THE EFFECTS OF DIETARY PROTEIN, FAT AND CHOLINE ON THE COMPOSITION OF THE LIVER CELL AND THE TURNOVER OF PHOSPHOLIPIN AND PROTEIN-BOUND PHOSPHORUS

by

ROSA M. CAMPBELL* AND H. W. KOSTERLITZ

Physiology Department, Marischal College, Aberdeen (Scotland)

INTRODUCTION

It was shown recently that the deoxyribonucleic acid (DNA) content of the liver of the adult rat varies linearly with the body weight and that it is not affected by variations in the protein content of the diet. DNA P was therefore suggested as a suitable reference standard for other liver constituents[1]. It was further found that, when in protein-deficiency part of the phospholipin P and ribonucleic (RNA) P had been lost from the liver, the turnover of the remaining phospholipin P and RNA P appeared to be increased[2].

In the present paper, further work is reported which confirms and extends the previous findings on the effects of diet on the composition of the liver. An examination is also made of the effects of variations in dietary protein, fat and choline on the renewal rate of phospholipin P and protein-bound P in the liver.

MATERIALS AND METHODS

Animals

Hooded male rats of the Rowett Institute strain, 3–4 months old, were fed on diets with different protein, fat and choline contents. The basal low-fat diet consisted of 2% agar, 3% salts No. 351[3], 10% cooking fat and 85% sucrose and the basal high-fat diet of 2% agar, 3% salts, 40% cooking fat and 55% sucrose. Where casein (vitamin-free, Glaxo) was given it replaced an equal weight of sucrose. The fat was supplemented with vitamins A, D and E[4]. Immediately before feeding time at 4 p.m. each rat was injected subcutaneously with 0.5 ml containing the following water-soluble vitamins: 5 μg biotin, 5 μg folic acid, 20 μg synkavit dicalcium phosphate (Roche), 30 μg aneurin, 30 μg pyridoxin, 50 μg riboflavin, 100 μg calcium d-pantothenate, 100 μg nicotinic acid, 100 μg *p*-aminobenzoic acid, 1 mg inositol, and 17.3 mg choline hydrochloride. The rats on the choline-deficient diet received a similar mixture, but without choline. The general management was the same as described previously[4].

Sampling of tissues for analysis and analytical methods

These have been described previously[4,5].

Turnover of phospholipin P and protein-bound P

The following terms have been used (HEVESY[6]). Specific activity (S.A.): ^{32}P (radioactive counts as % of injected counts)/^{31}P (mg). Relative specific activity (Rel. S.A.) of phospholipin P, etc.: S.A.

* Carnegie Senior Scholar, 1947–'49.

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of phospholipin P, etc./S.A. of inorganic P. Turnover time of phospholipin P (hr): (mean S.A. of precursor P—mean S.A. of phospholipin P)/increase in S.A. of phospholipin P per hr (ZILVERSMIT, ENTENMAN, AND FISHLER[7]). Turnover rate of phospholipin P (mg/hr): total phospholipin P (mg) in liver/turnover time (hr) of phospholipin P. Percentile turnover (%/hr): % of total phospholipin in liver turned over in 1 hr.

The effect of changes in the S.A. of inorganic P on the S.A. of phospholipin P can to a great extent be eliminated by calculating the Rel. S.A. of phospholipin P, a term which is a function of the percentile turnover of phospholipin P. In order to obtain a measure of the total amount of phospholipin turned over in a given time, and to be able to compare the values from rats with widely differing liver phospholipin contents, the Rel. S.A. of phospholipin P has to be multiplied either by the ratio phospholipin P/body weight (= total relative activity/body weight, CAMPBELL, Olley, AND BLEWETT[8]) or by the ratio phospholipin P/DNA P (= total relative activity, CAMPBELL AND KOSTERLITZ[2]). In view of the increasing evidence in favour of a constant DNA P content of the cell nucleus (VENDRELY AND VENDRELY[9], DAVIDSON AND MCINDOE[10], MIRSKY AND RIS[11]) and the fact that the number of liver cells and the DNA P content of the liver of the adult rat remain unaffected by dietary variations (KOSTERLITZ[4], MANDEL, JACOB AND MANDEL[12]), it is now proposed to call the product, (Rel. S.A. of phospholipin P) × (phospholipin P/DNA P), Rel. S.A. per unit of liver cells.

13–17 μc. $Na_2H^{32}PO_4$ with 1–1.5 mg ^{31}P in 0.4–0.6 ml were injected subcutaneously, usually 6 hr before the rats were killed at 2 p.m. When specific activity—time curves were constructed, rats were also killed 45, 90 and 180 min after administration of ^{32}P. In all experiments with ^{32}P, food left over at 9 a.m. was removed daily throughout the feeding period, to induce the animals to eat their food between the hours of 4 p.m. and 9 a.m. In this way, the livers were analysed in the post-absorptive state.

The ^{32}P determinations were carried out in most experiments on solid samples in small nickel dishes, with a Geiger-Müller counter. In a small number of experiments, ^{32}P was estimated in liquid samples with the liquid counter described by VEALL[13].

For estimation of inorganic ^{31}P and ^{32}P, 2 g liver were finely ground with 18.6 ml 5% (w/v) trichloroacetic acid (TCA) in successive amounts, to give a final dilution of 1 in 10. Inorganic P was precipitated in 5 ml of this extract with an ammonia-magnesium mixture (LOHMANN[14]) and then redissolved in 0.5 ml *N* H_2SO_4 and made up to 5 ml with water. ^{31}P was estimated in 2 ml and ^{32}P in 0.2 ml samples which were dried on a sand bath.

The liver residue was extracted another three times with 10 ml of 5% TCA; a fifth extraction yielded only negligible quantities of acid-soluble ^{32}P. The residue was then extracted twice with 20 ml cold ethanol, twice with 20 ml ethanol-ether (3:1, v/v) brought to boiling point for 1 min, and once with warm ether. The extracts were made up to 100 ml. Phospholipin ^{31}P was estimated in 3 ml of this extract, and phospholipin ^{32}P in 1 ml after drying on the sand bath.

After the solvent-extracted liver residue had been dried *in vacuo* over $CaCl_2$, DNA^{31}P and RNA^{31}P were estimated by the method of SCHMIDT AND THANNHAUSER[15], modified as follows. The liver residue was digested with 20 ml *N* KOH at 37° C for at least 15 hr. In 5 ml of the digest, DNA was precipitated by adding 1 ml 6*N* HCl and 5 ml 5% (w/v) TCA. After centrifuging, RNA^{31}P was estimated in 1 ml of the supernatant fluid (RNA fraction). The precipitate containing DNA was washed three times with 5% TCA, dissolved in 3 ml *N* KOH and the solution made up to 15 ml with water. DNA^{31}P was estimated in 5 ml samples.

Although this procedure is satisfactory for the estimation of RNA^{31}P and DNA^{31}P, it has been shown that the RNA fraction is contaminated with phosphorus-containing substances, present only in minute quantities but having a high concentration of ^{32}P (DAVIDSON, GARDNER, HUTCHISON, MCINDOE, RAYMOND, AND SHAW[16], JEENER[17], JEENER AND SZAFARZ[18], DAVIDSON, FRAZER, AND HUTCHISON[19]). For this reason, the ^{32}P present in the RNA fraction will be referred to as crude RNA^{32}P. It has been found convenient to estimate crude RNA^{32}P in the dried solvent-extracted liver residue before fractionation into RNA and DNA, the contribution made by DNA^{32}P being of the order of only 1% of the total activity. Therefore, the S.A. of crude RNA P is protein-bound ^{32}P/RNA^{31}P. A part of the contamination of the RNA fraction is due to the quantitatively negligible phosphoprotein P which has a very high specific activity (DAVIDSON *et al.*[16], JEENER[17]). It is likely that its presence is at least partly due to adsorption of inorganic P by protein (JEENER AND SZAFARZ[18]). In order to obtain an estimate of the activity of the phosphoprotein fraction, approximately 0.13 mg of ^{31}P were added as KH_2PO_4 to 5 ml of the RNA fraction, the inorganic P precipitated by the method of LOHMANN[14] and the ^{32}P determined in the precipitate. The activity found is taken as that of the phosphoprotein fraction. By deducting these counts from the total counts present in the RNA fraction, the S.A. of crude RNA P was considerably lowered. Although the new value is probably still greater than the true S.A. of RNA P, it gives a first approximation and will be designated S.A. of apparent RNA P.

In order to obtain a measure of DNA^{32}P, the following procedure was adopted: the precipitate obtained by adding 1 ml 6 *N* HCl and 5 ml 5% TCA to 5 ml of the alkaline digest to the dried liver

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residues was dissolved in 5 ml *N* KOH and reprecipitated by again adding 6 *N* HCl and 5% TCA. This procedure was repeated three times and finally the precipitate was dissolved in ethanol and transferred in successive small quantities to a nickel dish and dried.

Statistical procedure

The methods of FISHER were used[20]. The significance of a difference is expressed as the probability *P* of an observed difference occurring at random in a homogeneous population.

RESULTS

The DNA P content of the liver cell

It has been shown recently that the DNA P content of the liver of the adult male rat increases linearly with increasing body weight and is not affected by variations in the amount of dietary protein (CAMPBELL AND KOSTERLITZ[1]). The slope of the regression line obtained from the present results (138 rats) by plotting liver DNA P against body weight was not different from that obtained previously but the vertical distances from the abscissa were not identical. The regression equation for the present series was calculated to be DNA P (mg) = 0.00550 × body weight (g) + 0.77 (Fig. 1) and that for the previous series DNA P = 0.00550 × body weight + 0.66, the difference of the absolute terms, 0.11 ± 0.021, being highly significant. This means that the DNA P content of the liver of a 300 g rat of the same colony was now 5% greater than two years ago.

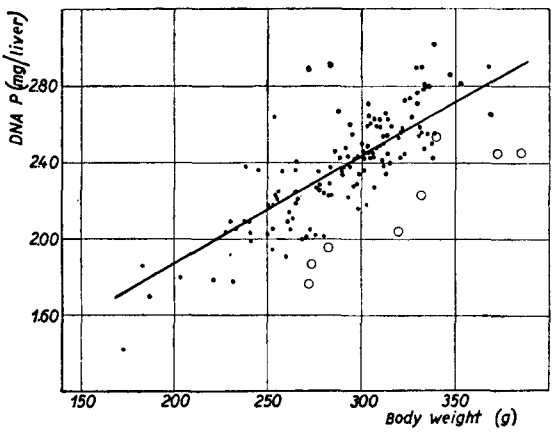

Fig. 1. Regression of DNA P in the livers of male rats on initial body weight. The line is drawn from the regression equation DNA P = (0.00550 ± 0.00028) × body weight + 0.77. The open circles indicate values obtained from rats which had been fed on the 32% casein 40% fat diet for 4 weeks. These values were not utilised for the calculation of the regression equation.

In order to discover any possible dietary effects on liver DNA P, variations in DNA P caused by variations in body weight had to be eliminated; *i.e.* the DNA P values had to be adjusted to the mean body weight. This was done by multiplying the differences between the actual and mean body weights with the regression coefficient and by adding to or subtracting from the actual DNA P values the products thus obtained. These adjusted values were then grouped according to the different dietary treatments (Table I). No effects were discernible which might have been due to fasting up to 48 hr, or to variations in the protein, fat or choline contents of the diet. The rats which had been fed on the protein-free diet for several weeks lost 40 to 50 g in body weight; this had no influence on the DNA P content of the liver.

The only results not fitting in were those obtained from rats which had been fed on the diet containing 32% casein and 40% fat for four weeks. These animals increased their weight during the experimental period by an average of 19 g per week, an abnormally high value. The carcass was not analyzed but it was considered likely that these

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rats formed more adipose tissue than animals on the stock diet and that therefore the observed body weight was in excess of the weight determining the DNA P content of the liver.

TABLE I

DNA P VALUES IN LIVERS OF RATS FED ON DIFFERENT DIETS

Diet (prior to fast in fasted rats)	*Duration Weeks (duration of fast in hr)*	*No. of rats*	*Mean max. body wt. (g)*	*Mean DNA P (mg/liver)*	*Mean DNA P adjusted to body wt. of 292 g (mg/liver)*
a. *High-fat (40%), with choline*					
32% casein	1	5	279	2.28	2.35
32% casein	4	4	327	2.22	2.03*
8% casein	1	4	300	2.44	2.40
8% casein	4	4	278	2.36	2.44
Protein-free	1	4	266	2.12	2.26
Protein-free	2	4	230	2.98	2.32
Protein-free	3	4	234	1.97	2.29
				Mean (without second value)	2.34
b. *High-fat (40%), without choline*					
32% casein	1	5	289	2.40	2.42
32% casein	4	4	318	2.10	1.96*
8% casein	1	4	292	2.34	2.34
8% casein	4	4	268	2.34	2.47
Protein-free	1	4	263	2.13	2.29
Protein-free	2	4	231	2.08	2.42
Protein-free	3	4	233	2.09	2.41
				Mean (without second value)	2.39
c. *Low-fat (10%), with choline*					
Stock	—	6	304	2.36	2.27
23% casein	1	8	317	2.56	2.42
40% casein	1	18	310	2.58	2.48
Protein-free	1	32	309	2.47	2.38
				Mean	2.40
d. *Fasted rats after 1 week on low fat diets*					
23% casein	24 (hr)	4	301	2.35	2.30
Protein-free	24	4	284	2.26	2.30
23% casein	48	4	299	2.47	2.43
Protein-free	48	4	299	2.38	2.35
				Mean	2.35

* The regression coefficient for (a) and (b) combined was 0.00678 ± 0.00077, for (c) 0.00595 ± 0.00085 and for (d) 0.00681 ± 0.00209. These coefficients did not differ significantly from each other, nor from a previous estimate of 0.00518 ± 0.00032. The final estimate therefore was 0.00550 ± 0.00028, which was used for the adjustment of the DNA P values to the mean body weight of 292 g. The body weight values were the final ones if there was no loss in weight; otherwise the initial values were taken. The values marked with an asterisk differ significantly ($P < 0.01$) from all others.

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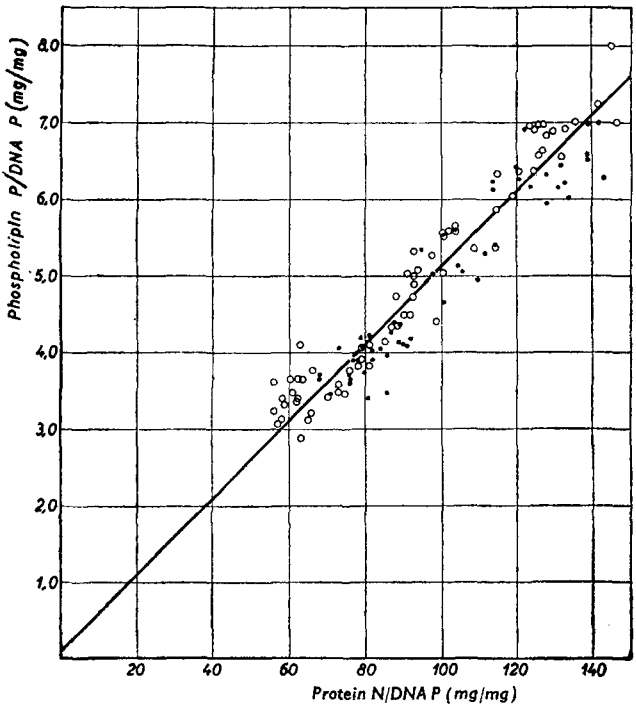

Fig. 2. Regression of the phospholipin P content of a unit of liver cells on its protein content. The open circles represent rats on a 40% fat diet and the dots rats on a 10% fat diet. The line is drawn from the regression equation Phospholipin P/DNA P = (0.0497 ± 0.0013) × protein N/DNA P + 0.114.

The absence of dietary effects on liver DNA P justifies its use as a reference standard for other liver constituents. Therefore, all analytical data in this paper will be expressed per 1 mg DNA P.

Effect of diet on the phospholipin P content of the liver cell

Previous findings that the phospholipin content of a liver cell (phospholipin P/DNA P) varies directly with its protein content (protein N/DNA P) and thus with the amount of protein in the diet have been confirmed (Fig. 2). The influence of dietary factors other than protein were tested by the principle employed for the examination of dietary effects on the DNA P content. After elimination of the effects of changes in the protein content by adjusting the values of phospholipin P/DNA P to the mean value of protein N/DNA P, a difference was found between the phospholipin contents of the livers of rats fed on the high and low fat diets (Table II). Thus, when the protein contents were identical, the liver cells of the rats fed on the high fat diets contained more phospholipin P than those fed on the low diets. There was however no significant difference between the rats fed on the diets with and without choline.

TABLE II

EFFECTS OF VARIATIONS IN DIETARY FAT AND CHOLINE ON THE PHOSPHOLIPIN P OF RATS' LIVER CELLS

Diet	*No. of rats*	*Mean phospholipin P/DNA P, adjusted to protein N/DNA P of 97.1 (mg/mg)*
10% fat with choline	30	4.71
40% fat with choline	30	5.03
40% fat without choline	30	5.19

The values phospholipin P/DNA P were adjusted to the mean value of protein N/DNA P of 97.1 by using the regression coefficient 0.0497 ± 0.0013 (S.E.). Values of P: 40% fat with choline v. 40% fat without choline: > 0.05; 10% fat with choline v. 40% fat with choline: < 0.001.

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Effect of diet on the RNA P content of the liver cell

It has been found previously that the RNA P content of the liver cell (RNA P/DNA P) is a linear function of its protein content (protein N/DNA P) and thus of the protein content of the diet (CAMPBELL AND KOSTERLITZ[1]). The slope of the regression lines calculated from the present results was not different from that obtained previously, the regression coefficients being 0.02816 ± 0.000761 (S.E.) and 0.02763 ± 0.00061 respectively. After adjustment of the values of RNA P/DNA P to to the mean value of protein N/DNA P, it was found that fasting up to 24 hr and variations in the fat content of the diet had no effect on the RNA P content of the liver cell. Choline deficiency caused a slight rise above the average, while fasting for 48 hr. led to a loss of RNA P which was greater than could be accounted for by the loss of protein N (Table III).

TABLE III

EFFECTS OF DIETARY VARIATIONS ON THE RNA P CONTENT OF RATS' LIVER CELLS

Diet	*No. of rats*	*Mean RNA P/DNA P, adjusted to protein N/DNA P of 97.1 (mg/mg)*
40% fat with choline	39	4.02
40% fat without choline	30	4.16
10% fat with choline	38	3.97
24 hr fast after 10% fat	8	3.99
48 hr fast after 10% fat	8	3.70

The values RNA P/DNA P were adjusted to the mean value of protein N/DNA P of 97.1 by using the regression coefficient 0.02816 ± 0.000761 (S.E.). The value for 40% fat without choline is significantly higher and that for 48 hr fast lower than any of the other values ($P < 0.01$). The value of RNA P/DNA P for protein N/DNA P of 97.1 calculated from the regression equation obtained previously, RNA P/DNA P = 0.02673 × protein N/DNA P + 1.210, is 3.89 (CAMPBELL AND KOSTERLITZ[1]).

Effect of diet on the uptake of ^{32}P by inorganic P, phospholipin P, crude RNA P and DNA P

A preliminary graphical examination of the data obtained for the S.A.'s of inorganic P, phospholipin P, crude RNA P (*cf.* Methods) and DNA P indicated that the S.A.'s of the first three compounds, but not of DNA P, varied inversely to the changes in protein N/DNA P. It was also apparent that the relationship was not linear but that such linearity could probably be attained by converting the S.A. values to their logarithms. Further, it seemed likely that changes in body weight affected the S.A.'s. These assumptions were confirmed by calculating the multiple regressions of the log. S.A.'s of inorganic P, phospholipin P, crude RNA P and DNA P on body weight and protein N/DNA P (Table 4). For a graphic presentation of the results, it became necessary to eliminate the effects on the log. S.A. values of variations in body weight or in protein N/DNA P. This was done in the manner already described by adjusting the log. S.A. values to either mean body weight or mean protein N/DNA P. The results (Fig. 3) indicate that the log. S.A.'s of inorganic P, phospholipin P and crude NRA P increase

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with falling values of protein N/DNA P and thus with decreasing protein intake, while the log. S.A. of DNA P is not affected. The log. S.A.'s of all four compounds diminish with increasing body weight.

It was important to examine whether the described variations of the log. S.A.'s of phospholipin P, crude RNA P and DNA P were solely determined by the variations of log. S.A. of inorganic P, *i.e.* whether they were of similar magnitude. However, as indicated by the partial regression coefficients, the slopes of the regression lines for inorganic P were significantly smaller than those for the other compounds. This finding led to the conclusion that the log. Rel. S.A.'s of phospholipin and of crude RNA P also vary inversely to the body weight and protein N/DNA P, since log. Rel. S.A. of phospholipin P = log. S.A. of phospholipin P — log. S.A. of inorganic P (Table IV).

In order to test whether variations in the diet other than its protein content had any effect on the ^{32}P uptake by the various compounds, the log. S.A. and log. Rel. S.A. values had to be adjusted to the mean body weight and mean protein N/DNA P. From the adjusted values (Table V) it was

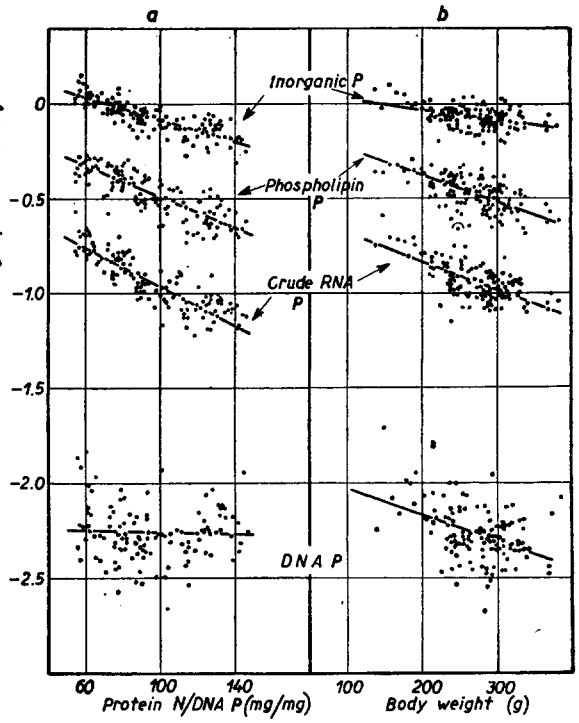

Fig. 3. Specific activities of inorganic P, phospholipin P, crude RNA P and DNA P in rats' liver cells: (a) shows the effects of varying protein contents of the liver cells, the log. S.A. values having been adjusted to a body weight of 272 g. (b) shows the effect of varying final body weights, the log. S.A. values having been adjusted to a protein N/DNA P ratio of 97.

TABLE IV

REGRESSIONS OF THE LOGARITHMS OF THE SPECIFIC ACTIVITIES OF INORGANIC P, PHOSPHOLIPIN P, CRUDE RNA P, DNA P AND OF THE RELATIVE SPECIFIC ACTIVITIES OF PHOSPHOLIPIN P AND CRUDE RNA P ON BODY WEIGHT (g) AND PROTEIN N/DNA P (mg/mg). (THE FIGURES IN BRACKETS ARE THE STANDARD ERRORS OF THE REGRESSION COEFFICIENTS)

log. S.A. of inorganic P	=	0.351 — 0.000538 × body wt. (± 0.000163)	— 0.002863 × protein N/DNA P (± 0.000297)
log. S.A. of phospholipin P	=	0.297 — 0.001357 × body wt. (± 0.000200)	— 0.004146 × protein N/DNA P (± 0.000367)
log. S.A. of crude RNA P	=	— 0.041 — 0.001491 × body wt. (± 0.000254)	— 0.005070 × protein N/DNA P (± 0.000465)
log. S.A. of DNA P	=	— 1.873 — 0.001339 × body wt. (± 0.000429)	— 0.000275 × protein N/DNA P (± 0.000785)
log. Rel. S.A. of phospholipin P	=	— 0.054 — 0.000819 × body wt. (± 0.000164)	— 0.001283 × protein N/DNA P (± 0.000300)
log. Rel. S.A. of crude RNA P	=	— 0.392 — 0.000953 × body wt. (± 0.000206)	— 0.002207 × protein N/DNA P (± 0.000377)

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concluded that variations in dietary fat and choline, and fasting up to 48 hr had no significant effect on the uptake of ^{32}P by phospholipin P and crude RNA P. On the other hand, when rats were allowed access to food during the 6 hr after injection of ^{32}P, a definite rise in the S.A. and Rel. S.A. of phospholipin P was observed, indicating that food intake as such increased the turnover of phospholipin P. A similar but less significant increase was found for the Rel. S.A. of crude RNA P in rats fed on a high fat diet and not fasted during the last 6 hr of the experiment. In the case of DNA P diet had no effect at all, with the exception of one value of rather doubtful significance.

TABLE V

THE EFFECTS OF VARIATIONS IN DIETARY FAT AND CHOLINE AND OF FASTING ON THE SPECIFIC ACTIVITIES AND RELATIVE SPECIFIC ACTIVITIES OF INORGANIC P, PHOSPHOLIPIN P, CRUDE RNA P AND DNA P IN THE LIVER. (THE MEAN S.A.'S AND REL. S.A.'S WERE OBTAINED BY ADJUSTING THE LOG. S.A.'S AND LOG. REL. S.A.'S TO THE MEAN VALUES OF BODY WEIGHT (271.7 g) AND PROTEIN N/DNA P (97.1) AND CONVERTING THE LOG.'S TO ANTILOG.'s)

Group No.	*Diet*	*No. of rats*	*Mean specific activity*				*Mean relative specific activity*	
			inorganic P	*phospho-lipin P*	*crude RNA P*	*DNA P × 100*	*phospho-lipin P*	*crude RNA P*
1	10% fat fed for 1 week or longer	22	0.87	0.329	0.116	0.50	0.378	0.133
2	10% fat fed for 1–4 days	8	0.75	0.370	0.111	0.49	0.494	0.149
3	10% fat food not removed when ^{32}P injected	8	0.91	0.407	0.126	0.56	0.448	0.138
4	40% fat without choline fed for 1 week or longer	30	0.84	0.311	0.113	0.66	0.370	0.135
5	40% fat with choline fed for 1 week or longer	30	0.87	0.326	0.116	0.55	0.376	0.133
6	40% fat with choline food not removed when ^{32}P injected	9	0.84	0.374	0.128	0.56	0.456	0.152
7	10% fat fed for 1 week followed by 24–48 hr fast	16	0.82	0.331	0.110	0.43	0.405	0.134

The following differences between the means are significant: S.A. of inorganic P: 1 v. 2 ($P < 0.01$); S.A. of phospholipin P: 1 v. 3 ($P < 0.001$), 4 and 5 v. 6 ($P < 0.001$); S.A. of DNA P: 4 v. 5 ($P < 0.05$); Rel.S.A. of phospholipin P: 1 v. 2 ($P < 0.001$), 1 v. 3 ($P < 0.01$), 4 and 5 v. 6 ($P < 0.001$); Rel.S.A. of crude RNA P: 4 and 5 v. 6 ($P < 0.05$).

It has already been stated in the section on Methods that the S.A. of crude RNA P, the RNA fraction of the Schmidt-Thannhauser method, must not be identified with the S.A. of RNA P. However, by deducting the counts due to "phosphoprotein" from the counts found for crude RNA P, values were obtained which were considered to be a first approximation to the true values of the S.A. of RNA P and were therefore designated the S.A. of apparent RNA P. When determined 6 hr after the injection of ^{32}P, the ratio, counts due to phosphoprotein/counts due to crude RNA P, appeared not to be affected by the protein content of the diet (Table 6). Therefore it is unlikely that the ^{32}P present in "phosphoprotein" had a significant influence on the values of the partial regression

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coefficients of log. S.A. and log. Rel. S.A. of crude RNA P on body weight and protein N/DNA P (Table 4). At any time between 45 and 360 min after the injection of ^{32}P, the Rel. S.A. of apparent RNA P was higher in rats fed on a protein-free diet than in those fed on a 40% casein diet (Table VI). The fact that variations in dietary protein affected the values of S.A. of apparent RNA P but not of DNA P, caused the ratio, S.A. of apparent RNA P/S.A. of DNA P, to change with changing protein contents of the diet.

TABLE VI

THE SPECIFIC ACTIVITIES OF INORGANIC P, APPARENT RNA P AND DNA P IN THE LIVERS OF RATS FED ON DIETS CONTAINING 40% OR NO CASEIN, DETERMINED AT DIFFERENT INTERVALS AFTER ADMINISTRATION OF ^{32}P

Time after injection of ^{32}P (min)	*No. of rats*	*Phosphoprotein/ crude RNA P counts/100 g liver*		*Specific activity*							
				Inorganic P		*apparent RNA P*		*DNA P*		*apparent RNA P/DNA P*	
		0% casein	40% casein	0% casein	40% casein	0% casein	40% casein	0% casein	40% casein	0% casein	40% casein
45	3	0.30	0.37	1.38	1.09	0.030	0.018	0.0014	0.0015	21	12
90	3	0.33	0.36	1.76	1.32	0.052	0.030	0.0023	—	23	—
180	4	0.26	0.29	1.65	1.09	0.091	0.046	0.0032	0.0033	28	14
360	4	0.15	0.16	0.96	0.67	0.111	0.056	0.0043	0.0042	26	13

Turnover of phospholipin P

For the calculation of the turnover time and rate of phospholipin P, specific activity—time curves were constructed for liver inorganic P, alkali-stable P and phospholipin P of rats fed on a protein-free diet and on a 40% casein diet (Figs. 4 and 5). Values were obtained 90, 180 and 360 min after injection of ^{32}P. When alkali-stable P, a measure of glycerophosphate P suggested by ZILVERSMIT, ENTENMAN AND CHAIKOFF[21], was used as precursor, the calculation of the turnover times gave 7.6 and 10.3 hr for the protein-free and 40% casein diets, respectively. When inorganic P

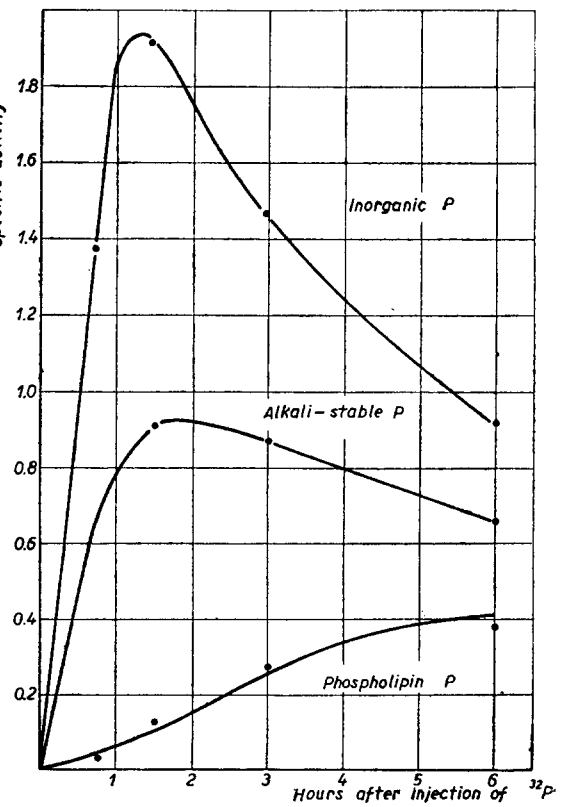

Fig. 4. Specific activity—time curves obtained from rats fed on a protein-free 10% fat diet for one week. Each value is the mean obtained from 4 rats. The turnover times, calculated for alkali-stable P as precursor, are 7.5 hr for the interval 1.5–3 hr and 7.6 hr for the interval 3–6 hr. The respective figures for inorganic P as precursor are 15.6 and 15.3 hr.

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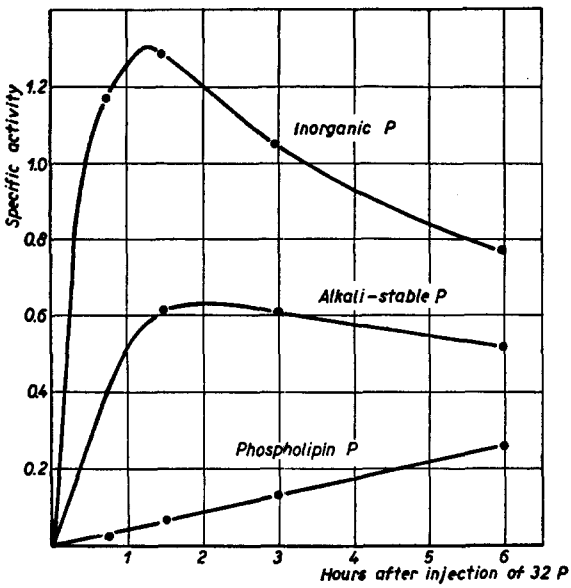

Fig. 5. Specific activity—time curves obtained from rats fed on a 40% casein, 10% fat diet for one week. Each value is the mean obtained from 4 rats. The turnover times, calculated fo alkali-stable P as precursor, are 10.6 hr for the interval 1.5–3 hr and 9.9 hr for the interval 3–6 hr. The respective figures for inorganic P as precursor are 23.1 and 17.6 hr.

was assumed to be the precursor, the figures were approximately twice as high, *viz.* 15.5 and 20.4 hr. Since the phospholipin P contents of the livers, calculated for a body weight of 300 g were 8.7 and 14.4 mg, respectively, the amount of phospholipin P renewed per hr, assuming alkali-stable P to be the precursor, was 1.15 mg in the rats on the protein-free diet and 1.4 mg in those on the 40% casein diet. It is impossible to state whether this difference is significant or not.

For this reason, another approach was made to this problem by calculating the Rel. S.A. of phospholipin P per unit of liver cells (*cf.* Methods). This term gives a measure of the turnover of phospholipin P in a given number of cells during a given time interval and thus makes it possible to assess approximately how far a loss of phospholipin P is compensated by an increased turnover. The effects of various dietary changes on the phospholipin P content, the Rel. S.A. and the Rel. S.A. per unit of liver cells are shown in Tables VII and VIII. It would appear that the loss of phospholipin P found after a fast of 24 hr, the intake of a protein-free diet for 4 days or of a diet containing 8% casein and 40% fat for one week was well compensated by an increased turnover of the remaining phospholipin P. However, a fast of 48 hr, a protein-free diet eaten for one week or longer or an 8% casein and 40% fat diet consumed for 4 weeks with or without choline led to a decrease in the Rel. S.A. per unit of liver cells. The only data which do not fit in are the normal values found in rats fasted for 24 hr after having been fed on a protein-free diet for one week. In the rats fed on the 40% fat diets, the presence or absence of choline had no effect, although the triglyceride content was considerably raised in the choline deficient rats.

DISCUSSION

The results reported in this paper give further support to the view that the DNA content of the liver of the adult rat is not readily influenced by changes in the composition of the diet. Previous work demonstrated the independence of liver DNA from the protein content of the diet (CAMPBELL AND KOSTERLITZ[1], MANDEL *et al.*[12]) and it has been shown now that neither dietary fat nor choline have any influence. The finding of DAVIDSON[22] that fasting has no effect on the DNA content of the liver has been confirmed. Further, variations of dietary protein, fat or choline do not affect the turnover of DNA P. Thus, DNA P is an excellent reference standard for investigations into the

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TABLE VII

THE INCORPORATION OF ^{32}P INTO LIVER PHOSPHOLIPIN P OF RATS FED ON LOW-FAT DIETS, WITH CHOLINE

Diet	*Phospholipin P*		
	/DNA P (mg/mg)	*Relative specific activity*	*Relative specific activity per unit of cells*
Stock	6.74	0.341	2.30
23% casein 1 week	6.29	0.334	2.10
23% casein 1 week, food not removed	6.24	0.360	2.25
23% casein 1 week, 1 day protein-free	5.22	0.417	2.18
4 days protein-free	4.15	0.532	2.21
Protein-free 1 week	3.83	0.399	1.53**
Protein-free 1 week, food not removed	3.84	0.485	1.86*
24 hr fast after 1 week 23% casein	5.23	0.389	2.03
48 hr fast after 1 week 23% casein	4.37	0.389	1.70**
24 hr fast after 1 week protein-free	3.99	0.507	2.02
48 hr fast after 1 week protein-free	3.79	0.449	1.70**

The values represent the means of 4 rats, or of 5 rats in the group on the protein-free diet for one week. The S.D. of the relative specific activity of phospholipin P per unit of cells was 0.255, determined with 78 degrees of freedom.

* and ** = significantly less than 2.22, the mean of 12 estimations in rats fed on the stock diet or 23% casein diet. * = $P < 0.05$ and ** = $P < 0.01$.

metabolism of the liver, an organ whose weight can increase or decrease very rapidly due to changes in its glycogen, protein, fat and water contents.

It has been shown by MANDEL *et al.*[12] that, in adult rats, prolonged protein-deficiency affects neither liver DNA nor the number of nuclei in the liver, so that the average DNA content of a liver nucleus remains constant. Recently, ELY AND ROSS[23] have found that, in the young growing rat, the average DNA content of a liver nucleus increases when growth is retarded or suppressed by a reduction or the absence of dietary protein. It would appear unlikely that these findings apply to the adult rat.

It is known that the quantity and quality of dietary protein determines the protein content of the liver cell and, since the ratio liver protein N/phospholipin P remains approximately constant over a wide range, also its phospholipin content (KOSTERLITZ[4], CAMPBELL AND KOSTERLITZ[1]). It has now been found that, in the post-absorptive state, dietary fat influences the phospholipin content of the liver cell much less than dietary protein, while the choline content of the diet has no effect at all. This latter fact is in agreement with the findings of FISHMAN AND ARTOM[24,25] who, however, found that under certain conditions choline supplementation may cause a rise in the choline-containing fractions of the phospholipins with a corresponding fall in the fraction containing no choline. The fact that the phospholipin content of the liver is mainly determined by the protein content of the diet may possibly be one of the reasons for the

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TABLE VIII

THE INCORPORATION OF ^{32}P INTO LIVER PHOSPHOLIPIN P OF RATS FED ON HIGH-FAT DIETS, WITH AND WITHOUT CHOLINE

Diet	*Phospholipin P*			
	/DNA P (mg/mg)	*Relative specific activity*	*Relative specific activity per unit of cells*	*Triglycerides/ DNA P (mg/mg)*
32% casein 1 week with choline	5.99	0.342	2.05	109
32% casein 1 week without choline	6.52	0.337	2.20	142
32% casein 4 weeks with choline	6.76	0.282	1.91	106
32% casein 4 weeks without choline	7.09	0.323	2.29	139
8% casein 1 week with choline	5.21	0.400	2.09	129
8% casein 1 week without choline	5.41	0.361	1.95	188
8% casein 4 weeks with choline	4.58	0.377	1.73**	268
8% casein 4 weeks without choline	4.94	0.350	1.73**	1660**
Protein-free 1 week with choline	3.74	0.458	1.71**	80
Protein-free 1 week without choline	3.58	0.464	1.66**	161**
Protein-free 2 weeks with choline	3.25	0.500	1.63**	119
Protein-free 2 weeks without choline	3.39	0.466	1.58**	194
Protein-free 3 weeks with choline	3.48	0.464	1.61**	270
Protein-free 3 weeks without choline	3.53	0.453	1.60**	592*

The values represent the means of 4 rats, or 5 rats in the groups fed on 32% casein for 1 week. The triglyceride values are geometric means. The S.D. of the relative specific activity of phospholipin P per unit of liver cells is 0.255, determined with 78 degrees of freedom, and that of the log. triglyceride 0.214, determined with 40 degrees of freedom.

* and **: in the case of relative specific activity of phospholipin P per unit of liver cells significantly less than 2.11, the mean of 18 estimations on rats fed on the 32% casein diet, and in the case of the triglycerides, significantly more than on the corresponding diets with choline. * = $P < 0.05$ and ** = $P < 0.01$.

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findings of BEST AND RIDOUT[26] and of CHANNON, MILLS AND PLATT[27], *viz.* that the lipotropic effect of casein cannot be explained by its methionine and cystine contents alone.

The determination of the absolute value of the turnover rate of phospholipin P is fraught with difficulties in view of the fact that the immediate precursor is not known with certainty. Assuming inorganic P to be the precursor, BOLLMAN, FLOCK AND BERKSON[28] found the percentile turnover of phospholipin P of rat's liver to be approximately 5%/hr, a figure which agrees with that obtained by us for rats fed on the 40% casein diet. ELLIOT AND HEVESY[29], determining the percentile turnover by a different method, found a value of approximately 8%/hr in 90 days old rats fed on the stock diet. Assuming that glycerophosphate is the immediate precursor of phospholipins, POPJÁK AND MUIR[30] found in rats weighing 200–250 g a turnover time of 6.75 hr, a value which agrees fairly well with that obtained by us in rats fed on a protein-free diet for one week. Our values of turnover time are likely to be somewhat too high since alkali-stable P, which was used for calculation following the suggestion of ZILVERSMIT *et al.*[21], may contain phosphorus compounds with a specific activity higher than that of glycerophosphate. However, even turnover time determinations using isolated glycerophosphate as precursor may give unreliable results, since OLLEY AND BLEWETT[31] have shown that the glycerophosphate fraction of rat's liver is not homogeneous.

Our finding that the turnover rate of phospholipin P is determined by the protein content of the diet while the effects of variations in dietary fat and choline are negligible, appears to be of importance for two reasons. First, in any work on the turnover rate of phospholipin P in the liver, the intake and utilisation of dietary protein must be carefully controlled, in order to exclude fortuitous results. Secondly, it is of interest that notwithstanding the considerable loss of phospholipin P from the liver in protein deficiency, the liver continues to maintain the rate of synthesis of phospholipin P at almost the normal rate.

The explanation of this phenomenon may possibly lie in the fact that the turnover rate of liver phospholipin P appears to a great extent to be governed by metabolic requirements of the body as a whole. The following facts support this view. The fall in metabolic rate after hypophysectomy reduces the Rel. S.A. of liver phospholipin P (FRAENKEL–CONRAT AND LI[32], GESCHWIND, LI AND EVANS[33]). Since in hypophysectomized rats the absolute amount of phospholipin is diminished, the turnover rate is also decreased. Administration of thyroxine raises the rate of phospholipin P synthesis in the liver while thiouracil lowers it (FLOCK, BOLLMAN AND BERKSON[34]). In pregnant rats, there is a steep rise in the Rel. S.A. as well as in the Rel. S.A. per unit of liver cells of phospholipin P during the third week of gestation. This rise is prevented by removal of the foetuses on the 14th day of pregnancy, even when the pregnant state of the maternal organism is maintained by allowing the placentae to continue to develop (CAMPBELL AND KOSTERLITZ[5]). The fact that the turnover rate in rats fed on a protein-free diet for longer than 4 days is lower than in those on the stock diet may possibly be due to the lowered metabolic rate found in such animals.

It is rather surprising that, when examined in the post-absorptive state, the amounts of fat and choline in the diet appear to have only little or no influence on the renewal of liver phospholipins. When the fat content of the diet is increased, the rate of ^{32}P uptake remains unaltered but the amount of phospholipin in the liver cells increases slightly so that the actual quantity of phospholipin synthesized per unit of time is raised. Our observations that choline has no effect, confirm similar results recently published by

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CAMPBELL *et al.*[8]. In contrast to these findings in the post-absorptive state, the single administration of a large dose of fat or choline increases the ^{32}P uptake by liver phospholipins (PERLMAN, RUBEN AND CHAIKOFF[35], PERLMAN AND CHAIKOFF[36]). It is possible that an increased synthesis may take place only during a relatively short time after the administration of fat or choline. As far as choline is concerned, however, there are substances which, without being lipotropic, increase the turnover rate of liver phospholipins, as *e.g.* cystine (PERLMAN, STILLMAN AND CHAIKOFF[37]), ethanolamine (PLATT AND PORTER[38], ARTOM AND CORNATZER[39]) and diethylethanolamine (CORNATZER AND ARTOM[40]).

It should be noted that the results presented in this paper deal with the overall synthesis of liver phospholipins. It is possible, however, that either the choline containing or the choline free phospholipins are preferentially affected. Further, in view of the differences found between the turnover rates of the phospholipin P in the nucleus, the large granules and the microsomes of the cytoplasm (ADA[41], ELLIOT AND HEVESY[29]), it would be of great interest to examine whether the phospholipins of these structures ar equally sensitive to variations in dietary protein or metabolic rate.

So far as the RNA P content of the liver is concerned, the results reported in this paper have, on the whole, confirmed our earlier findings, *viz.* that it is determined mainly by the protein content of the diet. When part of the RNA P is lost from the liver in rats fed on a protein-deficient diet, there is some evidence that the turnover of the remaining RNA molecules may be accelerated. However, these results have to be interpreted with caution since it is likely that the methods employed have not succeeded in excluding contaminants of high S.A. The Rel. S.A.'s of apparent RNA P (RNA fraction of the SCHMIDT–THANNHAUSER method minus phosphoprotein) and DNA P found by us are in fairly good agreement with those obtained by HAMMARSTEN AND HEVESY[42] for RNA P and DNA P isolated by the method of HAMMARSTEN[43], while JEENER[17] found somewhat lower values. The values of the ratio, S.A. of apparent RNA P/S.A. of DNA P, found by us are similar to those reported by HAMMARSTEN AND HEVESY[42] for isolated RNA and DNA, while DAVIDSON AND RAYMOND[44] and DAVIDSON *et al.*[19] found considerable lower values for the isolated nucleic acids. Although, for reasons stated above, our values are too high, it appears unlikely that this will vitiate the significance of our finding of an inverse relationship between the percentile turnover of crude RNA P (the P of the SCHMIDT AND THANNHAUSER RNA fraction) and the RNA P content of the liver.

The effect of body weight on the S.A.'s and Rel. S.A.'s of inorganic P, phospholipin P, crude RNA P and DNA P is of some interest. Although the experiments were not designed to test this particular point, it is rather striking that the partial regression coefficients of log. S.A. of phospholipin P, crude RNA P and DNA P on body weight are similar to each other and significantly greater than that of log. S.A. of inorganic P. In the latter case, the effect of body weight may possibly be explained by the fact that the rats fed on the protein-free diet lost weight and that the apparently increased rate of penetration of ^{32}P into the liver cells was due to an increase in the S.A. of plasma inorganic P which, however, was not determined. So far as phospholipin P, crude RNA P and DNA P are concerned, the most obvious explanation is a decrease in the turnover rates with increasing age. A similar interpretation was offered by ELLIOT AND HEVESY[29] and GESCHWIND *et al.*[33] for their findings, *viz.* a decrease in the Rel. S.A. of phospholipin P with increasing age.

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SUMMARY

1. The deoxyribonucleic acid P (DNA P) content of the liver of the adult rat and its turnover are not affected by variations in the protein, fat or choline contents of the diet. Thus, liver DNA P is well suited as a standard of reference for other liver constituents.

2. The phospholipin P content of a unit of liver cells is determined mainly by the dietary protein intake, a little by the fat intake and not at all by the choline content of the diet. The ratio, phospholipin P/protein N, is constant over a wide range of dietary protein intake.

3. The ribonucleic acid P (RNA P) content of a unit of liver cells is determined mainly by the protein content of the diet.

4. When measured in the post-absorptive state, the rate of uptake of ^{32}P by inorganic P, phospholipin P, crude RNA P (RNA fraction of the SCHMIDT AND THANNHAUSER method) and DNA P decrease with increasing body weight. The specific and relative specific activities of phospholipin P and crude RNA P rise with falling protein content of the diet but are unaffected by variations in dietary fat or choline. Calculation of the turnover time of phospholipin P indicates that, when in rats fed on a protein-free diet part of the phospholipin P has been lost from the liver cell, the remaining phospholipin P is turned over more frequently. The amount of phospholipin P turned over per unit of time in rats fed on a protein-free diet is nevertheless somewhat lower than in rats fed on a 40% casein diet.

5. Evidence is adduced for the view that the turnover rate of liver phospholipin P, when measured in the post-absorptive state, is not determined by intrahepatic conditions but by the metabolic requirements of the body as a whole.

RÉSUMÉ

1. La teneur en acide désoxyribonucléique P (DNA P) du foie de rat adulte et la quantité transformée de cet acide ne sont pas affectées par des variations de la teneur en protéine, graisse ou choline de la diète. Ainsi, le DNA P de foie est une substance de référence convenable pour d'autres constituants du foie.

2. La teneur en phospholipine P d'une unité de cellules hépatiques est déterminée surtout par la quantité de protéine de diète absorbée, un peu par la quantité de graisse absorbée, mais pas du tout par la teneur de choline de la diète. Le rapport phospholipine P/protéine N est constant dans un large domaine d'absorption de protéine de diète.

3. La teneur en acide ribonucléique P (RNA P) d'une unité de cellules hépatiques est déterminée surtout par la teneur en protéine de la diète.

4. Mesurée après l'absorption, la vitesse d'absorption de ^{32}P par le P inorganique, la phospholipine P, le RNA P brut (fraction RNA de la méthode de SCHMIDT ET THANNHAUSER) et le DNA P diminue lorsque le poids du corps augmente. L'activité spécifique et spécifique rélative de la phospholipine P et du RNA P brut augmentent lorsque la teneur en protéine de la diète baisse, mais elles ne sont pas affectées par des variations de la graisse et de la choline de la diète. Le calcul du temps de transformation de la phospholipine P indique que, lorsque, dans des rats nourris par une diète exempte de protéine, une partie de la phospholipine P a disparu de la cellule hépatique, la phospholipine P restante est transformée plus souvent. La quantité de phospholipine P transformée par unité de temps dans des rats nourris par une diète exempte de protéine est, cependant, un peu plus petite que dans des rats nourris par une diète à 40% de caséine.

5. Les auteurs apportent des arguments en faveur de l'idée que la vitesse de transformation de la phospholipine P du foie, mesurée après l'absorption, n'est pas déterminée par des conditions intrahépatiques, mais par les besoins métaboliques du corps tout entier.

ZUSAMMENFASSUNG

1. Der Gehalt der Leber der erwachsenen Ratte an Desoxyribonucleinsäure P (DNA P) und deren Umsatz werden durch Variationen des Protein-, Fett- und Cholingehaltes der Diät nicht beeinflusst. Deshalb ist Leber-DNA P als Vergleichssubstanz für andere Leberbestandteile sehr geeignet.

2. Der Phospholipin-P-Gehalt einer Einheit von Leberzellen wird hauptsächlich durch die Proteinaufnahme aus der Diät, ein wenig durch die Fettaufnahme und gar nicht durch den Cholin-

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gehalt der Diät bestimmt. Das Verhältnis Phospholipin-P/Protein-N ist in einem grossen Bereich von Proteinzufuhr aus der Diät konstant.

3. Der Ribonucleinsäure-P (RNA P)-Gehalt einer Einheit von Leberzellen wird hauptsächlich durch den Proteingehalt der Diät bestimmt.

4. Messungen im post-absorptiven Zustand haben gezeigt, dass die Geschwindigkeit der Aufnahme von ^{32}P durch anorganischen P, Phospholipin-P, rohe RNA P (RNA-Fraktion der Methode von SCHMIDT UND THANNHAUSER) und DNA P mit steigendem Körpergewicht abnimmt. Die spezifische und relative spezifische Aktivität von Phospholipin-P und roher RNA P steigen mit fallendem Proteingehalt der Diät, werden aber durch Variationen im Diät-Fett oder -Cholin nicht beeinflusst. Berechnungen der Umsatz-Zeit von Phospholipin-P zeigen dass, wenn in proteinfrei gefütterten Ratten ein Teil des Phospholipin-P aus den Leberzellen verschwunden ist, das restliche Phospholipin-P öfter umgestetzt wird. Die pro Zeiteinheit umgesetzte Menge Phospholipin-P in proteinfrei gefütterten Ratten ist trotzdem etwas niedriger als bei mit 40%-iger Casein-Diät gefütterten Ratten.

5. Es werden Beweise für die Ansicht angeführt, dass die Umsatzgeschwindigkeit von Leber-Phospholipin-P (im post-absorptiven Zustand gemessen) nicht durch intrahepatische Bedingungen sondern durch Stoffwechsel-Bedürfnisse des gesamten Körpers bestimmt wird.

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