columns has been examined this possibility must remain in doubt. The further possibilities that one of the new urinary fluorogens detected by Migeon (1953) and the fluorogenic impurity in crystalline oestriol detected by Braunsberg *et al.* (1954) may be identical with 16-epioestriol obviously require investigation.

SUMMARY

A new Kober-chromogen has been isolated from acid-hydrolysed human pregnancy urine in a yield of about 0.1 mg./l. It has been shown to be identical with 16-epioestriol (oestra-1:3:5-triene-3:16 β :17 β -triol).

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The Content and Distribution of Cytochrome *c* in the Fatty Liver of Rats

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Many authors have been interested in the study of the metabolic changes occurring in the liver during experimental fatty degeneration. Though the fundamental metabolic deviation is not yet clear, some phenomena have been described. One of these is the increase of oxygen uptake by liver slices, which was reported by several investigators (Meier & Thoenes, 1933; Califano, 1934; Ennor, 1942; Biagini, 1951). This increase has been recently attributed to a change which takes place in the mitochondria during the fatty degeneration (Dianzani, 1953) and is characterized by an increase of oxidations and by uncoupling of oxidations from phosphorylations. Prader (1947) reported a three-fold increase of the cytochrome *c* content of the rabbit liver which has undergone fatty degeneration as a result of phosphorus poisoning. He used for the determination of cytochrome *c* the method of Prader & Gonella (1947) and attributed the high oxygen uptake of the fatty liver to an increased rate of metabolism. The values for the cytochrome *c* content of normal liver reported by Prader were, however, lower than those obtained by other authors who employed different methods (Rosenthal & Drabkin, 1943; Stotz, 1939; Potter & Dubois, 1942).

The aim of the present study was to determine the content and the distribution of cytochrome *c* in cellular fractions of the liver by two independent methods.

EXPERIMENTAL

Material and methods. Adult albino rats weight 170–180 g. and rabbits weighing 1.5–1.8 kg. were used. They were fed *ad lib.* with a standard diet containing all vitamins and dietary factors. Fatty degeneration of the liver was produced in rats by daily subcutaneous injections of either 0.2 ml. of a 20% (v/v) soln. of CCl₄ in olive oil, or of 0.1 ml. of a 0.5% soln. of white phosphorus in olive oil. Phosphorus was administered for 1–4 days, CCl₄ for 1–60 days. Fatty degeneration in rabbits was produced by injecting 0.5 ml. 0.5% soln. of phosphorus in olive oil for 2–6 days. The animals were killed by bleeding 24 hr. after the last injection; they were kept fasting for 20 hr. before killing in order to reduce (i) the amount of glycogen, which can interfere with the fractionation procedure (Claude, 1946) and the determination of cytochrome *c* (Prader & Gonella, 1947), and (ii) the amount of protein in the mitochondria (Muntwyler, Seifter & Harkness, 1950).

Immediately after the death of the animals, the livers were dissected, weighed, transferred to the cold room at 2°, and 10% homogenates were prepared in a Potter-Elvehjem glass homogenizer with 0.25M sucrose. Separation of cellular fractions was carried out in the cold room by the procedure

of Schneider (1948). Four fractions ('nuclear fraction', 'mitochondria', 'microsomes', 'supernatant fluid') were collected. The 'nuclear fraction' was washed thrice with sucrose solution and it contained tissue debris, red cells, undamaged cells, nuclei and some mitochondria. The other fractions were only washed once, the washing fluids being added every time to the next fraction. Cytochrome *c* and N-content (Kjeldahl) of each fraction were determined and compared with those of the whole homogenate.

Determination of cytochrome *c*. Two methods have been used for the determination of cytochrome *c*. The first one was that developed by Prader & Gonella (1947) and the second one was that described by Potter & Dubois (1942).

In the first method cytochrome *c* is extracted by using 1.25 ml. $N-H_2SO_4$, followed by 0.5 ml. $2N-NH_3/g.$ tissue. Haemoglobin and some other proteins are removed from the extract by the addition of an equal vol. of sat. $(NH_4)_2SO_4$ and the mixture left at 55° for 20 min. before the precipitate is discarded by filtration. Next, cytochrome *c* is precipitated from the filtrate with trichloroacetic acid (TCA), 1 ml. of 90% (w/v) soln./10 ml. filtrate. After standing in the refrigerator for 16–20 hr. the precipitate is centrifuged, dissolved in water, the solution neutralized with 0.1 $N-NaOH$ and the cytochrome *c* content assessed by determining the difference between light absorption at 550 and 560 $m\mu$. ($E_{550}-E_{560}$), after reduction with sodium dithionite. The amount of cytochrome *c* was then extrapolated from a curve obtained by the use of a pure preparation of cytochrome *c*, containing 0.46% Fe and kindly supplied by Dr Sven Paléus.

The method of Potter & Dubois (1942) includes extraction of cytochrome *c* with diluted TCA at pH 3.5, neutralization with 1% $NaOH$, precipitation of cytochrome *c* from the filtrate with concentrated TCA and determination of cytochrome *c* in the dissolved precipitate by means of an enzyme preparation from rat kidney. Light absorption at 550 $m\mu$ is measured before and after addition of 0.01 ml. 0.2M sodium succinate and 0.03 ml. 0.1M-KCN. Calculation is made according to the formula: $c_t = (E_r - E_o)/1.91 \times 10^{-7}$, where c_t is the concentration of cytochrome *c* in moles/ml., E_r and E_o the extinctions of cytochrome *c* in the

reduced and in the oxidized forms respectively; 1.91×10^4 is the difference between the coefficients of molecular extinction of reduced and of oxidized cytochrome *c*. Spectrophotometric determinations were made with a Beckman Model DU apparatus. The amount of tissue used was 2.5 g. in the case of the method of Prader & Gonella and 1 g. in the case of that of Potter & Dubois. The lipid content of the liver was determined by weighing the dry material, before and after extraction with ether for 4 hr. in a Kumagawa-Suto apparatus. The drying was carried out at 85° to constant weight.

Other methods. Microscopic examination of mitochondria was made with the Zeiss-Winkel phase-contrast microscope. Small pieces of liver were fixed in formalin and used for the preparation of histological specimens, which were stained either with haematoxylin-eosin or with sudan III.

The analytical results were subjected to statistical analysis; standard deviation, standard error of the mean and student's *t* value (Fisher, 1948) were calculated for each group of experiments. The experiments with a '*t*' value corresponding to a probability $P < 0.05$ were considered to be significant.

RESULTS

The content and the distribution of cytochrome *c* in the liver fractions of normal rats is shown in Table 1. It is clear from this table that the values obtained with the method of Prader & Gonella are much lower than those obtained with the method of Potter & Dubois. An attempt was made to find the reason for this discrepancy. The method of Prader & Gonella is a spectroscopic one, while that of Potter & Dubois is based on the enzymic reduction of cytochrome *c*. Since, as it was shown by Margoliash (1952, 1954*a, b*), the purified preparations of cytochrome *c* contain always a certain amount of enzymically inactive material, the values obtained by the second method would be expected to be lower than those obtained by the first method.

Table 1. *Content of cytochrome c and nitrogen in cellular fractions obtained from the liver of normal rats*

The values, per g. fresh tissue, represent averages (range in brackets) obtained, *A*, by the method of Prader & Gonella (1947) in ten experiments and *B*, by the method of Potter & Dubois (1942) in seven experiments. The values expressed as % denote the percentage distribution of N and cytochrome *c* (Cyt. *c*) in the various fractions as compared with the whole homogenate. The total lipid content of fresh rat liver was 38.8 ± 3.0 mg./g. fresh tissue (range, 35.2–45.3).

Fraction	Nitrogen		<i>A</i>			<i>B</i>		
	mg./g.	%	Cyt. <i>c</i>		Cyt. <i>c</i> /N	Cyt. <i>c</i>		Cyt. <i>c</i> /N
			$\mu g./g.$	%		$\mu g./g.$	%	
Whole homogenate	33.0 ± 0.1 (31.2–34.3)	100	28.8 ± 3.8 (23.8–34.3)	100	0.87	113.0 ± 9.4 (99–125)	100	3.4
Nuclear fraction	6.4 ± 0.8 (5.8–7.8)	20.1	6.4 ± 1.1 (5.4–7.9)	26.3	1.0	26.8 ± 4.6 (22–35)	24.9	4.2
Mitochondria	7.3 ± 0.6 (6.2–8.0)	23.0	8.4 ± 2.3 (5.9–11.0)	34.1	1.1	51.9 ± 4.1 (46.0–59.0)	48.3	7.1
Microsomes	5.8 ± 0.6 (5.0–6.9)	18.2	6.7 ± 1.6 (4.5–9.4)	27.4	1.2	16.8 ± 4.0 (10.0–22.0)	15.6	2.9
Supernatant	12.3 ± 1.4 (10.3–15.0)	38.7	3.0 ± 0.8 (2.0–4.0)	12.2	0.24	12.0 ± 2.7 (10.3–18.0)	11.2	0.9
Sum of fractions	31.8	—	24.8	—	—	107.5	—	—
Recovery (%)	96.4	—	85.5	—	—	95.1	—	—

Known amounts of the cytochrome *c* preparation containing 0.46 % Fe were 'determined' by both methods. The values obtained with the procedure of Potter & Dubois were about 50 % lower than those obtained by the method of Prader & Gonella. This means that the percentage of biologically active cytochrome *c* in this preparation was about 50 %. Another preparation containing 0.37 % Fe, prepared according to the method of Keilin & Hartree (1945), contained about 80 % of enzymically active cytochrome *c*.

Since the determinations of cytochrome *c* content in the liver gave higher values with the method of Potter & Dubois than with that of Prader & Gonella, the following experiments were designed to discover at which stage of extraction or purification the losses were occurring. Spectroscopic estimations of the extinction difference between 550 and 560 m μ . in the presence of sodium dithionite were made on the liver homogenate before any treatment and after each stage of both extraction procedures. It was found that both methods extract 95–98 % of the substances absorbing at 550 m μ . In the method of Prader & Gonella a considerable loss, however, occurred during the stage of salting out with (NH₄)₂SO₄ at 55°. This agrees with the fact, reported by Paul (1951), that cytochrome *c* becomes less thermostable in the presence of salts. In another type of experiments, a known amount of cytochrome *c* was added to the liver homogenate before extraction. The amount of cytochrome *c* was checked at each stage by reading the difference of extinctions between 550 and 560 m μ . Also in this case a considerable loss occurred as a result of the treatment with (NH₄)₂SO₄ at 55° (Table 2).

Differences between the methods of Prader & Gonella and of Potter & Dubois were also observed when determinations of cytochrome *c* were carried out in the cellular fractions. The values for the distribution of cytochrome *c* obtained with the method of Prader & Gonella were 26.3 % in nuclear fraction, 34.1 in mitochondria, 27.4 in microsomes and 12.2 in the supernatant fluid. Those obtained, with the method of Potter & Dubois were 24.9, 48.3, 15.6 and 11.2, respectively. The last results agree with those reported by Beinert (1951), whose figures were 12.8, 56.1, 9.0 and 22.1. Beinert showed that some redistribution of cytochrome *c* takes place in the homogenates during the fractionation. In the present study, the cytochrome *c* of mitochondria never accounted for less than 43 %.

Table 2. *Comparison of two methods for the extraction and determination of cytochrome c in rat liver*

The homogenate contained 150 μ g. of cytochrome *c*; to this was added 200 μ g. of pure cytochrome *c*, and the mixture extracted and purified by *A*, the method of Prader & Gonella (1947), and *B*, the method of Potter & Dubois (1942).

Method <i>A</i>	Cytochrome <i>c</i>	
	μ g.	% recovery
Starting material	350	—
Extract	338	96.6
After heating with (NH ₄) ₂ SO ₄	105	30.0
After precipitation with TCA	100	28.6
Method <i>B</i>		
Starting material	350	—
Extract	344	98.3
After NaOH treatment	340	97.4
After precipitation with TCA	320	91.4

Table 3. *Content of cytochrome c and nitrogen in cellular fractions obtained from the liver of rats treated with CCl₄*

The values, per g. fresh tissue, represent averages (range in brackets) obtained, *A*, by the method of Prader & Gonella (1947) in seventeen experiments and *B*, by the method of Potter & Dubois (1942) in six experiments. The values expressed as % denote the percentage distribution of N and cytochrome *c* (Cyt. *c*) in the various fractions as compared with the whole homogenate. The total lipid content of fresh rat liver was 62.5 $\pm$ 6.6 mg./g. of fresh tissue (range 42.5–82.5).

Fraction	Nitrogen		A			B		
			Cyt. c		Cyt. c/N	Cyt. c		Cyt. c/N
	mg./g.	%	μg./g.	%		μg./g.	%	
Whole homogenate	29.3±1.8 (27.1–32.9)	100	27.3±3.2 (21.9–32.8)	100	0.93	80.8±5.8 (73.0–90.0)	100	2.72
Nuclear fraction	6.1±0.4 (5.2–7.1)	21.2	6.2±1.3 (3.0–11.9)	24.0	1.0	21.8±3.1 (18.2–27.0)	28.8	3.57
Mitochondria	6.6±0.8 (5.0–8.4)	22.9	7.2±1.9 (3.9–11.9)	27.7	1.1	19.7±3.6 (16.0–24.0)	26.0	2.9
Microsomes	5.1±0.3 (4.0–4.6)	17.7	7.9±1.9 (5.9–10.1)	30.6	1.5	12.6±1.5 (11.0–15.0)	16.6	2.4
Supernatant	11.0±1.2 (9.5–12.9)	38.8	4.6±1.4 (3.2–5.1)	17.7	0.4	21.6±3.9 (18.0–28.0)	28.6	1.96
Sum of fractions	28.8	—	25.9	—	—	75.7	—	—
Recovery (%)	98.2	—	95.0	—	—	93.5	—	—

Table 4. *Content of cytochrome c and nitrogen in cellular fractions obtained from the liver of rats treated with phosphorus*

The values, per g. fresh tissue, represent averages (range in brackets) obtained, *A*, by the method of Prader & Gonella (1947) in nine experiments and *B*, by the method of Potter & Dubois (1942) in five experiments. The values expressed as % denote the percentage distribution of N and cytochrome *c* (Cyt. *c*) in the various fractions as compared with the whole homogenate. The total lipid content of fresh rat liver was 61.7 ± 1.8 mg./g. of fresh tissue (range 50.4–76.8).

Fraction	Nitrogen		<i>A</i>			<i>B</i>		
	mg./g.	%	Cyt. <i>c</i>		Cyt. <i>c</i> /N	Cyt. <i>c</i>		Cyt. <i>c</i> /N
			μg./g.	%		μg./g.	%	
Whole homogenate	30.1 ± 1.2 (26.9–31.8)	100	23.7 ± 2.2 (13.5–28.3)	100	0.92	76.6 ± 12.3 (59.0–92.0)	100	2.5
Nuclear fraction	6.2 ± 0.7 (5.0–7.0)	20.7	6.1 ± 0.9 (5.4–9.8)	27.2	0.98	19.1 ± 5.6 (12.0–25.0)	23.7	3.1
Mitochondria	6.3 ± 0.4 (5.4–7.0)	21.1	5.0 ± 0.8 (3.1–8.0)	22.3	0.79	21.7 ± 3.3 (16.5–25.0)	27.0	3.44
Microsomes	4.2 ± 0.4 (3.2–4.8)	14.0	6.9 ± 1.5 (4–10.0)	30.8	1.64	11.9 ± 1.6 (10.0–14.0)	14.8	2.8
Supernatant	13.2 ± 0.9 (11.4–14.6)	44.2	4.4 ± 1.7 (2.5–10.0)	19.7	0.33	27.7 ± 2.0 (25.0–29.0)	34.5	2.1
Sum of fractions	29.9	—	22.4	—	—	80.4	—	—
Recovery (%)	99.3	—	94.6	—	—	104.9	—	—

Table 5. *Content of cytochrome c in the liver of normal rabbits and of rabbits treated with phosphorus*

The values, expressed in μg./g. (fresh tissue), are averages (range in brackets) obtained, *A*, by the method of Prader & Gonella (1947) in three experiments and *B*, by the method of Potter & Dubois (1942) in three experiments.

	<i>A</i> (μg./g.)	<i>B</i> (μg./g.)
Normal rabbits	27.0 ± 4.5 (22.0–31.0)	65.2 ± 6.6 (59.6–72.5)
Rabbits treated with phosphorus	18.7 ± 10.3 (12.5–30.0)	32.6 ± 6.9 (25.5–39.4)

Table 3 shows the distribution of cytochrome *c* in cell fractions from rats treated with CCl_4 . The differences between the values obtained with the two methods are quite striking. While the method of Potter & Dubois reveals a significant decrease of cytochrome *c* content of the liver, the values obtained by the Prader & Gonella's method are those encountered in the normal livers. Some difference exists also with respect to the distribution among the cell fractions; with the method of Prader & Gonella the amount of cytochrome *c* found in the supernatant fluid is lower, whereas that found in microsomes is higher than the amounts found in the corresponding fractions analysed by the method of Potter & Dubois. A marked decrease of cytochrome *c* in mitochondria and an increase in the supernatant fluid was found, however, to occur with both methods.

Table 4 shows the results obtained in rats treated with phosphorus. Again in this case a marked

difference was observed between the values obtained by the two methods. The main result of the experiments on rats treated with CCl_4 or with phosphorus was that their cytochrome *c* content was not higher than that of normal animals. Since the difference between our results and those of Prader (1947) might have been due to a species difference, some experiments have been carried out on rabbits. Table 5 shows, however, that also in this animal species the cytochrome *c* content of the fatty liver is diminished rather than increased. However, also in this case the values obtained by the method of Prader & Gonella were lower than those obtained by the method of Potter & Dubois.

Experiments were carried out to investigate the cause for the decrease of the cytochrome *c* content in the mitochondria and the increase in the 'supernatant fraction'. Mitochondria from a fatty liver are known to be more frail than those present in the normal liver (Dianzani & Bahr, 1954), and they are known to release into the surrounding medium more ribonucleic acid on 30 min. incubation at 38° than normal mitochondria (Dianzani, 1953). The change in the distribution of cytochrome *c* in the fatty liver may thus be a direct consequence of an increased release of cytochrome *c* from mitochondria. Experiments were made to examine this possibility.

Mitochondria isolated from 2.5 g. of normal and of fatty liver were incubated at 38° for 1 hr. with 10 ml. 0.25M sucrose. They were then centrifuged 30 min. at 12000 *g* and the cytochrome *c* content of both the sediment and the supernatant fraction was determined. Table 6 shows that the amount of

Table 6. *Release of cytochrome c from normal and from fatty liver mitochondria after incubation at 38° for 1 hr.*

Mitochondria were isolated from 5 g. liver and suspended in 20 ml. 0.25 M sucrose. The suspension was divided into two equal parts. One was immediately analysed for cytochrome *c* by the method of Potter & Dubois, the other after incubation at 38° for 1 hr. The values for normal rats, expressed as $\mu\text{g./g.}$ (fresh tissue), are averages obtained in three experiments. The values for rats treated with phosphorus were obtained in single experiments.

No. of injections of phosphorus	Nitrogen (mg./g.)	Non-incubated sample ($\mu\text{g./g.}$)	Cytochrome <i>c</i>		
			Incubated sample		Released into the supernatant
			Sediment ($\mu\text{g./g.}$)	Supernatant ($\mu\text{g./g.}$)	(%)
None	7.2 $\pm$ 0.1	64 $\pm$ 6	50 $\pm$ 2.5	12 $\pm$ 2.1	19.3
1	7.4	60	40	18	31.0
2	7.6	56	35.1	19	35.1
4	7.6	40	17	15.4	47.5

cytochrome *c* released from mitochondria isolated from fatty livers is higher than that released from normal mitochondria.

DISCUSSION

The results described in this paper do not favour the conclusion reached by Prader (1947) that the cytochrome *c* content of the fatty liver is higher than that of the normal liver. On the contrary, the content of cytochrome *c* was found lower both in whole homogenates and in mitochondria from fatty livers. Moreover, some redistribution of cytochrome *c* takes place during the fractionation of liver homogenates, and it seems that this redistribution occurs at a higher rate in the fatty liver than in the normal one. Incubation at 38° produces a greater loss of cytochrome *c* from mitochondria isolated from the fatty liver than those isolated from a normal liver. This phenomenon is probably related to the morphological changes occurring in mitochondria as a result of fatty degeneration of the liver.

SUMMARY

1. The content of cytochrome *c* is lower in the liver of rats and rabbits treated with either carbon tetrachloride or phosphorus than in normal animals.

2. Mitochondria separated from a liver which has undergone fatty degeneration contain a smaller amount of cytochrome *c* than those obtained from a normal liver.

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