Effect of refeeding diets containing cottonseed flour with traces of gossypol on rat liver and testis

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The aim of the present work was to show that cottonseed flour introduced into rehabilitation diets is not nutritionally harmful. The cottonseed flour obtained from glandless seeds contains traces of gossypol. As gossypol is known to have pathological hepatic and testicular actions, the effects on hepatic variables and on the histological appearance of liver and testis of diets containing cottonseed flour were compared with those without cottonseed flour. Seventy growing male Wistar rats were divided into two groups. The control group (C) received a balanced diet (200 g casein + 3 g methionine/kg) for 78 d and the experimental group was fed on a low-protein diet (20 g casein + 3 g methionine/kg) for 29 d. After the period of protein deprivation, the depleted rats were divided into four groups and each group was refed with a different diet, for 49 d: a balanced diet (200 g casein + 3 g methionine/kg; rC), maize–soya-bean flour–milk (60:30:5, by wt; MSM), maize–cottonseed flour–milk (60:30:5, by wt; MCM) or rice–cottonseed flour–milk (60:30:5, by wt; RCM). Each diet was supplemented with a vitamin mixture (10 g/kg) and a salt mixture (40 g/kg). In the liver, protein, total lipid, fatty acid composition and nucleic acid contents were determined after 29 d of protein deprivation and after 2, 14, 29, 49 d of refeeding. The refeeding of rats with either diet MSM or RCM promoted a higher growth than that with diet MCM or diet rC. In all groups, a progressive decrease in relative liver weights with age was observed. The highest values were obtained with diets rC and MSM. After 49 d of refeeding, the lowest values for lipid, DNA and protein:RNA and the highest values for RNA and RNA:DNA were obtained with the cottonseed diets (MCM and RCM). Rats fed on the cottonseed-flour diets for a 49 d period after severe protein malnutrition showed no change in size or shape of their hepatocytes compared with those obtained with the control casein diet, and all stages of spermatogenesis occurred normally and spermatozoa were accurately formed. Therefore, our findings show that a low level of gossypol administrated during 49 d, even to an organism weakened by protein malnutrition, does not affect hepatic variables and the histological appearance of liver and testis.

Cottonseed flour: Gossypol: Liver: Testes: Rat

Cottonseed flour contains polyphenol gossypol (1,1′,6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl-2,2′ binaphthalene-8,8′ dicarboxaldehyde). This compound, in the free state, is toxic to non-ruminant animals. Tolerance to gossypol toxicity varies according to the species. Hence, as pigs cannot safely tolerate diets containing more than 0·1 g free gossypol/kg, the use of cottonseed flour as a source of protein for this non-ruminant species is largely restricted (Smith & Clawson, 1965). However, it has been shown that high levels of dietary protein either alleviate or counteract the toxic effects of gossypol in diets for pigs and rats. Hale & Lyman (1957) reported that pigs fed on a 155 g protein/kg diet containing 0·1 g free gossypol/kg showed no signs of gossypol toxicity. At the 0·15 g/kg level, toxicity signs were observed in pigs and death occurred at 0·19 g free gossypol/kg and above. Withers & Carruth (1915) reported that animals fed on cottonseed flour with

gossypol lost appetite, and post-mortem observations revealed oedema, excessive abdominal fluid, haemorrhagic intestine, and congested livers and kidneys.

In rats, clear toxic effects are recorded only at very high doses (Raghunah & Giridharan, 1987). In fact, rats are sensitive to lower doses of gossypol than man (7·5 mg/kg body-weight *v.* 20 mg/kg). So, rat is a good model to demonstrate that glandless cottonseed in rehabilitation diets is not nutritionally harmful. Ambrose & Robbins (1951) showed that in rats, gossypol intake caused a loss of appetite, and that body-weight depression was proportional to the quantity administered. When single doses of 5 mg ^{14}C-labelled gossypol were administered to rats the highest radioactivity was found in liver, muscle and kidney, and the highest specific and total activities were observed in the liver (Sharma *et al.* 1966; Abou-Donia *et al.* 1970; Smith & Clawson, 1970).

It has been shown that gossypol can be a specific inhibitor of DNA synthesis in mammalian cells (Wang & Rao, 1984), but it has no effect on RNA and protein synthesis at a concentration of 10 μg/ml dimethyl sulphoxide (diluting medium) and, hence, has no effect on cell division. In the presence of the drug, cells can enter the S phase and gossypol does not increase chromosome aberrations. Using Chinese hamster (*Cricetulus griseus*) ovary cells and human lymphocytes, Ye *et al.* (1983) showed that gossypol treatment causes no increase in chromosome breakage or polyploidy, but reduces the mitotic index, the synthesis rates of DNA, RNA and protein, and the percentage of viable cells as revealed by the dye exclusion method. In vitro, gossypol is found to be genotoxic by inducing degradation of rat liver DNA (Srivastava & Padmanaban, 1987). Gross lesions, including hepatomegaly (Holmberg *et al.* 1988) and acute toxic hepatitis (Rogers *et al.* 1975), have been observed with gossypol in calves. Lesions reported in young calves include delayed clotting of blood with fatty liver degeneration (Hollon *et al.* 1958).

Gossypol is cytotoxic to spermatocytes and spermatids, while Sertoli cells, Leydig cells and spermatogonia remained unaffected (National Coordinating Group on Male Infertility, 1978). Gossypol, when given orally to human subjects, induces an infertility effect of 99·9 % (Wichmann, 1982). Gossypol inhibits spermatozoal glycolysis and fructolysis, and immobilizes the sperm cells (Pösö *et al.* 1980). In human subjects 10 mg gossypol/d induces, after 3 months, a decrease in sperm density and motility (Wichmann *et al.* 1983; Frick *et al.* 1988; Stephens *et al.* 1988). A dose of 20 mg gossypol/d, given for a 3-month period, reduces sperm motility. When this treatment is followed by a weekly dose of 50–60 mg, sperm production is blocked (Kalla, 1982). A daily oral dose of 20 mg/kg for 62 d to male rats damages epididymal epithelium and sperm (Reyes *et al.* 1984). A dose of 25 mg gossypol/kg per d inhibits sperm motility by blocking ATP production and utilization and inhibits pyruvate dehydrogenase (*EC* 1.2.2.2, 1.2.4.1) and ATPase (*EC* 3.6.1.3, 3.6.1.8) activities (Reyes *et al.* 1988; Ueno *et al.* 1988).

The present study was designed to demonstrate that cottonseed-flour diets are not nutritionally harmful. With this intention, investigations were conducted to determine the effects of cottonseed flour on the liver and testis, since these organs are particularly sensitive to gossypol toxicity. As gossypol toxicity varies with the level of protein in the diet, the hepatic and testicular effects of a low gossypol level (0·012 g/21 g protein) were studied in rats subjected to severe protein malnutrition, hence on weakened organisms, over a long period of time.

EXPERIMENTAL METHODS

Seventy male Wistar rats (Iffa-Credo, Lyon, France) weighing 70 (SE 5) g at the beginning of the study, were allowed free access to a balanced diet, containing 200 g casein/kg supplemented with 3 g methionine/kg for 5 d. After this adaptation period to familiarize the rats with a diet in the form of a gruel, they had reached an average body-weight of 100

(SE 10) g and they were randomized into two groups. The control group (C; eighteen rats) was allowed free access to a balanced diet for 78 d, whereas the experimental group (fifty-two rats) was fed on a low-protein diet (M; 20 g casein + 3 g methionine/kg) for 29 d. The composition of control and experimental diets is shown in Table 1. At the end of this period, the rats subjected to protein malnutrition (PM) were randomized into four equal groups (thirteen rats) fed on different diets for 49 d. This period corresponded to balanced refeeding (BR): balanced diet (200 g casein + 3 g methionine/kg; rC), maize–soya-bean flour–milk diet (60:30:5, by wt; MSM), maize–cottonseed flour–milk diet (60:30:5), by wt; MCM), rice–cottonseed flour–milk diet (60:30:5, by wt; RCM). Each diet was supplemented with a vitamin mixture (10 g/kg) and a salt mixture (40 g/kg). The composition of these diets is described in Tables 1 and 2. Maize and rice were tested in association with cottonseed flour because these flours, when associated with soya-bean flour, constitute classical rehabilitation mixtures; by testing the effect of cottonseed flour in comparison with that of soya-bean flour, classical rehabilitation diets could be compared with potential new diets.

Each diet was boiled for 0·5 h immediately before consumption (diet–water; 1:2, w/w) and was given as a gruel, to simulate the conditions of human consumption. Heating with water caused a decrease (less than 52%) in free gossypol level, in a diet containing 360 g cottonseed flour/kg (Bressani *et al.* 1966). The raw diets with cottonseed flour used in the present study contained about 0·12 g free gossypol/kg. After heating, the free gossypol content was reduced by about 50% (0·06 g/kg). Cottonseed flour originating from Bouake (Ivory Coast) was rendered fat-free at 60° by light petroleum (b.p. 67–70°) extraction. The vitamin losses were not determined after heating, but they were assumed to be similar for each diet.

Maize, rice and soya-bean flours and skimmed milk were commercial products. Soya-bean flour had been heated and defatted (20 g lipid/kg).

Rats were kept in wire-bottomed cages at constant temperature (25°) and humidity 60 (SE 5)% with a 07.00–19.00 hours light cycle. Water and food were supplied *ad lib.* The food intakes and body-weights were determined daily in each group. After an overnight fast, rats were killed at day 29 of PM, and at days 2, 14, 29, 49 of BR. At each time-point, three rats from each group were anaesthetized using an intraperitoneal injection of sodium pentobarbital (65 mg/kg body-weight) and then bled from the abdominal aorta.

The livers were quickly removed, washed with cold saline (9 g sodium chloride/l) and weighed. The same hepatic lobe was used to determine protein, nucleic acid and lipid levels.

Protein was assayed according to Lowry *et al.* (1951). Total hepatic lipids were extracted according to the procedure of Folch *et al.* (1958) and a known amount of pentadecanoic acid ($C_{15:0}$) was added before preparation of the butyl esters. The amount of each fatty acid was calculated by comparison with the amount of internal standard ($C_{15:0}$). The fatty acid compositions were determined using a Becker-Packard Model 419 gas–liquid chromatograph equipped with a 30 m × 0·4 mm i.d. glass capillary column (coated in the laboratory with Carbowax 20 M), at a constant temperature of 195° and a nitrogen flow-rate of 3 ml/min. The column was equipped with a ROS injector and the apparatus with a flame-ionization detector. The peak areas were measured using an ENICA 21 integrator. DNA was assayed according to Dische's (1930) method and RNA according to Hatcher & Goldstein's (1969) technique.

For histological studies only rats fed on BR diets for 49 d were used. Tissues were sampled, fixed in alcoholic Bouin's solution (Duboscq-Brasil), washed several times in alcoholic solution, and rinsed in ethanol (Gabe, 1968). Tissues were processed for histological examination by routine paraffin-embedding procedures and cutting 5 μm sections using an ultramicrotome. Testis and livers were stained by Masson's trichrome

Table 1. *Composition (g/kg) of control and low-protein diets*

	Control	Low-protein
Ingredients		
Milk casein*	200	20
Wheat gluten†	597	777
Saccharose‡	50	50
Maize oil‡	40	40
Agar-agar (Fibres)*	50	50
Salt mixture§	40	40
Vitamin mixture‖	20	20
Methionine*	3	3
Composition		
Lipid	40	40
Protein (nitrogen × 6·25)	189	19
Fibre	50	50
Carbohydrate	658	828

* Prolabo, Paris.
† Etbs Louis François SA, 94100 St Maur, France.
‡ Commercial product.
§ UAR B205 (Villemoisson 91360 Epinay-S/Orge). The salt mixture provided the following amounts (g/kg diet): calcium 4·0, potassium 2·4, sodium 1·6, magnesium 0·4, iron 0·12, manganese 0·032, copper 0·005, zinc 0·018, cobalt 0·0004, iodine 0·002.
‖ UAR 200 (Villemoisson 91360 Epinay-S/Orge). The vitamin mixture provided the following amounts (mg/kg diet): retinol 12, cholecalciferol 0·125, thiamin 40, riboflavin 30, nicotinic acid 140, pyridoxine 20, pyridoxal 300, cyanocobalamin 0·1, ascorbic acid 1600, α-tocopherol 340, menadione 80, calcium pantothenate 200, choline 2720, folic acid 10, *p*-aminobenzoic acid 100, biotin 0·6.

Table 2. *Composition (g/kg) of experimental diets*

	Maize–soya-bean flour–milk	Maize–cottonseed flour–milk	Rice–cottonseed flour–milk
Ingredients			
Cottonseed flour*	—	300	300
Maize‡	600	600	—
Rice‡	—	—	600
Soya-bean flour†	300	—	—
Milk‡	50	50	50
Salt mixture§	40	40	40
Vitamin mixture‖	10	10	10
Composition			
Lipid	30	48	28
Protein (nitrogen × 6·25)	201·7	210·4	217·5
Fibre	29	24	35
Carbohydrate	684	668	670

* Bouaké Oil mill (Ivory Coast).
† Soyapan; Lecithos.
‡ Commercial products; skimmed milk.
§ USB, Biological salt mixtures, 21 325 salt mixture Bernhart-Tomarelli modified. The salt mixture provides the following amounts (g/kg diet): calcium 17·20, potassium 4·00, sodium 4·00, magnesium 2·42, iron 0·320, manganese 0·098, copper 0·020, zinc 0·080, cobalt 0·016, iodine 0·032.
‖ Eurobio, Bd St Germain, Paris. The vitamin mixture provides the following amounts (mg/kg diet): retinol 45, cholecalciferol 2·5, thiamin 10, riboflavin 10, nicotinic acid 45, pyridoxine 10, cyanocobalamin 0·0135, α-tocopherol 50, menadione 22·5, calcium pantothenate 30, choline chloride 750, folic acid 0·9, *p*-aminobenzoic acid 50, biotin 0·2, ψ-inositol 50.

Table 3. *Body-weight and food intake of rats refed on diets containing cottonseed flour with traces of gossypol after a period of protein depletion*

(Values are means with their standard errors for three rats/group)

Stage of experiment	Diet†	Body-wt (g) Mean	Body-wt (g) SE	Food intake (g/kg body weight) Mean	Food intake (g/kg body weight) SE
PM 29 d	C	252·33	4·59	77·0	2·6
	M	98·75***	5·82	112·7***	5·6
BR 2 d	C	288·75[a]	8·55	76·1[a]	4·9
	rC	124·75[b]	5·61	106·0[b]	0·8
	MSM	137·33[b]	2·72	109·7[b]	3·2
	MCM	126·25[b]	3·02	86·3[ac]	9·1
	RCM	127·00[b]	6·18	97·3[bc]	7·7
BR 14 d	C	322·25[a]	12·73	66·0[a]	3·6
	rC	176·67[b]	10·24	101·6[b]	9·4
	MSM	201·50[b]	2·27	129·1[b]	30·2
	MCM	170·08[b]	2·55	105·4[b]	4·9
	RCM	182·17[b]	9·70	117·0[b]	7·3
BR 29 d	C	368·83[a]	15·90	55·1[a]	4·2
	rC	248·75[b]	16·61	73·3[b]	7·5
	MSM	288·50[b]	3·98	82·2[b]	1·6
	MCM	247·33[b]	10·63	83·1[b]	5·9
	RCM	269·50[b]	20·33	78·8[b]	14·1
BR 49 d	C	421·00[a]	21·77	46·4[a]	0·7
	rC	323·17[b]	22·16	58·0[b]	4·7
	MSM	356·75[b]	11·70	60·2[b]	3·1
	MCM	312·50[b]	13·50	62·2[b]	4·2
	RCM	345·00[b]	33·54	59·5[b]	3·6

[a, b, c] Means in vertical columns with different superscript letters were significantly different ($P < 0·05$).

Means for group C rats were significantly different from those of group M rats (Student's t test), *** $P < 0·01$.

C, rC, control diets; M, low protein diet; MSM, maize–soya-bean flour–milk diet; MCM, maize–cottonseed flour–milk diet; RCM, rice–cottonseed flour–milk diet; PM, protein malnutrition; BR, balanced refeeding.

† For details, see Tables 1 and 2.

(Gabe, 1968). The classification of Leblond & Clermont (1952) was used for identification of the different stages of the spermatogenic cycle which were observed under light microscope with magnifications of 300-fold and 1150-fold.

Statistical analysis

Results are expressed as the arithmetical mean of each group with their standard errors. The significance of the difference between mean values was calculated by Student's t test for paired samples. During BR, statistical evaluation of the data was carried out by variance analysis and by classification of the means using Duncan's new multiple-range test (Duncan, 1955). Differences were considered statistically significant at $P < 0·05$.

RESULTS

Body-weight and food intake

The rats were stunted from the beginning of PM. After 29 d of PM, weights of protein-depleted rats were only 39% of those of control rats (Table 3), whereas food intakes per kg body-weight were higher in the protein-depleted group (+46%). Growth recovery was rapid with the four BR diets. The greatest body-weight gains were observed with MSM and RCM diets and the lowest with rC and MCM diets. The MCM group consumed more

Table 4. *Relative liver weight, hepatic lipid and protein content of rats refed on diets containing cottonseed flour with traces of gossypol after a period of protein depletion*

(Values are means with their standard errors for three rats/group)

Stage of experiment	Diet†	Liver weight (g/kg body weight) Mean	SE	Lipid (mg/g liver) Mean	SE	Protein (mg/g liver) Mean	SE
PM 29 d	C	48·5	2·9	57·54	2·79	200·00	4·04
	M	48·1	5·1	86·37***	15·54	174·97***	20·39
BR 2 d	C	44·8^{a}	2·9	54·77^{a}	9·37	189·24	7·50
	rC	52·6^{a}	4·9	55·37^{a}	8·19	248·63	37·55
	MSM	47·9^{a}	3·1	51·60^{a}	4·67	220·81	24·96
	MCM	50·2^{a}	3·4	53·98^{a}	10·89	228·89	36·64
	RCM	44·7^{a}	3·3	39·80^{b}	1·94	214·28	22·16
BR 14 d	C	38·2^{a}	2·0	52·47	7·19	199·13^{a}	10·50
	rC	44·7^{b}	0·6	53·43	2·86	291·86^{c}	20·63
	MSM	41·0^{abc}	2·3	52·07	4·16	267·71^{ac}	10·31
	MCM	43·1^{bc}	2·4	49·13	2·26	284·95^{c}	42·90
	RCM	38·9^{ac}	1·9	48·83	1·65	212·20^{ac}	55·41
BR 29 d	C	36·0^{a}	2·2	53·22^{a}	2·74	280·67^{a}	10·49
	rC	41·3^{b}	0·3	49·73^{ab}	2·76	206·39^{b}	6·88
	MSM	41·3^{b}	1·0	49·43^{ab}	3·74	288·35^{a}	19·00
	MCM	38·8^{ab}	3·0	46·03^{b}	1·25	206·09^{ab}	13·79
	RCM	37·9^{ab}	1·4	39·53^{c}	3·00	180·35^{b}	5·82
BR 49 d	C	31·1^{a}	0·8	53·00^{a}	4·20	205·96^{a}	18·15
	rC	34·0^{bc}	1·5	51·30^{ab}	0·99	179·76^{b}	8·67
	MSM	36·0^{b}	0·7	45·97^{bc}	3·60	193·39^{ab}	2·12
	MCM	32·1^{ac}	1·2	45·37^{bc}	1·39	210·15^{a}	6·71
	RCM	32·4^{ac}	1·4	40·93^{c}	2·96	205·17^{a}	9·55

$^{a, b, c}$ Means in vertical columns with different superscript letters were significantly different $P < 0·05$.

Means for group C rats were significantly different from those of group M rats (Student's t test) *** $P < 0·01$.

C, rC, control diets; M, low protein diet; MSM, maize–soya-bean flour–milk diet; MCM, maize–cottonseed flour–milk diet; RCM, rice–cottonseed flour–milk diet; PM, protein malnutrition; BR, balanced refeeding.

† For details and composition see Tables 1 and 2 and pp. 270–271.

lipids due to the higher dietary content. On the other hand, weight gains were not correlated with food intakes. At the end of the experiment, body-weights with MSM, RCM, rC and MCM diets were still lower (15·2, 18·1, 23·3 and 25·9 respectively), than those of group C. A significant difference between group C and the experimental groups was recorded, but weights in the four BR groups showed no marked differences.

Liver weight

At the end of PM, relative liver weight (g/kg body-weight) was the same for control and protein-depleted groups (Table 4). At the beginning of BR, there were no significant differences in relative liver weight between group C and the four experimental groups. After the first period of BR, the lowest values were observed in group C rats and this continued throughout the experiment. In each group, relative liver weight decreased with age. At the end of BR, the highest values were observed with diet MSM.

Total lipids

In the protein-depleted group, PM increased hepatic lipid contents (mg/g liver) by 50% (Table 4). At the beginning of BR, with the four refeeding diets, total lipids per g liver decreased rapidly and were, generally, lower than group C values. The different groups

presented approximately the same hepatic lipid contents from the beginning to the end of BR excepted for diet RCM which produced the lowest significant values after 29 d of BR.

Protein

At the end of PM, liver protein levels (mg/g liver) were similar for group C and protein-depleted rats (Table 4). Until day 14, the liver protein contents were higher in the four BR groups than in group C. At the end of experiment, the lowest values were observed in group rC.

Fatty acid composition of hepatic total lipids

The present study was restricted to 29 d of PM and days 2 and 14 of BR in the five groups (Table 5). PM modified the amounts of some fatty acids: 20:4 $n-6$ and 22:6 $n-3$ decreased to 40·6 and 54·8 % of the control values respectively, while 14:0, 16:0, 18:1 $n-7+$ 18:1 $n-9$, 18:2 $n-6$, 22:4 $n-6$ and 22:5 $n-6$ increased respectively by 200, 163, 260, 229, 367 and 279 %. After 2 d of BR, 14:0, 16:0, 16:1 $n-7$, 18:0, 18:1 $n-7+18:1$ $n-9$, and 24:1 $n-9$ had similar values in all BR groups, whereas 22:4 $n-6$ and 22:5 $n-6$ values were higher than group C values. After 14 d of BR, 14:0, 16:1 $n-7$, 18:1 $n-7$ and 18:1 $n-9$ levels were lower in diets containing vegetable proteins than in diets rC and C, and in contrast, the 18:0 level was higher. The 22:5 $n-6$ levels were higher in groups fed on cottonseed flour and 22:6 $n-3$ reached the highest values in MSM group. Similar 20:4 $n-6$, 22:4 $n-6$ and 24:0 values were observed in the five groups.

DNA

This value gives an indication of the number of nuclei and, therefore, of the number of cells per g liver. After 29 d of PM, liver DNA values of protein-depleted rats did not differ from those of group C rats (Table 6). After 2 d of BR, values were significantly lower with diet RCM. After 14 d of BR, liver DNA of rats fed on both cottonseed-flour diets were significantly lower than those of rats fed on casein or soya-bean-flour diets. At the end of the experiment, values did not differ between the groups.

Protein:DNA

This value indicates the amount of protein per cell; PM decreased this value by 24 % (Table 6). At day 2 of BR, group C values decreased by 26·5 % in comparison with group C values obtained at the end of PM. This could indicate that hypertrophy or cellular maturation was followed by a hyperplasia phase, as we have previously shown in pancreas (Prost *et al.* 1988). From day 14, the highest values were obtained with both cottonseed-flour diets due to their lower liver DNA levels. After 29 d of BR, diet RCM presented significantly lower values than those of diets MCM and MSM and diet MSM values were significantly higher than those of diets rC and C. At the end of the experiment, values did not differ significantly from one group to another.

RNA

Values were not significantly different between group C and protein-depleted rats after 29 d of PM (Table 7). At day 2 of BR, diet RCM values were higher than those of diet MCM (Table 7). At day 14 of BR, the highest values were observed in group RCM and the lowest ones in group rC. At the end of the experiment, the findings obtained with both diets containing cottonseed flour showed higher concentrations of RNA (mg/g liver).

Table 5. *Hepatic fatty acids (mg/g liver) of rats refed on diets containing cottonseed flour with traces of gossypol after a period of protein depletion*

(Values are means with their standard errors for three rats/group)

Fatty acid....		14:0		16:0		16:1 *n*−7		18:0		18:1 *n*−9 +18:1 *n*−7		18:2 *n*−6	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
0 d	C	0·40	0·10	13·51	2·64	0·98	0·15	9·71	2·14	5·83	1·07	5·36	2·88
PM 29 d	C	0·42	0·03	13·53	0·24	2·43	0·17	7·49	0·69	7·44	0·75	8·62	0·80
	M	0·84*	0·17	22·02*	3·24	2·97	0·61	7·62	0·18	19·36**	3·45	19·75*	3·43
BR 2 d	C	$0·32^{a}$	0·10	10·59	1·74	1·61	0·46	6·96	0·47	8·58	1·98	8·00	1·92
	rC	$0·22^{a}$	0·02	12·74	1·88	1·11	0·20	8·13	0·83	7·11	1·89	5·51	1·57
	MSM	$0·31^{a}$	0·07	12·05	1·09	1·00	0·27	7·60	0·20	6·62	1·57	6·55	1·16
	MCM	$0·43^{a}$	0·28	13·59	4·01	1·43	0·96	7·53	0·93	8·16	4·83	8·16	4·77
	RCM	$0·21^{a}$	0·01	9·47	0·42	0·62	0·07	6·96	0·35	4·16	0·27	4·25	0·11
BR 14 d	C	$0·36^{ab}$	0·10	$12·13^{ac}$	1·49	$1·82^{a}$	0·44	$6·90^{a}$	0·66	$6·69^{a}$	1·24	$8·81^{a}$	2·11
	rC	$0·43^{a}$	0·05	$12·90^{c}$	0·81	$2·29^{a}$	0·28	$7·63^{ab}$	0·43	$7·17^{a}$	0·45	$6·99^{ab}$	0·56
	MSM	$0·26^{b}$	0·04	$11·34^{abc}$	0·95	$1·21^{b}$	0·18	$8·76^{bc}$	0·58	$3·95^{b}$	0·77	$7·16^{ab}$	0·61
	MCM	$0·25^{b}$	0·06	$9·80^{b}$	0·45	$0·93^{b}$	0·16	$9·39^{c}$	0·63	$4·21^{b}$	1·10	$5·73^{b}$	0·13
	RCM	$0·26^{b}$	0·00	$10·69^{ab}$	0·16	$0·86^{b}$	0·02	$9·18^{c}$	0·31	$5·39^{ab}$	0·12	$5·70^{b}$	0·16

Fatty acid....		20:4 *n*−6		22:4 *n*−6		22:5 *n*−6		22:6 *n*−3		24:0		24:1 *n*−9	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
0 d	C	4·43	3·56	0·04	0·04	0·11	0·11	1·83	1·63	0·61	0·19	0·16	0·08
PM 29 d	C	11·86	1·16	0·45	0·03	0·63	0·17	2·61	0·25	0·42	0·02	0·18	0·02
	M	4·81*	3·27	1·65**	0·23	1·76**	0·16	1·43**	0·13	0·33	0·04	0·18	0·02
BR 2 d	C	$10·97^{ab}$	1·23	$0·46^{a}$	0·04	$0·67^{a}$	0·07	$2·23^{a}$	0·17	$0·34^{ab}$	0·04	0·14	0·02
	rC	$12·18^{a}$	0·94	$0·92^{ab}$	0·23	$3·51^{c}$	0·64	$2·00^{a}$	0·45	$0·48^{c}$	0·04	0·18	0·03
	MSM	$8·81^{bc}$	1·05	$1·29^{b}$	0·37	$2·54^{b}$	0·20	$2·95^{b}$	0·23	$0·29^{a}$	0·04	0·16	0·02
	MCM	$8·31^{bc}$	1·63	$1·14^{b}$	0·19	$2·53^{b}$	0·36	$2·55^{ab}$	0·30	$0·34^{ab}$	0·09	0·12	0·04
	RCM	$7·72^{c}$	0·67	$0·86^{ab}$	0·03	$2·06^{b}$	0·37	$1·96^{a}$	0·22	$0·42^{bc}$	0·01	0·12	0·03
BR 14 d	C	$10·69^{ab}$	1·06	$0·44^{a}$	0·08	$0·62^{a}$	0·06	$2·29^{a}$	0·20	$0·33^{a}$	0·05	$0·09^{a}$	0·02
	rC	$10·11^{b}$	0·81	$0·46^{a}$	0·04	$1·27^{b}$	0·19	$2·18^{a}$	0·20	$0·36^{ab}$	0·01	$0·21^{b}$	0·09
	MSM	$12·21^{a}$	0·53	$0·55^{ab}$	0·09	$0·99^{ab}$	0·14	$3·30^{b}$	0·15	$0·40^{ab}$	0·05	$0·20^{b}$	0·04
	MCM	$11·57^{ab}$	0·68	$0·69^{b}$	0·06	$2·38^{c}$	0·35	$2·39^{a}$	0·50	$0·39^{ab}$	0·01	$0·11^{ab}$	0·02
	RCM	$10·52^{ab}$	0·56	$0·49^{a}$	0·07	$1·98^{c}$	0·39	$1·89^{a}$	0·11	$0·41^{b}$	0·01	$0·14^{ab}$	0·01

[a, b, c] Means in vertical columns with different superscript letters were significantly different ($P < 0·05$).

Means for group C rats were significantly different from those of group M rats (Student's *t* test) * $P < 0·05$; ** $P < 0·02$.

C, rC, control diets; M, low protein diet; MSM, maize–soya-bean flour–milk diet; MCM, maize–cottonseed flour–milk diet; RCM, rice–cottonseed flour–milk diet; PM, protein malnutrition; BR, balanced refeeding.

For details and composition see Tables 1 and 2 and p. 270–271.

Table 6. *Liver DNA content and liver protein: DNA of rats refed on diets containing cottonseed flour with traces of gossypol after a period of protein depletion*

(Values are means with their standard errors for three rats/group)

Stage of experiment	Diet†	Liver DNA (mg/g tissue) Mean	SE	Liver protein: DNA (mg/mg) Mean	SE
PM 29 d	C	2·47	0·09	80·97	3·60
	M	2·83	0·30	60·87***	2·39
BR 2 d	C	3·18	0·10	59·51[a]	0·72
	rC	3·07	0·06	80·99[bc]	12·42
	MSM	3·25	0·23	67·94[ac]	3·03
	MCM	3·28	0·09	69·78[ac]	9·48
	RCM	2·32	0·40	92·36[b]	7·54
BR 14 d	C	3·15[a]	0·27	63·22[a]	5·27
	rC	3·44[a]	0·25	84·84[a]	6·55
	MSM	3·64[a]	0·09	73·54[a]	1·35
	MCM	1·35[b]	0·35	211·07[b]	87·69
	RCM	1·82[b]	0·45	116·59[b]	98·26
BR 29 d	C	3·34[a]	0·14	84·03[ac]	6·48
	rC	2·93[ab]	0·22	70·44[ac]	5·41
	MSM	2·68[bc]	0·23	107·59[b]	15·63
	MCM	2·25[c]	0·27	91·59[bc]	13·20
	RCM	2·76[b]	0·23	65·34[a]	5·59
BR 49 d	C	2·91	0·78	70·68	17·85
	rC	1·78	0·13	100·99	11·93
	MSM	2·48	0·65	77·98	21·96
	MCM	2·09	0·07	100·55	6·33
	RCM	1·79	0·33	114·62	31·71

[a, b, c] Means in vertical columns with different superscript letters were significantly different ($P < 0·05$). Means for group C rats were significantly different from those of group M rats (Student's t test) *** $P < 0·01$.

C, rC, control diets; M, low protein diet; MSM, maize–soya-bean flour–milk diet; MCM, maize–cottonseed flour–milk diet; RCM, rice–cottonseed flour–milk diet; PM, protein malnutrition; BR, balanced refeeding.

† For details and composition see Tables 1 and 2 and pp. 270–271.

Protein: RNA

The highest values indicated the most elevated protein synthesis per RNA unit. Malnutrition decreased this ratio by 23·5% (Table 7). At the beginning of BR, values increased quickly, except in group RCM which showed the lowest values. At the end of experiment, the lowest values were observed with both cottonseed diets and the highest with diet MSM.

RNA: DNA

This ratio indicates the RNA content per cell. After 2 d of BR, the greatest value was obtained with diet RCM and with both diets containing cottonseed flour after 14 d of experiment. After 29 d of BR, the lowest values were achieved with diets rC and RCM and the highest ones with diet MSM. After 49 d of BR, the highest values were observed in both groups consuming cottonseed flour.

Liver

Livers of experimental rats before sampling were as well irrigated as the control rats. Hepatic lobules of animals which consumed diets MCM and RCM had a morphological appearance similar to that of group C rats. The number, size and shape of hepatocytes were similar for all groups (Plate 1). Portal areas, hepatic artery, portal vein and bile duct

Table 7. *Liver RNA content, liver protein:RNA and liver RNA:DNA of rats refed on diets containing cottonseed flour with traces of gossypol after a period of protein depletion*

(Values are means with their standard errors for three rats/group)

Stage of experiment	Diet†	Liver RNA (mg/g tissue) Mean	SE	Liver protein:RNA (mg/mg) Mean	SE	Liver RNA:DNA (mg/mg) Mean	SE
PM 29 d	C	4·20	0·80	47·62	13·25	1·70	0·09
	M	5·94	0·88	29·46	2·79	2·09	0·13
BR 2 d	C	2·84^{a}	0·43	66·63^{a}	9·53	0·89^{a}	0·13
	rC	4·83^{ab}	1·53	51·48^{ab}	20·50	1·58^{a}	0·53
	MSM	3·52^{a}	0·36	62·73^{ab}	12·31	1·08^{a}	0·19
	MCM	4·20^{ab}	1·13	54·50^{ab}	14·58	1·28^{a}	0·37
	RCM	6·06^{b}	0·62	35·36^{b}	3·95	2·61^{b}	0·30
BR 14 d	C	2·93^{a}	0·10	67·96^{a}	4·42	0·93^{a}	0·10
	rC	2·11^{b}	0·75	138·32^{b}	66·38	0·61^{a}	0·27
	MSM	3·00^{a}	0·23	89·24^{ab}	8·68	0·82^{a}	0·06
	MCM	3·33^{a}	0·03	85·57^{ab}	13·46	2·47^{b}	0·70
	RCM	4·25^{c}	0·17	49·93^{c}	15·05	2·34^{b}	0·76
BR 29 d	C	5·53^{a}	1·17	50·75^{ab}	14·30	1·65^{ac}	0·29
	rC	3·14^{b}	0·42	65·73^{a}	9·05	1·07^{d}	0·22
	MSM	6·54^{a}	0·15	44·09^{b}	2·76	2·45^{b}	0·21
	MCM	3·86^{b}	0·08	53·39^{ab}	4·49	1·72^{a}	0·25
	RCM	3·22^{b}	0·34	56·01^{ab}	7·14	1·17^{cd}	0·07
BR 49 d	C	3·84^{a}	0·06	53·57^{ab}	5·49	1·32^{a}	0·48
	rC	3·79^{a}	0·58	47·43^{b}	7·44	2·13^{ac}	0·46
	MSM	3·17^{a}	0·29	61·01^{a}	5·81	1·28^{a}	0·37
	MCM	6·25^{b}	0·67	33·62^{c}	3·73	2·99^{bc}	0·28
	RCM	5·96^{b}	0·58	34·42^{c}	3·75	3·33^{b}	0·66

$^{a, b, c, d}$ Means in vertical columns with different superscript letters were significantly different ($P < 0·05$).

C, rC, control diets; M, low protein diet; MSM, maize–soya-bean flour–milk diet; MCM, maize–cottonseed flour–milk diet; RCM, rice–cottonseed flour–milk diet; PM, protein malnutrition; BR, balanced refeeding.

† For details and composition see Tables 1 and 2 and pp. 270–271.

presented no sign of physiological impairment, and no sign of necrosis was observed. Hepatocyte nuclei and nucleoli from rats fed on cottonseed diets were similar to those of group C rats. There were no traces of intracellular or extracellular alteration.

Testis

Groups C and MCM presented tubules at stage VI while group RCM presented tubules at stage V (Plate 2). Testes of experimental animals presented the same size and morphological aspect as those of group C rats with active seminiferous tubules. After 49 d of BR with diets MCM and RCM there was no sign of seminiferous tubule degeneration: tubule lumen appeared similar to that of group C, germinal cells were against the basal membrane, spermatozoa were seen in the seminiferous lumen, and at high magnification, the seminiferous epithelium cycle was active. At each stage, the spermatogenic cells observed were normal: Sertoli cells, spermatids, spermatogonia (SA and SB) and spermatocytes were present. Spermatozoa were formed without dissociation between head and flagels as described by Radigue *et al.* (1988). Other microscopy sections were observed (100 for each group), and all stages of the seminiferous epithelium cycle were comparable with those of group C sections. The stages observed, after 49 d, indicated that the spermatogenic cycle presented no abnormalities (different size or number of cells or incomplete steps) and that the morphological pattern was unchanged.

DISCUSSION

Our results showed that group C values varied with age; therefore, it was necessary to compare the values of experimental groups with group C values on the same day of experiment. Moreover, as the animals were different from one time-point to another, inter-individual variations could explain some of the changes in each group during the experiment and between the groups at the same time-point. Nevertheless, the comparisons are relevant because the values of the five groups were measured in parallel, under the same conditions.

In previous papers (Bertrand & Belleville, 1988, 1989) we have shown that nutritional efficiency varied with diet, though the energetic intake per kg body-weight was similar between the four experimental groups. The highest nitrogen digestibility coefficients were obtained with diets C and rC, the lowest with diet RCM followed by diet MCM and then diet MSM. Apparent lipid digestibility coefficient values were of the same order. The highest N balances were obtained with diets C and rC, followed by diets MSM and RCM and the lowest values with diet MCM. Moreover, diets MSM and RCM involved the highest protein intake, while diets MCM and rC promoted a more elevated lipid consumption. These findings might explain why the highest body-weight gain occurred with diets MSM and RCM and the lowest with diets MCM and rC.

Relative liver weights were not modified by PM and increased more in the experimental groups during the BR period than in group C. This indicates that liver growth was facilitated by the four rehabilitation diets. However, relative liver weights decreased with age in all groups. At the end of the experiment, rats on diets MSM and rC had the highest relative liver weights, in comparison with those of rats on the diets containing cottonseed flour. These findings might result from higher levels of isoleucine and leucine in diets rC and MSM. However, at this time, the highest relative liver weights were associated with the lowest protein concentrations.

The liver lipid content was increased in the protein-depleted group. It has already been shown that liver steatosis induced by PM is due to reduced serum very-low-density-lipoprotein apolipoprotein levels which impair the transport of liver triacylglycerols and lead to an accumulation of liver lipids (Meghelli-Bouchenak *et al.* 1987). From day 2 of refeeding, the lowest lipid hepatic level in group RCM might be due to a lower lipid intake because diet RCM had the lowest lipid content (Table 2).

After 1 month of a low-protein diet, liver DNA and RNA levels in group C and protein-depleted rats were similar. In the protein-depleted group a normal level of hepatic RNA with a normal hepatic protein level might indicate that the mRNA was accurately translated due to decreased amino acid bioavailability. At the end of the experiment, in rats fed on both diets containing cottonseed flour, the hepatic RNA levels and RNA:DNA values were highest, while the protein:RNA values were the lowest, suggesting that, at this time, transcription of RNA might have been raised. These diets supported an increase in hepatic RNA synthesis or a decrease in RNA catabolism, or both, as confirmed by the rise in RNA per cell. Protein and RNA levels were not impaired but protein:RNA was decreased, indicating that transduction was diminished with these diets. However, transcription was not impaired since the hepatic protein contents were not decreased with either cottonseed-flour diet.

In the liver, according to sample times, our results illustrate three different patterns: when DNA per g liver was increased, for example, in group MCM at day 2 of refeeding, cell number per tissue unit was higher and consequently cell size was decreased. In this case, DNA was high whereas RNA was low. These variations indicate that cell division prevailed over protein synthesis. This characterizes hyperplasia. After 2 d of refeeding, with other

diets such as diet RCM the opposite phenomenon was observed, cells were large but less numerous. Their RNA content was elevated, proving that protein synthesis prevailed over cell division. This characterizes hypertrophy. In intermediate cases such as diet MSM, after 2 d of refeeding, medium protein and nucleic acid levels were observed. The relative importance of cell division and protein synthesis were equilibrated.

In previous reports (Narce *et al.* 1988; Meghelli-Bouchenak *et al.* 1989) we have discussed the changes in fatty acid composition of total hepatic lipids in protein-depleted groups, and in the present study the same results have been obtained. In particular the level of 18:2 $n-6$ was increased and that of 20:4 $n-6$ was diminished. A period of 2 d of BR was sufficient to restore most of the fatty acid levels in total lipids to group C values, except for 22:4 $n-6$ and 22:5 $n-6$ levels which were raised again, probably because the 18:2 $n-6$ intake was increased by the four rehabilitation diets. Moreover, these results indicate that PM has immediate effects on $\triangle$ 6- and $\triangle$ 5-hepatic microsomal desaturases which are involved in the transformation of 18:2 $n-6$ into 20:4 $n-6$. Narce *et al.* (1988) have proved that protein malnutrition decreases the activities of $\triangle$5- and $\triangle$6-desaturases, but at day 2 of refeeding, changes in metabolic state involve a rebound effect of hepatic desaturase activities which are higher than group C values. The lowest (not significant) concentrations of 18:2 $n-6$, 20:4 $n-6$, 22:4 $n-6$ and 22:6 $n-3$ in group RCM rat liver at day 2 of refeeding might be explained by the reduced levels of 18:2 $n-6$ and 18:3 $n-3$ in these diets and a lower intake. At day 14 of refeeding, in rats fed on diets containing vegetable proteins, the decreases in 14:0, 16:0, 16:1 $n-7$, and 18:1 $n-9+18:1$ $n-7$ levels in hepatic lipids might be due to their hepatic lipid contents and their lipid digestive efficiencies which were lower than those promoted by diet rC. The richest 22:6 $n-3$ level obtained with diet MSM was probably attributable to its higher 18:3 $n-3$ content.

Liver micrographs observed after 49 d of refeeding, revealed no cytotoxic effects of gossypol at the low dose of 0·6 g/kg heated cottonseed flour in diets MCM and RCM. Size and form of hepatocytes were unchanged compared with hepatocytes of rats receiving the casein diet without gossypol. Our histological study confirmed the absence of impairment on hepatic variables caused by glandless cottonseed flour.

At the end of the experiment, rats had become sexually mature. Ghafoorunissa (1980) studied PM effects on testis which indicated that malnutrition inhibits testis weight gain. Seminiferous scores were decreased compared with group C, seminiferous tubules were degenerated and spermatocytes were located in the seminiferous lumen. At sexual maturity, the spermatogenesis cycle was blocked at the stage reached previously. Gossypol at 20 mg/kg body-weight is highly effective in reducing sperm (Hadley *et al.* 1981). Rats treated with increasing daily doses of gossypol (0, 1, 5, 10 mg/kg) for 1 week revealed that gossypol has a direct inhibitory effect on steroidogenesis (Lin *et al.* 1981). Gafvels *et al.* (1984) have shown that gossypol at 1 mg/kg body-weight, for 5 weeks, had no effect on sex organ weights, blood flow to testes and morphology of testes. A 10 mg/kg dose caused signs of tubular degeneration, reduced testosterone concentration, and involution of the ventral prostate and seminal vesicles.

The findings of Chinese scientists on the anti-fertility effect of sub-toxic doses of gossypol (Sang, 1983; Quian & Wang, 1984) in man and male animals have changed the concept of gossypol toxicity. Its use as a contraceptive for humans has been confirmed but its toxicity and various side-effects on humans have hampered further human and animal studies. Studies regarding its mechanism as an anti-fertility compound have shown that gossypol decreases or inhibits sperm motility (Hadley *et al.* 1981; Liu *et al.* 1981; Chongthammakun *et al.* 1986), degenerates tubular epithelium in rats (Xue, 1981), alters the ultrastructure of mature spermatozoa (Hadley *et al.* 1981; Hoffer, 1982) and reduces spermatozoal substrate utilization (Tso & Lee, 1982).

Raghunah & Giridharan (1987) stated that gossypol is cytotoxic to spermatocyte and spermatozoon.

In our study, analysis of testis micrographs, showing various developed stages indicated that the spermatogenesis cycle was complete. These stages of seminiferous tubule cycles were not sensitive in the long-term to traces of gossypol. Another study on sperm motility is required before we can conclude that no apparent effect is observed at low doses. Presently, it can be stated that after PM, low intakes of gossypol do not involve any toxic effects, therefore the type of cottonseed flour used in the present investigation can be added to rehabilitation diets.

Conclusion

BR by diets containing cottonseed flour, shows that 0·06 g gossypol/kg in heated flours, with a high level of cottonseed protein, is too low to develop severe toxicity symptoms in liver weakened by a long period of PM. More especially, cottonseed diets do not impair hepatic division and protein synthesis. Only hepatic RNA levels are higher in rats fed on diets containing cottonseed flour for 49 d, in comparison with those containing casein or soya-bean flour. Moreover, in our experimental conditions, no change in liver and seminiferous tubule morphology was observed after 49 d of the recovery phase with cottonseed diets containing a low gossypol content.

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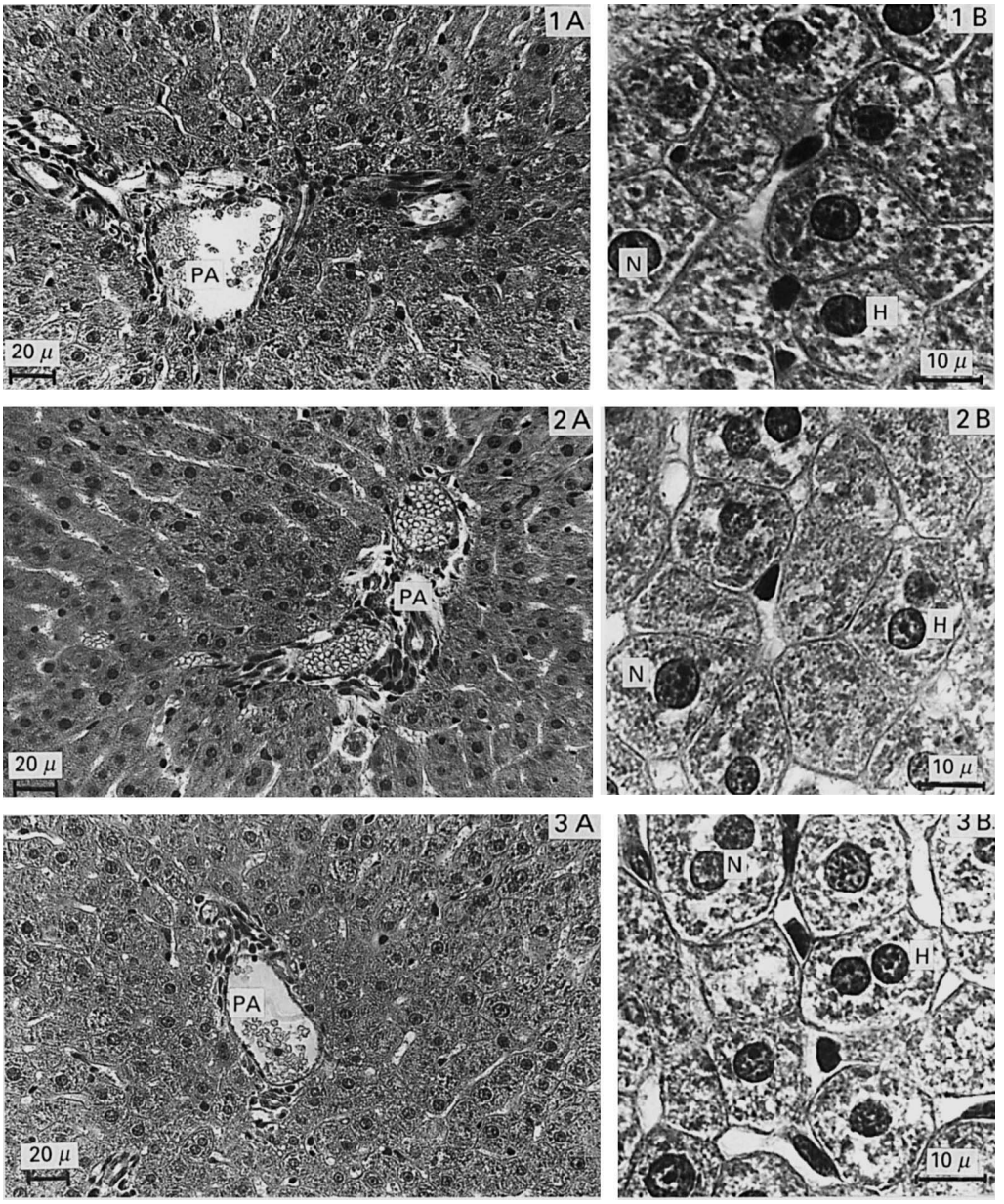

Plate 1. Liver structure of rats fed on control (1 A, 1 B), maize–cottonseed flour–milk (2 A, 2 B) and rice–cottonseed flour–milk (3 A, 3 B) diets (for details of composition, see Tables 1 and 2 and pp. 270–271) after a period of protein depletion. PA, portal area formed by a hepatic artery, bile duct and a hepatic portal vein; N, nucleus; H, hepatocyte.

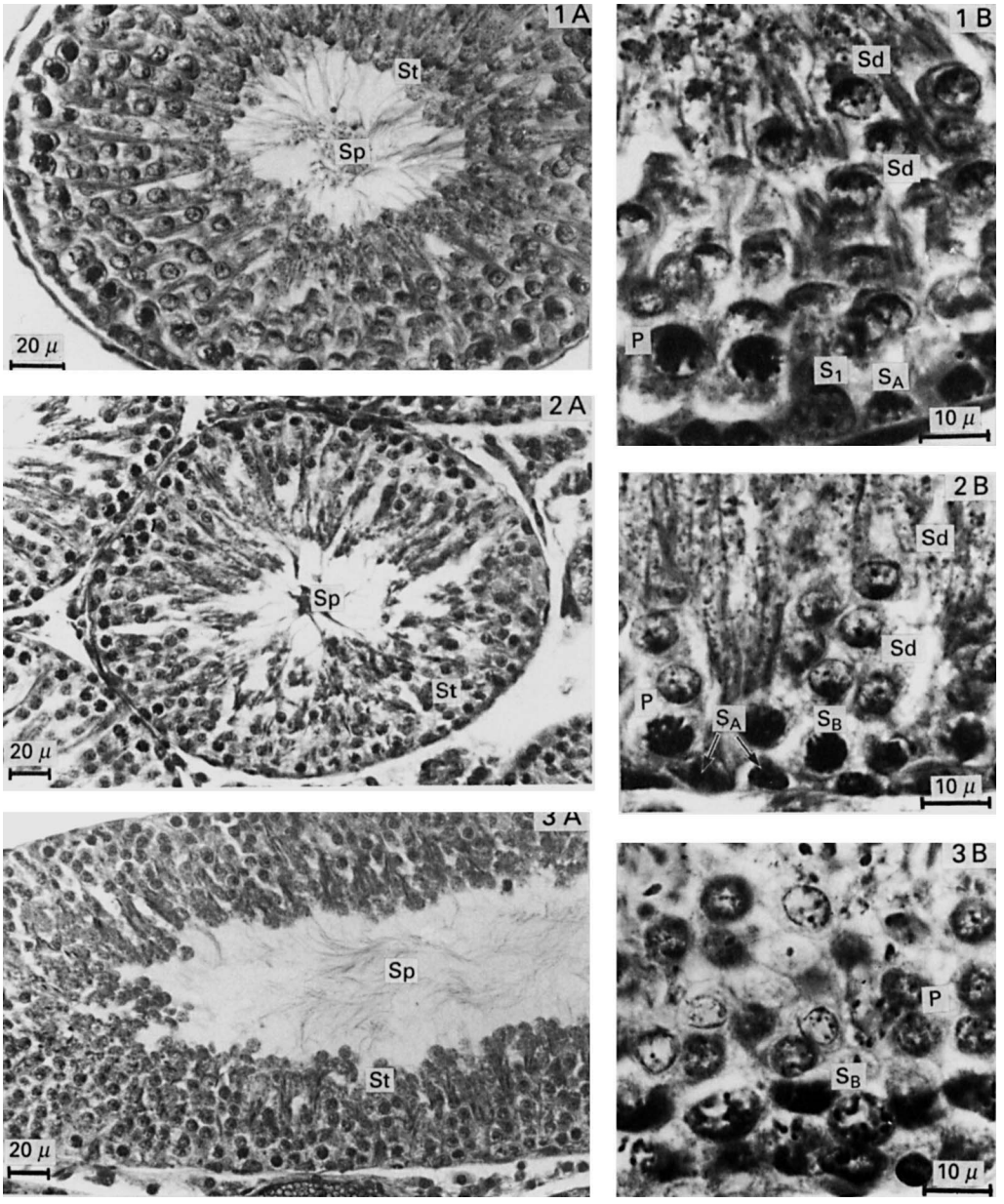

Plate 2. Testis seminiferous tubule of rats fed on balanced control diet (1 A, 1 B), maize–cottonseed flour–milk (2 A, 2 B) and rice–cottonseed flour–milk (3 A, 3 B) diets (for details of composition, see Tables 1 and 2 and pp. 270–271) after a period of protein depletion. St, seminiferous tubule; S_A, spermatogonia A; S_B, spermatogonia B; S_1, primary spermatocyte; P, pachytene stadis; Sd, spermatid; Sp, spermatozoa.

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