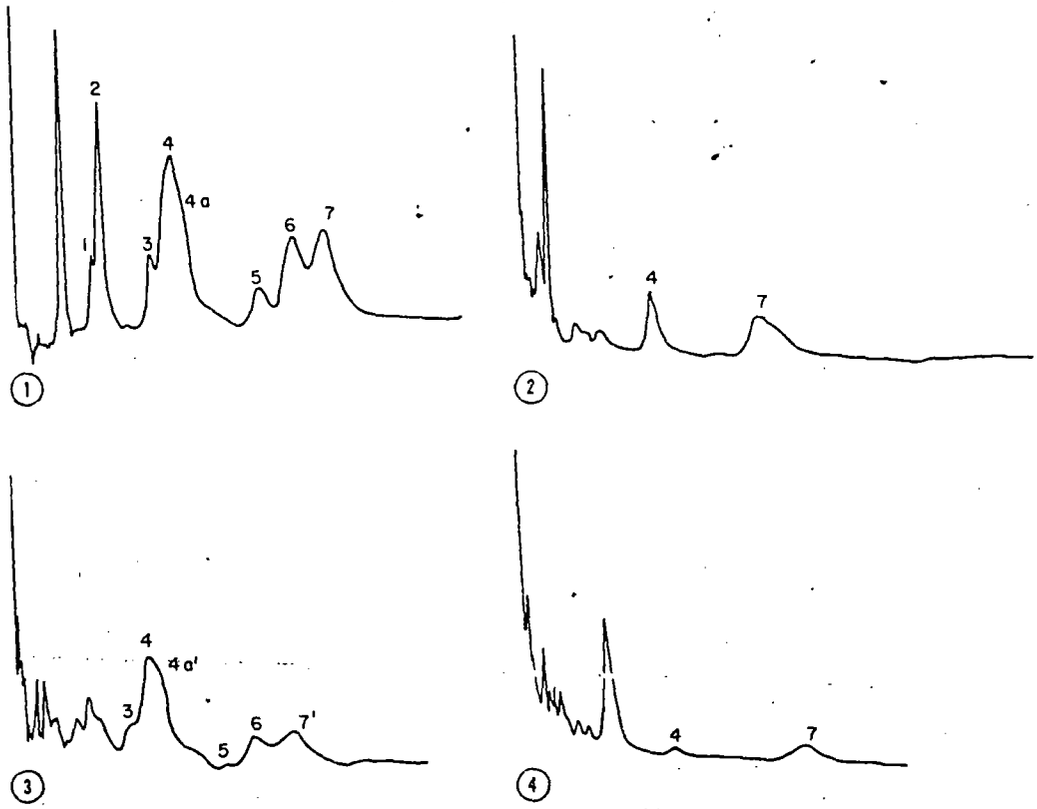


FIG. 1. Toxic CEF components found in unsap used in this study.
FIG. 2. Rat liver extract showing 2 CEF peaks.
FIG. 3. Rat feces extract showing CEF peaks, with altered Nos. 4a and 7.
FIG. 4. Chick liver extract showing 2 CEF peaks. (Large peak just before No. 4 found to be contaminant leached from liner of sample vial.)

DSW 255068

STLCOPCB4060759

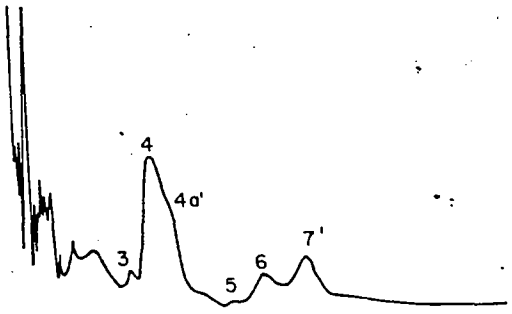


FIG. 5. Chick feces extract showing CEF peaks, with altered Nos. 4a and 7.

made on previously studied birds in this laboratory are of interest. Hematocrit values are depressed. Of a total of 43 birds, we have observed that, with an average of 0.08 ml HPF in control birds (considered normal), there was a packed cell volume of 33.0%, while for diseased birds having 0.73 ml HPF, the packed cell volume was 27.9%.

Also, we observed that whereas control birds will show disappearance of a given amount of an I.V. injected dose of T-1824 dye (Evans Blue) from their vascular system of approximately 1%/min, poisoned birds will show a disappearance of approximately 2%/min, supporting the observation of Allen (4), that the permeability of the vascular wall appears to be increased.

Distribution of the CEF in animal body. Of the rat tissues and samples examined, CEF was detected only in the liver and feces. Fig. 2 through 5 show typical chromatograms of purified extracts of feces and liver of both species. Control animals not receiving CEF did not show these peaks. These chromatograms show that only peaks 4 (or 4a?) and 7 were present in the liver. In the fecal extracts all peaks were found, with the exception that instead of peaks 4a and 7 showing their original retention times, these were slightly increased in each case and have been designated 4a′ and 7′.

These retention time increases in the fecal components were measured by noting their retention times in relation to their neighbors, as shown in Table IV. Whether the 2 components of the liver are the products of components 4 and 7 or the original unaltered substances cannot be accurately determined from these chromatograms, since the rest of the CEF chromatographic pattern is missing. It may be concluded, however, that there is a selective absorption of peaks 4 (or 4a) and 7, with metabolism by the liver and excretion into the intestine.

It appears, on the basis of the tissues analyzed, that the liver is the target organ. Furthermore, the pattern of CEF chromatographic peaks presented here, Nos. 4 and 7 are the key components. We have succeeded in partially separating the CEF components such that 4a and 7 crystallized together, indicating a certain chemical as well as physiological similarity.‡ There is no way of differentiating 4 from 4a with regard to its absorption and metabolism on the basis of the available evidence.

Summary. Unsaponifiable matter isolated from a toxic fat and containing an estimated 10 ppm chick edema factor (CEF) was force-fed daily to adult rats at levels of 2.0 cc and 1.0 cc/kg body weight/day in 2 studies of 14 and 6 days, respectively. Feed consumption, body weight, and digestibility were depressed. Heart and spleen weights were unaffected, kidney weights seemed to be slightly increased, and adrenal and liver weights were significantly increased. In the chick, typical hydropericardium, ascites, and subcutaneous edema were observed. There were no significant changes in heart or spleen weights. Liver weights were significantly increased. The rat was as sensitive as the chick to CEF according to increase in liver weight. Of the 8 or 9 CEF components shown to be in this unsap, 2 (Nos. 4 and 7) were found to be absorbed and located in the liver, while the other com-

TABLE IV. Changes in Chromatogram Retention Times of CEF Peak Nos. 4a and 7.

Material analyzed	No. of runs	Relative retention times $R_{7:6}$	$R_{4a:3}$
Firestone Standard	31	1.10 ±.01‡	4a not found
Unsap*	2	1.11 ±.01	1.22 ±.01
Feces†	13	1.14 ±.03	1.27 ±.04

* Unsaponifiables administered to animals in this study.

† Includes feces of 11 individual rats and the composite feces of 2 groups of 6 chicks each.

‡ The deviation includes the total range of values.

DSW 255069

STLCOPCB4060760

ponents were not detected. In place of Nos. 4 and 7, there were 2 new peaks in the feces with slightly increased retention times. This suggests that the 2 active CEF components are metabolized in the liver and excreted into the intestine *via* the bile, both in the chick and the rat. No CEF-like material was found in kidneys, adrenals, or urine.

1. Harman, R. E., Davis, G. E., Ott, W. H., Brink, N. G., Kuehl, F. A., J. Am. Oil Chem. Soc., 1960, v82, 2078.

2. Wootton, J. C., Courchene, W. L., Jr. Agric. Food Chem., 1964, v12, 94.

3. Yartzoff, A., Firestone, D., Banes, D., Horwitz, W., Friedman, L., Nesheim, S., J. Am. Oil Chem. Soc., 1961, v38, 60.

4. Allen, J. R., Ph. D. Thesis, 1961, Univ. of Wisconsin.

5. Hogben, C. A. M., J. Lab. Clin. Med., 1964, v64, 815.

Received January 13, 1966. P.S.E.B.M., 1966, v121.

Oxygen Consumption and NADPH Oxidation in Microsomes from Vitamin K-Deficient, Warfarin- and Dicumarol-Treated Rats.* (31030)

ROBERTA BLEILER HILL, FLORENCE PAUL† AND B. CONNOR JOHNSON‡

Division of Nutritional Biochemistry, Department of Animal Science, University of Illinois, Urbana

In the pathway of electron transport for cytochrome P450 reduction and activation of oxygen in mixed function oxidases, a related reaction, that of lipid peroxidation, has been described. When reduced nicotinamide adenine dinucleotide phosphate (NADPH) added to rat liver microsomes was oxidized, the consumption of oxygen was greatly enhanced by various pyrophosphates in the presence of ferric iron(1,2,3,4,5). Oxygen consumption with concurrent lipid peroxidation by microsomes has been observed upon the addition of only ferrous iron, suggesting that lipid peroxidation is catalyzed by a pyrophosphate chelate of $Fe^{2+}O_2$(6). Lipid peroxidation is not inhibited by carbon monoxide(4).

In this paper, the effect of vitamin K deficiency upon oxygen consumption and lipid peroxidation in rat liver microsomes is reported. Since menadione is known to be an acceptor in the NADPH-cytochrome c reductase system(7,8), our investigations included the possible role of vitamin K in the lipid peroxidation pathway.

Materials and methods. Control (+K) rats were given vitamin K-deficient diets plus the diphosphosodium ester of menadione(9). The deficient group (−K) received the same diet without vitamin supplementation and were housed in tubular, coprophagy-preventing cages(10). Warfarin-treated (W) and dicumarol-treated (D) animals were controls injected with 20 mg of sodium warfarin or dicumarol per 100 g of body weight, 18-24 hours prior to sacrifice. All animals were fasted overnight.

The animals were killed by decapitation and plasma prothrombin times determined by the method of Quick(11). Livers§ were homogenized in 3 times their volumes of 0.25 M sucrose or 1.15% KCl,‖ and a fraction containing primarily heavy microsomes was obtained(12). Final suspensions were made in 1.15% KCl in 0.02 M Tris buffer, pH 7.4 at 37.5°C and contained 5 mg of microsomal protein per ml(13). Oxygen consumption at 37.5°C was measured by the Warburg technique and by an oxypolarograph with a vi-

* Work supported by grants from Nat. Inst. Health (A-6005) and from U. S. Army Surgeon General's Office (MD 544).

† Fulbright fellow, Commissariat à l'Energie, Atomique, Saclay, S/O, France.

‡ Present address: Oklahoma Medical Research Foundation, Dept. of Biochemistry, Oklahoma City.

§ When livers were taken from treated animals and microsomal fractions prepared, livers were removed from control rats and handled similarly.

‖ Preliminary work on oxygen consumption was done in 0.25 M sucrose, but 1.15% KCl was substituted when malonaldehyde measurements were made(1).

DSW 255070

STLCOPCB4060761