CREATINE SYNTHESIS BY TUMOUR-BEARING RATS

by

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The numerous studies reported in the literature on malignant animal tissue have revealed few qualitative differences in its metabolic behaviour from that of normal tissue. However, the greatly increased *rates* of reactions involving the utilization of exogenous and endogenous metabolites can certainly be considered as one of the characteristic features of such rapidly proliferating tissue and as a prerequisite for the maintenance of its fast rate of growth. Associated with the development of malignant tissue, an increased excretion of waste products, particularly of nitrogenous end products, might be expected, since protein represents the principal building material for the newly formed cells. It was therefore envisaged that the nature and distribution of the nitrogenous components of the urine excreted by tumour-bearing animals might possibly reflect the malignant state. Except for an early report by ORDWAY AND MORRIS[1] on the increased creatinuria of rats bearing the Jensen sarcoma, the majority of investigators in this field have in the past confined their attention to analyses of tissues rather than of urine (*cf.* GREENSTEIN[2], BOYLAND[3]). A quantitative study of the excretion of total nitrogen, urea, ammonia, creatine and creatinine by normal rats and by rats bearing Jensen sarcomas was therefore undertaken. From the results of the initial experiments in this work it soon became evident that no significant differences existed between normal and sarcomatous animals with respect to the excretion of the majority of nitrogenous urinary components, but that, parallelling the development of the tumour, the excretion of creatine by sarcomatous animals was to a considerable extent increased. The object of later experiments was to investigate the origin of this excess creatine excretion. Determinations of the muscle creatine during tumour development led to the conclusion that the augmented creatinuria reflects an increased rate of *creatine synthesis* which may possibly be facilitated by a preferential utilization of arginine as one of the synthesizing materials.

METHODS

Experimental animals. Male black-hooded rats, weight 100–170 g, were used except where otherwise stated. In experimental series 1a–1c the animals were housed individually in metabolism cages; in all other experiments they were grouped in batches of three, the animals used in each experiment being of approximately equal weights. After a preliminary 4–6 day period in the cages, batches of rats were injected with finely minced Jensen sarcoma tissue under sterile conditions and one batch

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of animals was kept as a control group. Most of the tumours grew rapidly and within three weeks after inoculation attained a considerable size (8000–15,000 ml). Not infrequently spontaneous regression of tumours was observed, in which case the particular animals were rejected.

Diet. In the experimental series 1a–7 the animals were fed on a stock laboratory diet containing some creatine. In the experimental series 8 a synthetic creatine-free diet was used containing casein, starch, margarine and vitamin supplements. Spillage of food was obviated by the use of appropriate food containers. In certain experiments the food consumption of the animals was determined.

Urine collection. Urine was collected free of faeces by means of a separator, as described by MAW[4]. Each 24 h output was collected under 2 ml of toluene throughout the experimental period, made up to a convenient volume with water (100 ml for individual rats, 250 ml for groups of three rats), and freed from hair, etc. by filtration.

Determination of urinary components. Total nitrogen (after incineration) and ammonia-nitrogen were determined on samples of the diluted urine by distillation, using the Markham apparatus. Urea was estimated by Cole's modification of the SCOTT method[5]. Creatine was converted to creatinine by the FOLIN method[6] and the creatinine estimated with a Spekker absorptiometer.

Estimations on muscle tissue and on whole carcass. One gram samples of skeletal muscle from the hind legs or 1 g of minced carcass were used. Creatine was determined by the method of ROSE, HELMER AND CHANUTIN[7], using a Spekker absorptiometer. Inorganic phosphate, creatine phosphate and ATP were estimated according to FISKE AND SUBBAROW[8].

Tumour sizes. Tumours were measured with calipers and their sizes expressed in ml as the product of length, breadth and thickness.

RESULTS

For a simple presentation of the data of the excretion of total nitrogen and creatine by tumour-bearing rats, several periods in the development of the tumours were selected and all 24 h excretion values (expressed as mg nitrogen/rat/day) falling within each chosen period grouped together and averaged. With urinary constituents which showed no significant changes during the experimental period, as with urea, ammonia and creatinine, the mean excretions were determined for the whole experimental period. In one experiment the creatine and creatinine excretion has been presented graphically (Fig. 2).

Excretion of total nitrogen. Table I shows the total nitrogen excretion of control and tumour-bearing rats in two typical experiments. That of the control rats increased as to be expected over the experimental period. The tumour-bearing animals, however, excreted less during this time than did the control rats. At least three factors may be considered which could influence the excretion of total nitrogen. First, the breakdown of nitrogenous biological material caused by the wastage of muscles and organs, as observed in the later stages of tumour development; second, a reduction in food intake, and third, a possible nitrogen retention caused by the demands of the rapidly growing tumour. Reduction in food intake appeared to be an important factor contributing to the difference between the nitrogen outputs of normal and tumour-bearing rats. In Table II are given the average food intakes of groups of such animals over a 23-day period. Parallelling the increasing excretion of total nitrogen (Table I), the average food intakes of the control rats rose from 10.1–12.5 g/day over the first to the fourth 5-day periods, whereas the food intakes of the tumour-bearing rats, after showing an initial rise of 10.0–11.3 g/day during the first 10 days, dropped to 7.1 g/day over the last 5-day period when the tumours had reached a large size (average of 8300 ml).

Excretion of ammonia, urea and creatinine (Table III). There were no significant differences in the outputs of ammonia (both absolute and relative to total nitrogen) by normal and sarcomatous rats. Less urea was excreted by the sarcomatous rats than by the control animals over the whole period, but the ratio urea-N/total-N was remarkably constant throughout with both types of animals. The urea excretion thus closely followed

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TABLE I

EXCRETION OF URINARY TOTAL-N BY NORMAL AND TUMOUR-BEARING RATS

*Number of experiment**	*Rats used*	*Urinary total-N excretion (mg/rat/day)*		
		*4th–6th day***	*9th–11th day***	*14th–17th day***
4 (130–150)	Controls	127	146	150
	Tumour-bearing	115	91	123
	Tumour-bearing	132	133	107
5 (140–170)	Controls	253	304	341
	Tumour-bearing	242	274	266
	Tumour-bearing	245	301	254

* weight of rats at beginning of experiment given in parenthesis.
**after tumour inoculation.

TABLE II

AVERAGE FOOD INTAKES OF NORMAL AND TUMOUR-BEARING RATS (Expt. 7) DURING FOUR SUCCESSIVE 5-DAY PERIODS (g/day)

	1st period	*2nd period*	*3rd period*	*4th period*
Normal rats	10.1	11.8	11.1	12.5
Tumour-bearing rats	10.0	11.3	8.7	7.1

TABLE III

EXCRETION OF URINARY AMMONIA-N, UREA-N AND CREATININE-N BY NORMAL AND TUMOUR-BEARING RATS

(Expressed as mg N/rat/day)

Number of experiment	*Experimental period** (days)*	*Rats used*	*Ammonia-N*		*Urea-N*		*Creatinine-N*
			Average excretion	*% total nitrogen excretion*	*Average excretion*	*% total nitrogen excretion*	*Average excretion*
1a (70–85)	11	Controls	6.9	3.9	131	84.6	1.07
		Tumour-bearing	4.8	3.4	114	85.6	1.07
1b (100–110)	22	Controls	6.4	3.3	164	84.6	—
		Tumour-bearing	6.3	3.4	152	84.5	—
1c (120–130)	18	Controls	10.0	4.0	229	84.7	1.41
		Tumour-bearing	9.9	4.0	215	84.4	1.51
3 (100–120)	15	Controls	—	—	—	—	1.34
		Tumour-bearing	—	—	—	—	1.04
5 (140–170)	23	Controls	—	—	247	88.3	3.53
		Tumour-bearing	—	—	211	85.2	3.34
		Tumour-bearing	—	—	204	85.0	3.42

* weights of rats on day of tumour inoculation given in parenthesis,
** after tumour inoculation.

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the excretion of total nitrogen, and the factors responsible for the decrease of the latter in tumour-bearing animals (see above) appear to have affected the urea output to the same extent. Since the urea content of urine generally reflects the quantity and composition of the protein in the diet, the decrease in urea excretion could have been caused by the reduced food intake of the sarcomatous animals. The excretion of creatinine was essentially the same in both types of experimental animals. This was in notable contrast to that of creatine, as will be seen below. The relative constancy of creatinine excretion is not easily reconcilable with the concept of a common origin and of an identical mode of formation of creatine and creatinine, nor with the view of BLOCH *et al.*[9] that creatinine is derived exclusively from exogenous and endogenous creatine. If this were the case, similar changes in creatinine excretion to those observed for creatine might be expected. BOYLAND[3] found considerable quantities of creatine but not of creatinine in human malignant tumours, and in experiments with fasting animals the excretion of creatinine, but not of creatine has been reported to be constant (see Discussion).

Excretion of creatine. Experiments with normal and with tumour-inoculated rats conducted over periods of three weeks showed a distinct rise in the excretion of creatine nitrogen by the tumour-bearing rats as compared with the controls. This rise generally became apparent by the 12–14th day after inoculation. The use of a creatine-containing diet (rat cake) in these experiments masked the effect of the sarcoma on creatinuria, since the reduced food intake of the tumour-bearing rats must have caused a considerable diminution in the urinary creatine derived exogenously. The influence of dietary creatine on creatine excretion is indicated by the high urinary levels of the control rats (2–3 mg creatine-N/rat/day), compared with levels of 0.1–0.24 mg creatine-N/rat/day excreted by rats maintained on a creatine-free diet. The latter values agree well with those of other workers using normal rats (ORDWAY AND MORRIS[1], TIDWELL[10]). When a creatine-free diet was used, as shown in Fig. 1A, the masking effect of exogenous creatine was avoided and the creatinuria of sarcomatous rats was found to increase by 6–8 times their excretion at the commencement of the experimental period.

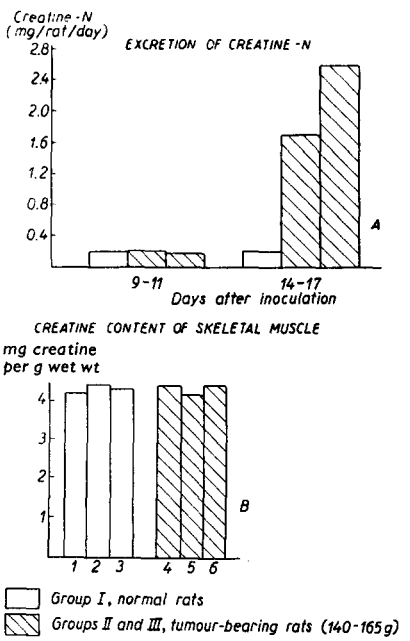

Fig. 1 (Exp. 8). A, excretion of creatine-N by groups of normal and tumour-bearing rats fed on a creatine-free diet, during two periods after tumour inoculation. B, creatine content of skeletal muscle from the same rats, killed 18 days after tumour inoculation and before the onset of cachexie.

Creatine content of muscle and carcass (Table IV). Further experiments were conducted with a view to investigating the cause of the creatinuria observed during the development of the tumour. The most likely possibilities were considered to be either a depletion of the body creatine, in particular that of the muscle, with the subsequent appearance of the released creatine in the urine, or an increased synthesis of creatine leading to excretion of the excess. If the first possibility is valid, the creatine content of the muscle of tumour-bearing animals should be greatly reduced, if the second is

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the case, the muscle creatine should be unchanged or increased. Creatine determinations on samples of skeletal muscle and of the minced carcass of sarcomatous rats were carried out at two distinctly different stages of tumour development. In experiments 5 and 6 the animals were killed after the onset of a markedly cachectic state, manifested by a general wastage of muscle, while in experiments 7 and 8 the creatine estimations were carried out before this stage had been reached. *In both cases, a period of increased creatinuria had immediately preceded the killing of the animals.* In Table IV are given the average creatine contents of muscle and carcass for each group of rats. A comparison between the creatine content of the muscle of tumour-bearing and of normal rats led to noticeably different results. In experiments 5 and 6 the creatine content of the muscle and carcass of tumour-bearing, cachectic animals was considerably lower than that of the control rats. On the other hand, in experiments 7 and 8, there was a slight increase in the muscle creatine of the sarcomatous, non-cachectic animals over that of the normal rats. Some evidence for the presence or absence of cachexie may be deduced from the total nitrogen content of the muscle of the animals. This was, in the first case (experiment 6), greatly reduced as compared with the controls, but was practically unchanged or higher in experiments 7 and 8. The reduction in creatine content in the cachectic animals was far greater than that of the total nitrogen. Consequently, the ratio creatine-N/total N in experiment 6 was almost halved as compared with that of the controls, while the ratio was practically unchanged in experiments 7 and 8, *i.e.* when the animals had been killed before the onset of cachexie.

Creatinuria and muscle creatine (Fig. 1). Two groups of rats were maintained on a creatine-free diet and one group was then inoculated with Jensen sarcoma tissue. Analysis of the urine of this group showed an almost dramatic increase in creatine excretion as compared with that of the control rats. In the later stages of tumour

TABLE IV

CREATINE-N CONTENT OF CARCASS AND SKELETAL MUSCLE OF NORMAL AND TUMOUR-BEARING RATS

(Expressed as mg/g wet weight of tissue. Figures in columns 4–6 represent the averages for each group of animals, duplicate analyses being carried out on each rat)

Number of experiment **	*Age of tumour (days)* **	*Rats used* ***	*Carcass creatine-N*	*Muscle creatine-N (A)*	*Muscle total-N (B)*	*100 A/B*
After onset of cachexie						
5 (140–170)	23 (27,000)	Controls (3)	0.70	—	—	—
		Tumour-bearing (3)	0.48	—	—	—
6 (150–170)	21	Controls (2)	0.64	1.44	32.3	4.46
		Tumour-bearing (2)	0.56	0.65	27.1	2.42
Before onset of cachexie						
7 (130–160)	23 (8300)	Controls (6)	—	1.28	33.1	3.83
		Tumour-bearing (6)	—	1.31	32.8	3.99
8 (140–170)	17 (5700)	Controls (3)	—	1.30	32.9	3.96
		Tumour-bearing (3)	—	1.40	35.2	3.99
		Tumour-bearing (3)	—	1.38	35.7	3.87

* weights of rats on day of inoculation given in parenthesis.
** average size of tumours at termination of experiment given in parenthesis (ml).
*** number of rats in each group given in parenthesis.

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development (14th–17th day after inoculation) the urinary creatine level of the tumour-bearing rats was 8–12 times that of the controls. The latter remained practically unchanged over the experimental period. Both groups of animals were killed on the 18th day, before the onset of cachexie in the sarcomatous animals, and muscle creatine determinations carried out. In Fig. 1 the changes in creatine excretion taking place are shown together with the muscle creatine values. The fact that the creatine content of the muscle of the two groups of rats remained unchanged, while the tumour-bearing group were excreting considerably more creatine suggests an *increased synthesis* of creatine associated with the development of the tumour.

Urinary components and tumour regression. The variations in excretion of urinary components were followed in the case of a rat (S_2) bearing a regressing tumour and were compared with those of its litter-mate (S_1) which bore a fully developing tumour.

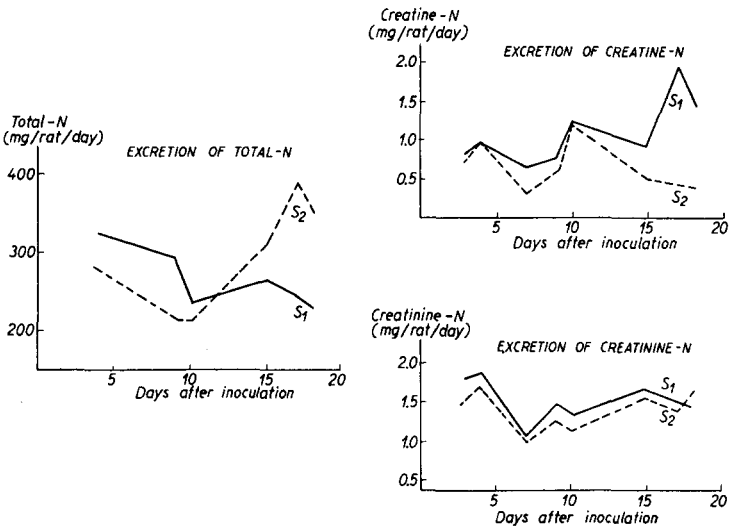

Fig. 2. Excretion of nitrogenous urinary components by rats bearing developing (S_1) and regressing tumours (S_2). Tumour regression observed in the case of rat S_2 between the 10th and 15th day after inoculation.

It will be seen from Fig. 2 that the extent of creatine excretion of the two animals differed little during the period up to the 10th day after inoculation. Signs of tumour regression were seen in rat S_2 at about the 12th day. This was accompanied by a drop in creatine excretion and a rise in the output of total nitrogen by this rat, while the other rat with a steadily developing tumour showed an increased creatine output and a decreased excretion of total nitrogen*. Although the results of this single experiment do not allow any definite conclusions to be drawn, they add value to the above findings with groups of tumour-bearing and normal animals.

Control experiments with injections of muscle tissue. The possibility had to be considered that the described effect on creatinuria resulting from tumour growth could also be produced by the introduction of foreign protein into the animal, irrespective of the former's malignant nature. A group of rats was inoculated with minced muscle tissue obtained from the rectus abdominis of normal rats. The distribution of the

* The excretion of creatinine was practically identical in both cases.

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nitrogenous components of the urine of the inoculated rats and of a group of normal rats was followed as in previous experiments for a period of 18 days after the inoculation. The data obtained indicated no significant differences between the urinary nitrogen partition of the two groups of animals.

DISCUSSION

Two principal results are apparent from the experiments described. First, that the development of Jensen sarcoma in rats is accompanied by a steadily increasing creatinuria, which on the 17th–23rd day after inoculation with tumour tissue reaches a level up to 8–12 times higher than that prevailing in normal animals. This is in line with the findings of KARNOFSKY *et al.*[11], who reported a large excretion of creatine by mice bearing a mammary carcinoma. Second, that neither the excretion of ammonia, urea and creatinine, nor especially the creatine content of skeletal muscle of tumour-bearing, non-cachectic animals differs appreciably from that of the control rats. This implies that the excessive excretion of creatine by the tumour-bearing animals is caused by an increased rate of creatine synthesis.

Increased creatinuria has been reported to be associated with a variety of abnormal conditions (*cf.* ZIERLER *et al.*[12]) but the number of these in which the relationship appears to be specific or of consistent occurrence is relatively small. A condition in which creatinuria has been frequently observed is starvation. It can be argued that the loss of appetite observed with tumour-bearing rats in the present work may have produced conditions similar to that prevailing after mild starvation. However, results reported in the literature on the occurrence of creatinuria in starvation are inconsistent (*cf.* SZAWSON[13], MCCOLLUM AND STEENBOCK[14] on pigs, MYERS AND FINE[15] on rabbits, PALLADIN AND EPELBAUM[16] on cats). The findings reported in the present work were, on the other hand, fully reproducible. They were made over a number of years on different strains of rats fed on different types of diet. Furthermore, no significant differences were found between the creatine content of muscle of non-cachectic, sarcomatous rats on the one hand, and that of the control animals on the other. In contrast to the results reported here, studies by other workers on the change of muscle creatine during fasting have shown rises in some cases (CHANUTIN AND SILVETTE[17], MENDEL AND ROSE[18]) and fluctuations in others (MYERS AND FINE[15], PALLADIN AND EPELBAUM[16]). Another important difference was the absence of any change in the output of other nitrogenous urinary constituents investigated during the tumour development. During fasting, the excretion of ammonia (MENDEL AND ROSE[18]), urea (HOWE AND HAWK[19]) and creatinine (PALLADIN AND EPELBAUM[16]) is noticeably decreased. From the foregoing data it appears, therefore, that the excess creatinuria coupled with an unchanged level of muscle creatine can be considered as characteristic of the malignant condition. There is some resemblance between the phenomena reported here and those found in muscular dystrophy produced by E avitaminosis (MACKENZIE AND MCCOLLUM[20]). A dramatic increase of urinary creatine has been observed during the development of the dystrophic condition (MORGULIS AND SPENCER[21]) and this is accompanied, according to the experiments of MELVILLE AND HUMMEL[22], by an increase in creatine synthesis. However, in nutritional muscular dystrophy, the muscle creatine rapidly decreases in proportion to the degree of muscle degeneration (GOETTSCH AND BROWN[23]).

Perfusion, isotope and tissue slice experiments (FISHER AND WILHELMI[24], BLOCH

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AND SCHOENHEIMER[25], BORSOOK AND DUBNOFF[26]) have established that glycine, methionine and arginine are precursors of creatine. Of these, for the purpose of the present study, arginine deserves special consideration for two reasons. First, because its participation in creatine synthesis has been confirmed by *in vivo* experiments (GROSS AND STEENBOCK[27], MOUROT[28]), and second, because it has been shown to play a special role in the metabolism of malignant tissue (GILROY[29]). The latter was further confirmed by the fact that arginine, of all amino acids investigated, when administered in small amounts to the medium of tissue cultures of mouse mammary carcinoma, increased the mitotic index by 100–200% over the controls. Arginase, the arginine-destroying enzyme, exerted the opposite effect (BACH AND LASNITZKI[30], BACH AND SIMON-REUSS[31]).

As a result of these investigations, two actions of arginine become apparent; its stimulating effect on the growth of certain types of malignant tissue, amongst them Jensen sarcoma, and its effect on the synthesis of creatine. Since it can be deduced from the present work that the development of the sarcoma causes an increased synthesis of creatine, it is believed that in malignant conditions of this type arginine may be preferentially metabolized in the direction of creatine formation. The synthesis of creatine obviously depends on the state of equilibrium between the urea-forming and the creatine-forming capacities of arginine, and a shift of this balance in the direction of creatine synthesis is envisaged to take place during the development of the tumour.

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SUMMARY

1. The excretion of urinary total-N, ammonia-N, urea-N, creatine-N and creatinine-N of groups of normal rats and rats inoculated with Jensen sarcoma has been determined during the development of the tumour.

2. A diminished total-N excretion by tumour-bearing animals as compared with normal rats has been explained, in part at least, by a decrease in the food intake of these animals, which develops during tumour growth.

3. Ammonia-N, urea-N and creatinine-N excretion of tumour-bearing rats was not significantly different from that of normal rats.

4. The principal difference in the nitrogen partition of the urine was found in the creatine component. Sarcomatous rats excreted considerably more creatine than did normal rats, the urinary output of tumour-bearing animals rising to a level 8–12 times that of controls.

5. The creatine content of the skeletal muscle of sarcomatous rats was found to be markedly reduced after the onset of cachexie, as was the total-N content. However, before the onset of cachexie no significant changes in muscle creatine content were apparent.

6. Since the increase in creatinuria exhibited by non-cachectic, tumour-bearing rats took place without any concomitant changes in the concentration of muscle creatine, it is concluded that the extra creatine excretion was the result of an increased endogenous synthesis of creatine associated with tumour growth. The role of arginine and arginase in this synthesis is discussed.

RÉSUMÉ

1. Nous avons déterminé les quantités excrétées dans l'urine d'N total, N ammoniacal, N d'urée, sous forme de créatine et de créatinine chez des groupes de rats normaux et de rats ayant reçu une injection de sarcome-Jensen pendant le développement de la tumeur.

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2. Une diminution de l'azote total excrété par des animaux porteurs d'une tumeur par rapport aux animaux normaux s'explique, en partie du moins, par le fait que ces animaux absorbent moins de nourriture au fur et mesure que la tumeur se développe.

3. Les quantités d'N ammoniacal et d'N sous forme d'urée et de créatinine excrétées par les animaux sarcomateux ne différaient pas considérablement de celles excrétées par les rats normaux.

4. La différence principale de la répartition de l'azote dans l'urine concerne la créatine. Les rats sarcomateux en excrétaient jusqu'à 8 à 12 fois plus que les rats normaux.

5. La teneur en créatine de muscle strié de rats sarcomateux se trouvait considérablement réduite après le début de la cachexie, de même que la teneur en N total. Cependant, avant le début de la cachexie, nous n'avons pas observé de changements significatifs dans la teneur en créatine du muscle.

6. Comme l'augmentation de la créatinurie chez les rats atteints de tumeurs non cachectiques avait lieu sans changements de la concentration de créatine dans le muscle, nous supposons que l'excrétion supplémentaire de créatine résulte d'une augmentation de la synthèse endogène de créatine, associée à la croissance de la tumeur. Le rôle que jouent l'arginine et l'arginase dans cette synthèse est discuté.

ZUSAMMENFASSUNG

1. Die Gesamtstickstoff-, Ammoniakstickstoff-, Harnstoffstickstoff-, Kreatinstickstoff- und Kreatininstickstoffausscheidung im Harn wurde bei Gruppen normaler Ratten und Ratten, die mit Jensen-Sarkom geimpft waren, während der Entwicklungszeit der Tumoren bestimmt.

2. Eine verminderte Gesamtstickstoffausscheidung bei den tumortragenden Tieren verglichen mit der normaler Ratten wurde, zum Teil zumindest, durch das Abnehmen der Nahrungsaufnahme dieser Tiere erklärt, das während des Tumorwachstums beobachtet wurde.

3. Die Ammoniak-, Harnstoff- und Kreatininstickstoffausscheidung der tumortragenden Tiere war nicht wesentlich von der normaler Tiere verschieden.

4. Der Hauptunterschied in der Stickstoffverteilung im Urin wurde bei der Kreatinkomponen te gefunden. Sarkomatöse Ratten schieden beträchtlich mehr Kreatin aus als normale Ratten; die Ausscheidung war im Harn der tumortragenden Tiere bis zu 8–12 mal so gross wie bei den Kontrolltieren.

5. Der Kreatingehalt der Skelettmuskeln von sarkomatösen Ratten war — wie gefunden wurde — nach dem Einsetzen der Kachexie beträchtlich reduziert; das gleiche gilt für den Gesamtstickstoffgehalt. Jedoch waren vor dem Eintritt der Kachexie keine bedeutungsvollen Veränderungen im Muskelkreatingehalt zu bemerken.

6. Da das Anwachsen der Kreatinurie, wie es sich bei nicht-kachetischen, tumortragenden Ratten zeigt, ohne gleichzeitige Veränderungen in der Konzentration des Muskelkreatingehaltes stattfand, wird geschlossen, dass die besondere Kreatinausscheidung das Ergebnis einer angestiegenen endogenen Synthese des Kreatins war, die das Tumorwachstum begleitete. Die Rolle des Arginins und der Arginase bei dieser Synthese wird besprochen.

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