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The Problem of Tissue Sampling from Experimental Animals with Respect to Freezing Technique, Anoxia, Stress and Narcosis[1]

A New Method for Sampling Rat Liver Tissue and the Physiological Values of Glycolytic Intermediates and Related Compounds

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The problems of the measurement of physiological concentrations of glycolytic intermediates and related compounds in rat liver were investigated. Five hundred milligrams, tissue samples, immersed in liquid nitrogen, remained at a temperature of more than 30° for about 15 sec. Not till then temperature dropped rapidly. When samples up to 24 g were cooled only a disproportionately small prolongation of the initial phase was observed.

The dependency of freezing time on tissue weight was investigated for liquid nitrogen, $CClF_3$ and isopentane. Using the latter two the freezing time could be shortened significantly. Yet, even for isopentane, it remained still longer than 12 sec for a 500 mg tissue sample. A mathematical approach proved that—even under ideal conditions—500 mg of tissue immersed should need about 7 sec to freeze through.

A new method of liver sampling from unanaesthetized rats was developed and the physiological substrate concentrations were determined. AMP values were found to be as low as 15 nmoles/g liver. The time course of the alterations of substrate concentrations during anoxia was investigated and found to be nearly linear during the first 13.6 sec. Nembutal was shown to influence substrate concentrations severely, independent on narcosis-induced anoxia. AMP was found to be a highly sensitive parameter to stress.

Many metabolites have been proved to be effectors of key enzymes, which—in turn—influence the concentration of physiological substrates. Following the literature in this field, we were surprised to find an extreme diversity even of results, obtained under identical metabolic conditions. As an example substrate concentrations published for the liver of normal, well-fed Albino rats are listed in Table IV. We assumed this fact to be caused by the different experimental procedures, preceeding the spectrophotometric or fluorometric analysis. Our results, confined to rat liver, have confirmed this working hypothesis. We now offer another method of animal killing and tissue sampling,

[1] Dedicated to Prof. Dr. J. Kühnau at the occasion of 70th birthday.

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which makes it possible to avoid or minimize the three main factors falsifying real substrate values: anaesthesia, anoxia, and stress.

MATERIALS AND METHODS

Rats. White, male Wistar rats, weighing 160–180 g (in anoxia experiments 250–300 g), kept for breeding in our institute and fed ad libitum with altromin (Altromin GmbH, Lage/Lippe, Germany) were used.

Gaining of liver samples. In anoxia experiments animals were anaesthetized with Nembutal (sodium pentobarbital ABBOTT, 60 mg/ml, ad us. vet.), 0.1 ml = 6 mg/100 g body wt, i.p., 30 min later they were fixed to a cork board. The abdomen was opened by a V-formed cut up to the costal arch. In each case the portal vein, the aorta abdominalis and the vena cava abdominalis were cut through simultanously by two assistant persons at zero time. In experiments, in which the first liver sample was taken at zero time, the right lobe of the organ was slightly elevated by tweezers and immediately fixed between the aluminum blocks (each 6 × 3 × 1 cm) of a freeze stop clamp, previously cooled in liquid nitrogen (1). If no substrate values were wanted at zero time, liver pieces were excised and immediately frozen between precooled aluminum blocks at the time indicated (Fig. 1).

From unanesthetized rats livers were obtained by a *new method.* The animals were beaten through with a double hatchet, shown in Fig. 1, the distance of the blades being the same as that between the neck and the height of the lateral caudal margin of the chest. Using the double hatchet the liver usually fell out on the chopping block and a part of it could be cut and frozen between precooled aluminum blocks within 2.5–3.5 sec. Animals were always unstressed. Compared with other methods of animal killing the double hatchet method is exceptionally rapid and painless. Time was always taken by stop watches. The frozen tissues were preserved in liquid nitrogen until deproteinisation.

Tissue preparation. Deproteinisation was done in a cooling chamber at +4°. The frozen tissue was pulverized in a porcelain mortar by aid of a porcelain pestle, while liquid nitrogen was continually added. The powder was transferred to a centrifuge tube standing on a balance and previously filled with 5 ml of ice-cold $HClO_4$ (0.7 N). It was weighed and immediately homogenized with a high speed homogenizer (Ultraturrax, Janke u. Kunkel KG., Staufen i.Br., Germany) for 15 sec. Under these conditions the temperature of the suspension rose up to +2°. $HClO_4$ (0.7 N) was added to establish a tissue: acid ratio of 1 + 7 (w/v). After rehomogenisation for 3 sec the sample was centrifuged for 20 min (8,000 *g*, 0°). The Ultraturrax was cleaned by running in about 5 ml

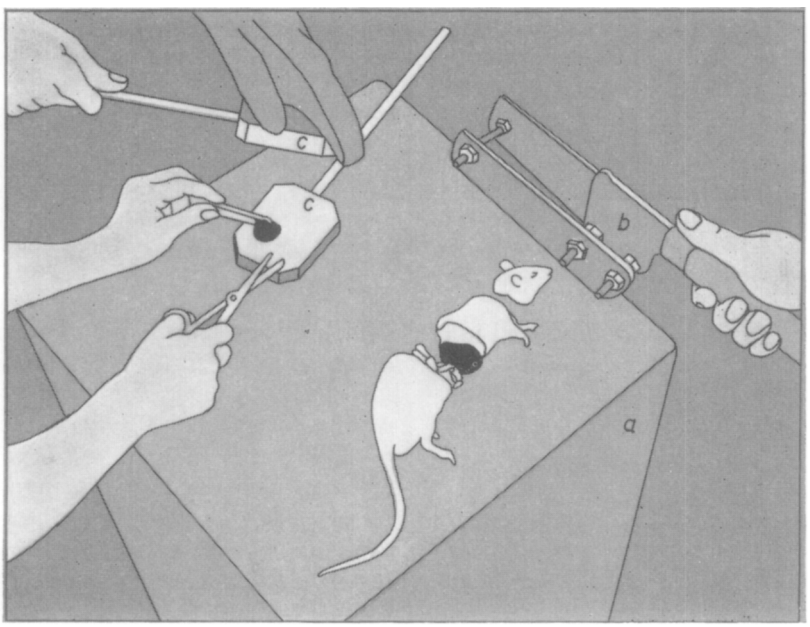

FIG. 1. Double hatchet method. (a) chopping block; (b) double hatchet; (c) aluminum blocks, 10 × 8 × 2 cm, precooled in liquid nitrogen. A fourth person is needed to take the time between killing and freeze stop.

of ice-cold $HClO_4$ (0.7 N) for 10 sec. The rinsing fluid was stored in ice and later added to the sample sediment for DNA determination. An aliquot of the supernatant was sucked up and transferred to a double walled glass vessel, through which precooled methanol (−2°) from a kryostat was passed. 5% (v/v) of a TRA-buffer (0.4 M, pH 7.2) was added and the acid extract was neutralized to pH 6.8–7.2 with ice-cold KOH (2 N) in about 30 sec. During neutralisation the liquid was severely stirred by a magnetic agitator. It has been established that under these conditions substrate concentrations were the same when compared with those obtained after 10 min neutralisation. The neutral solution was centrifuged for 15 min (8,000 *g*, 0°). All substrates with exception of DNA were determined in the supernatant.

Biochemical analysis. Many analytical methods cited below were modified with respect to cosubstrate or coenzyme concentrations. Since these modifications are not essential, they are not described in detail. If not otherwise stated TRA-buffer (0.1 M, pH 7.2) was used in the tests. It has been verified that for all determinations the progress curve was linear with respect to substrate concentration and passed through the origin. A defined amount of the substrate was quantitatively recovered if added to the tissue extract at the end of the spectrophotometric measurement. Moreover, the stability of all substrates was determined under the following conditions: (a) Control: The acid extract was neutralized immediately after homogenisation during 3 min. No determination was done later than 2.5 hr after tissue sampling. (b) All conditions were as described in (a) with the exception of neutralization, which was accomplished very rapidly during 3 sec. (c). After homogenization the acid extract was kept at +4° for 4 and 24 hr, respectively, and then neutralized as described for condition (a). (d) Neutral tissue extract was prepared as mentioned for condition (a). It was immediately frozen, stored for 24 hr at −20°, thawed and two times frozen again. According to the results of these experiments all substrates, which have been found to be stable under condition (d), i.e., glucose-1-*P*, glucose-6-*P*, fructose-6-*P*, glycerol-1-*P*, 3-phosphoglycerate, lactate, citrate, and UDP-glucose were determined next day. For the other substrates the following maximal time between deproteinisation of the liver tissue and determination was observed: *P*-enolypruvate, pyruvate, and malate: 9 hr, fructose-1.6-di*P* and dihydroxyacetone-*P*: 6 hr, acetyl-CoA: 4 hr; ATP, ADP, AMP, and oxaloacetate: 2–3 hr.

Spectrophotometric measurements were performed at 334 nm either in a recording photometer Eppendorf or at 340 nm in a recording spectrophotometer Zeiss PMQ II at room temperature. After each four determinations one blank was read, in which the liver extract was replaced by water.

Substrate determinations were performed as follows: ATP according to Jaworek *et al.* (2) at pH 7.2, d = 1 cm. ADP and AMP according to Adam (3) at pH 7.2, d = 2 cm; each value was determined at three different extract concentrations producing a linear progress curve passing through the origin. UDP-glucose according to Keppler and Decker (4), using the original buffer without EDTA, d = 1 cm. We could not confirm the findings of these authors that UDP-glucose values are higher if EDTA is omitted. Glucose-1-*P*, glucose-6-*P* and fructose-6-*P* according to Hohorst *et al.* (5) at pH 7.2, d = 2 cm. Fructose-1.6-di*P* and dihydroxyacetone-*P* according to Hohorst (6) at pH 7.2, d = 4 cm. Glycerol-1-*P* according to Hohorst *et al.* (6), using the original buffer, d = 2 cm. 3-phosphoglycerate according to Czok (7) at pH 7.2, d = 1 cm. P-enolypyruvate and pyruvate according to Hohorst *et al.* (6) at pH 7.2 without EDTA, d = 2 cm. Lactate according to Hohorst *et al.* (6) with the original buffer, but at pH 9.2, d = 1 cm. We found the reproducibility of this method to be unsatisfying. Therefore each value was measured at three different extract concentrations producing a linear progress curve. Acetyl-CoA according to Tarnowski and Seemann (8), d = 4 cm. Citrate according to Gruber and Möllering (9), d = 2 cm. Malate according to Hohorst *et al.* (6) with the original buffer, d = 1 cm. Oxaloacetate according to Hohorst and Reim (10), d = 4 cm. DNA according to Schneider (11): the starting material was the sediment of the $HClO_4$-extract, combined with the rinsing fluid of the Ultraturrax. The reaction has been proved to be linear up to 1.8 g of liver tissue.

Calculation of results. For substrates, which are present in the blood as well as in the liver cell, no attempt was made to separate the concentrations in these two compartments. Statistical comparisons were based on the unconnected t test from gross data.

Reagents. The following compounds were purchased from Boehringer Mannheim GmbH, Germany. Substrates and cosubstrates: ATP (free acid); ADP (tri-Na-salt); NAD (free acid); NAD, reduced form (di-Na-salt), AMP-free; NADP (di-Na-salt); creatine-phosphate (di-Na-salt); 3-phosphoglycerate (tricyclohexylammonium-salt); *P*-enolpyruvate (mono-Na-salt). Enzymes: glycerolaldehyde-*P*-dehydrogenase (10 mg/ml), creatine kinase (lyophylized dry powder), lactate-dehydrogenase (5 mg/ml), pyruvate kinase (10 mg/ml), *P*-glucomutase (2 mg/ml),

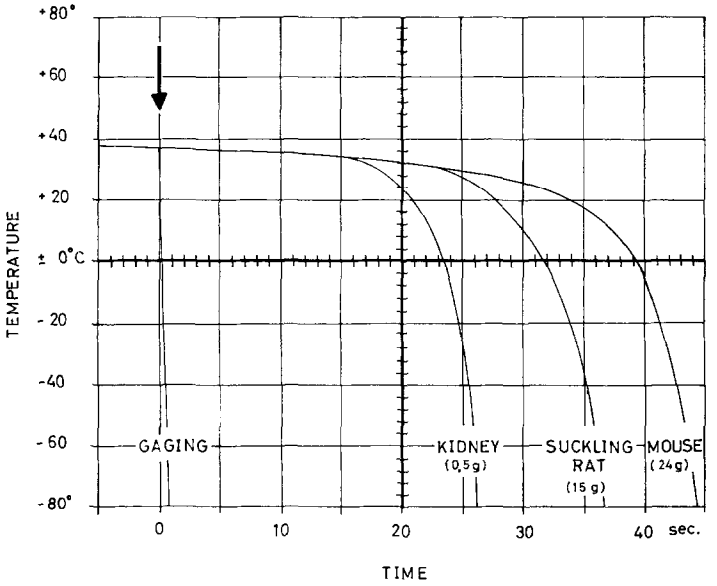

FIG. 2. Time course of deep freezing of different tissues after immersion in liquid nitrogen. The point of an injection needle was placed into the middle of an isolated kidney or into the middle of the liver of a whole animal. The thermocouple (Thermocoax miniature thermocouple, 0.25 mm, Philips AG) was pushed forward through the metal channel up to the point, the needle was drawn back and the kidney, whole mouse or whole rat were immersed at zero time and stirred in liquid nitrogen. Tissue temperature was recorded by a cathoden-oszillograph. Split kidneys were discarded.

glycerol-*P*-dehydrogenase (10 mg/ml), triose-*P*-isomerase (10 mg/ml), aldolase (10 mg/ml), all from rabbit muscle; myokinase (2 mg/ml) from pig muscle; malate dehydrogenase (5 mg/ml), citrate synthase (2 mg/ml) from pig heart; UDP-glucose dehydrogenase (5 mg/ml) from beef liver; *P*-glycerate kinase (10 mg/ml), hexokinase (1 mg/ml), glucose-6-*P* dehydrogenase (2 mg/ml), *P*-glucose isomerase (2 mg/ml) from yeast; glycerokinase (10 mg/ml) from candida mycoderma; citrate lyase (lyophilized dry powder) from aerobacter aerogenes. The following compounds were purchased from Merck AG, Darmstadt, Germany: Triethanolamine; glycine; ethylendiaminetetraacetate (di-Na-salt); hydrazine sulfate; hydrazine hydrate (supra pure); malate (K-salt); diphenylamine.

RESULTS

Immersion techniques. The relationship between freezing time and tissue weight has been studied using different refrigerants. As shown in Fig. 2 the innermost parts of a tissue sample as light as 500 mg, immersed in liquid nitrogen and stirred, remained at a temperature of more than 30° for about 15 sec. It took another 5 sec until 0° were reached. Not till then temperature dropped rapidly. The time course of the tissue temperature remained similar, when bigger samples or whole animals were cooled (Fig. 2). In the latter two cases only a disproportionately small prolongation of the initial phase of temperature drop was observed. In order to minimize the heat isolation of immersed tissues, which is caused by the development of a gaseous wrap of N_2 (phenomenon of Leidenfrost), experiments were carried out with liquid $CC1F_3$ (Frigen 13)[4] and isopentane[4]. Using these refrigerants the freezing time could be shortened significantly (Fig. 3). Yet, even for isopentane it remained still longer than 7 sec with tissue samples as small as 200 mg.

[4] Physical data: $CC1F_3$ (Frigen 13 Hoechst AG, Frankfurt/M.-Hoechst): boiling point at 1 atm: −81.4°; freezing point: −181.0°; specific heat at 1 atm: 0.203 kcal/kg·grd—Isopentane: boiling point at 1 atm: 27.9°; freezing point: −160.0°; specific heat at 8°: 0.527 kcal/kg·grd.

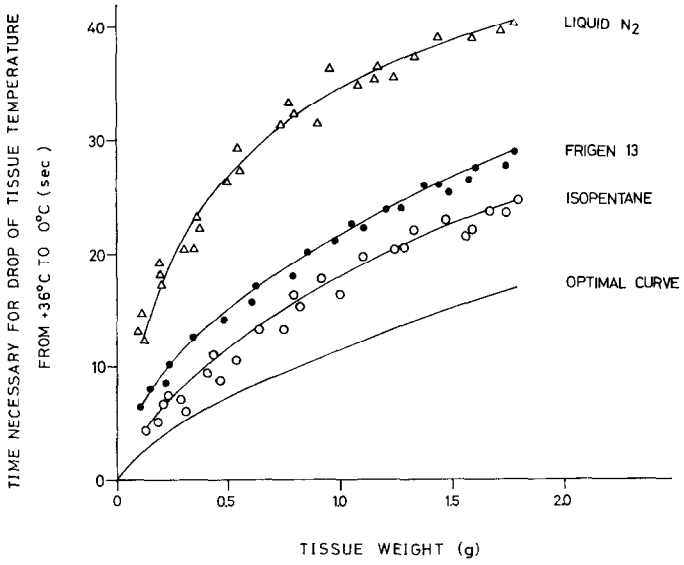

FIG. 3. Relation between freezing time of isolated rat kidneys and tissue weight. For experimental details: see legend of Fig. 2. Frigen 13 and isopentane were cooled down in liquid nitrogen to about −150°. The "optimal curve" representing the freezing time, which cannot be shortened on physical grounds, was deduced mathematically as described in the appendix.

On the base of the principal physical parameters a mathematical approach was made to evaluate, whether the freezing time of immersed tissues could be further reduced (for details: see Appendix). The result is shown in Fig. 3 ("optimal curve"). Apparently, even under ideal conditions, 1 g of tissue immersed should need more than 10 sec to freeze through. The low thermal conductivity of water—the main constituent of animal tissue —principally limits any further reduction of freezing time.

Anoxia. The time course of alteration of substrate concentrations under the influence of anoxia was studied. Most of the experiments were performed with normal fed rats, anaesthetized with Nembutal 30 min prior to killing. It has been verified that the time course was the same, when livers were taken from unanaesthetized animals, killed by the double hatchet method (Fig. 4).

In rat liver all substrate concentrations examined proved to be sensitive to anoxia (Fig. 4). During the first 30 sec there was an increase in substrate levels for: AMP (+700 %), glucose-6-*P* (+150 %), glucose-1-*P* (+140 %), glycerol-3-*P* (+130 %), fructose-6-*P* (+120 %), ADP (+100 %), fructose-1.6-di*P* (+100 %), lactate (+100 %), malate (+90 %) and acetyl-CoA (+20 %). There was a decrease for: oxaloacetate (−70 %), pyruvate (−65 %), ATP (−36 %), *P*-enolpyruvate (−32 %), glycerate-3-*P* (−30 %), dihydroxyacetone-*P* (−25 %), citrate (−24 %), and UDP-glucose (−7 %). Besides the ratio of [malate]:[oxaloacetate], [lactate]:[pyruvate], and [glycerol-1-*P*]:[dihydroxyacetone-*P*] (Fig. 5), AMP was by far the most sensitive parameter for tissue anoxia.

After ceasing blood supply substrate concentrations immediately changed without detectable lag period. All alterations were nearly linear with time during the first 13.6 sec (Table I). Therefore, it was possible to estimate zero values by extrapolation.

Animal irritation. In an attempt to evaluate the possible influence of stress, the concentrations of glycolytic intermediates and related compounds were determined under the following conditions: "absolute tranquility", "tranquility", "slight irritation" and "maximal irritation" (for details: see legend of Table II). Again AMP proved to

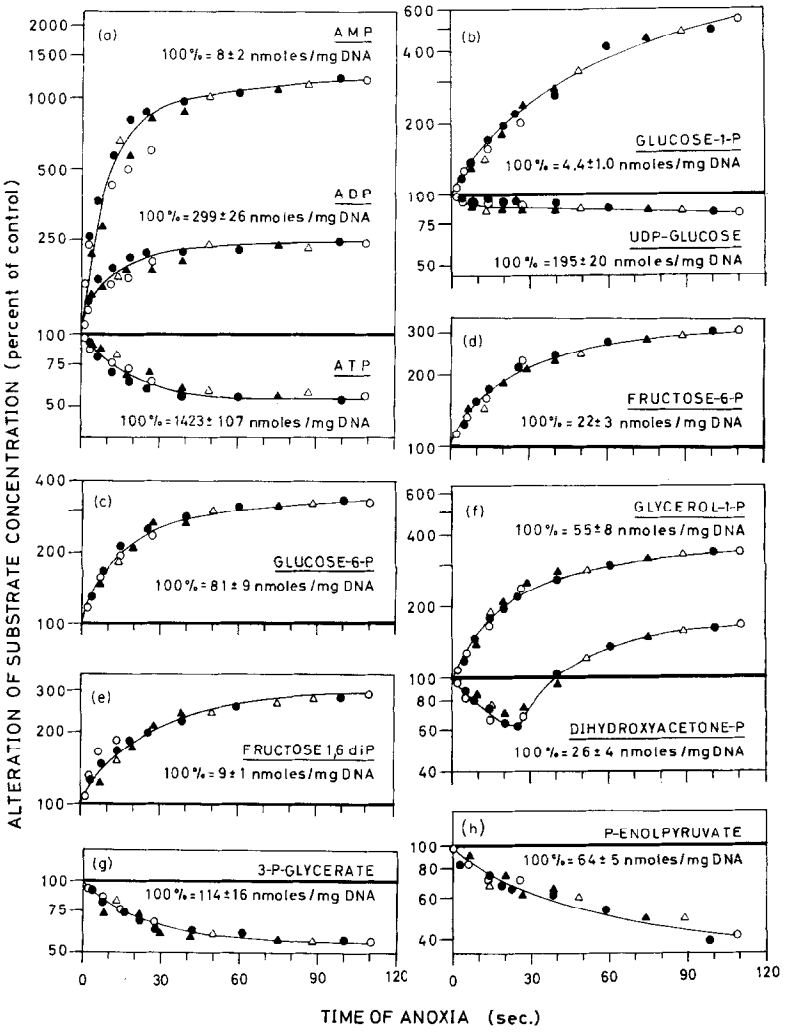

FIG. 4. Relative alteration of rat liver substrate concentrations induced by anoxia. Tissue samples were fixed by freeze stop as described in Materials and Methods. The livers were taken either from unanaesthetized animals, killed by the double hatchet method (○-○-○) or from animals, anaesthetized with Nembutal (●-●-●, ▲-▲-▲, △-△-△). The measured anoxia curves, indicating the absolute alterations of substrate concentrations (nmoles/mg DNA) with time, were related graphically to the extrapolated normal value, those values representing the starting points = 100% at zero time.

be a very sensitive parameter for stress (Table II). There were smaller differences for ADP and ATP, while the concentrations of other substrates (most of them are not represented in the table) did not change significantly. Obviously the change of adenine nucleotide concentration was not caused by anoxia, for substrate levels, which are highly sensitive to oxygen deficiency (i.e., glucose-6-*P* and the cytoplasmatic redox pairs) remained practically constant (Table II). If animals were killed by cervical dislocation the concentration of adenine nucleotides was altered as after "maximal irritation", i.e., ATP decreased, while ADP and AMP increased (Table II).

Nembutal narcosis. Substrate concentrations in livers, taken from normal, well-fed, unanaesthetized, unstressed Albino rats, killed by the double hatchet method, are

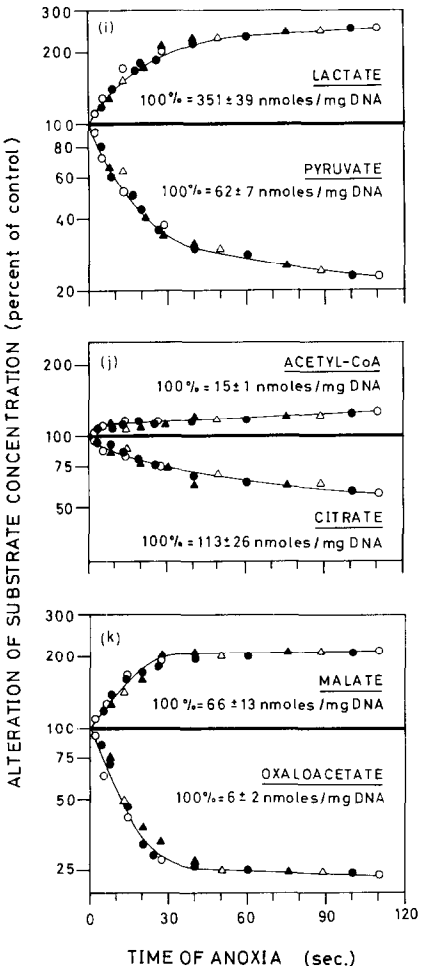

FIG. 4. i–k.

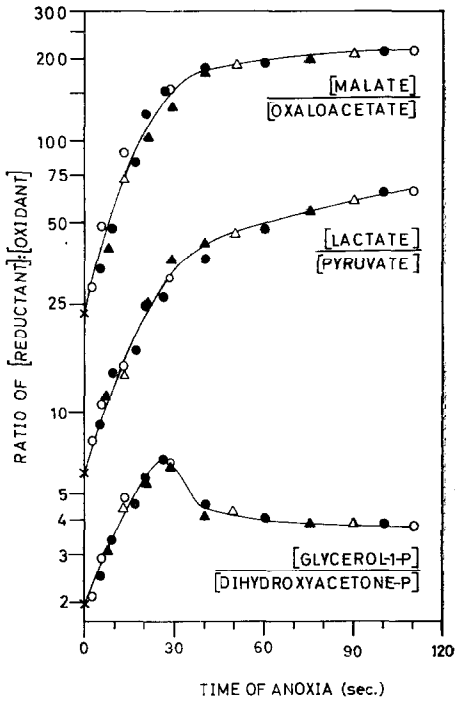

FIG. 5. Alteration of the ratio of [malate]:[oxaloacetate], [lactate]:[pyruvate], and [glycerol-1-*P*]:[dihydroxyacetone-*P*] during anoxia. For experimental details and symbols see legend of Fig. 4.

given in Table III. These values were compared with those from animals anaesthetized with the often used narcotic Nembutal. Under Nembutal nearly all substrate levels changed significantly (Table III). Probably, this is a specific effect of the anaesthetic, for the observed changes of concentrations of some anoxia-sensitive substrates (i.e. hexose-*P*, malate/oxaloacetate) were reversed.

DISCUSSION

Immersion techniques. As has been shown already by Wollenberger *et al.* (1) for liquid nitrogen, 16.3 sec are necessary to decrease the temperature of an immersed, 800 mg weighing guinea pig heart from 38 to 0°, the thermocouple being placed 1 mm beneath the surface. These results are now confirmed by our experiments. There are two causes for this long freezing time: the low thermal conductivity of the tissue and the phenomenon of Leidenfrost. Unfortunately only the latter can be influenced by using refrigerants other than liquid nitrogen or air. These should combine the following physical characteristics: high boiling point, low freezing point, high specific heat and high thermal conductivity. However, as our experiments demonstrate, even if suitable compounds are used, the immersion technique remains insufficient, if very short freezing times are required. The only way to overcome the low thermal conductivity of animal tissue is to minimize distances by flattening the tissue between precooled metal blocks, i.e., by applying the principles of the original freeze stop technique developed by Wollenberger *et al.* (1) in 1960. The immersion of clumps of tissue (12–19) or whole animals

TABLE I

LINEARITY OF ALTERATION OF METABOLITE CONCENTRATION DURING THE INITIAL PHASE OF ANOXIA[a]

Metabolite	Substrate concentration (nmoles/mg DNA) at 0 sec	Direction of alteration	Alteration of metabolite concentration induced by anoxia (nmoles/mg DNA sec)[a]			Mean change of concentration (%/sec) within the first 13.6 sec of anoxia
			Within 0–5.6 sec	Within 1.8–7.8 sec	Within 6.3–13.6 sec	
ATP	1423 ± 107	↓	20.60 ± 5.61	24.31 ± 9.22	22.52 ± 3.70	1.2
ADP	299 ± 26	↑	13.24 ± 2.36	12.63 ± 1.53	13.49 ± 1.81	5.1
AMP	8 ± 2	↑	4.44 ± 0.81	4.03 ± 0.47	4.35 ± 0.28	26.4
UDP-Glucose	195 ± 20	↓	0.11 ± 0.02	0.08 ± 0.02	0.09 ± 0.02	0.05
Glucose-1-*P*	4 ± 1	↑	0.14 ± 0.02	0.15 ± 0.02	0.15 ± 0.02	2.9
Glucose-6-*P*	81 ± 9	↑	2.04 ± 0.18	1.86 ± 0.21	2.04 ± 0.24	6.6
Fructose-6-*P*	22 ± 3	↑	0.94 ± 0.09	0.81 ± 0.10	0.90 ± 0.10	6.3
Fructose-1,6-di*P*	9 ± 1	↑	0.19 ± 0.01	0.16 ± 0.01	0.18 ± 0.01	3.5
Dihydroxyacetone-*P*	26 ± 4	↓	0.81 ± 0.08	0.69 ± 0.08	0.73 ± 0.06	3.4
Glycerol-1-*P*	55 ± 8	↑	2.89 ± 0.74	2.89 ± 0.67	3.03 ± 0.67	4.0
3-*P*-Glycerate	114 ± 16	↓	1.11 ± 0.29	1.33 ± 0.22	1.26 ± 0.22	1.7
P-Enolpyruvate	64 ± 5	↓	0.53 ± 0.03	0.50 ± 0.03	0.59 ± 0.05	2.0
Pyruvate	62 ± 7	↓	3.76 ± 0.24	3.84 ± 0.32	3.92 ± 0.32	4.8
Lactate	351 ± 39	↑	34.70 ± 5.34	32.73 ± 4.67	34.71 ± 4.67	5.1
Acetyl-CoA	15 ± 1	↑	0.13 ± 0.06	0.12 ± 0.04	0.12 ± 0.04	0.7
Citrate	113 ± 26	↓	0.78 ± 0.25	0.92 ± 0.19	0.92 ± 0.19	1.3
Malate	66 ± 13	↑	1.91 ± 0.11	1.80 ± 0.11	1.94 ± 0.14	5.3
Oxaloacetate	6 ± 2	↓	0.26 ± 0.03	0.25 ± 0.03	0.27 ± 0.03	4.6

[a] All values are given as means ± SD ($n = 6$)

(14, 20) into a refrigerant as well as the infusion of a refrigerant into the abdominal cavity (21) ought to be abandoned. With these methods freezing is too slow to prevent considerable changes of substrate concentrations (Fig. 4). Therefore, values obtained by immersion techniques should no longer be discussed.

Influence of narcosis. Since the original method of Wollenberger *et al.* (1) makes it necessary to expose the organ to be frozen, most authors use anaesthetized animals. Apparently, they suppose the influence of narcotics on substrate concentration to be negligible or low. Yet, this assumption is doubtful. Reinauer and Hollmann (22), who compared the concentration of ATP, ADP, glucose-6-*P*, pyruvate and lactate in the liver of rats, anaesthetized with ether, halothane, evipan, urethan and chloralose, found—especially for glucose-6-*P*, pyruvate and lactate—differences between these types of narcosis up to 500 %. For pyruvate and lactate similar results were obtained by Dale (20) and for glucose-6-*P* by Threlfall and Heath (12). Moreover, the concentration of dihydroxyacetone-*P* and fructose-1.6-di*P* in rat liver was about 50 % lower, when the animals were anaesthetized with phenobarbital instead of ether (20). After application of phenobarbital glucose-6-*P* levels decreased with duration of narcosis (12, 18). Though the studies cited above only compare the effects of several types of narcoses, they justify the assumption that most narcotics deviate the substrate concentrations in rat liver of normal well-fed animals. This can also be deduced from the data in Table IV, which were compiled from the literature.

In the case of phenobarbital the influence of anaesthetic is proved by our experiments, in which substrate concentrations in the liver of anaesthetized animals were compared with the more physiological values, obtained by the double hatchet method. From the results (Table III) it must be concluded that phenobarbital, which seems to

TABLE II

INFLUENCE OF ANIMAL IRRITATION AND NECK FRACTION ON METABOLITE CONCENTRATIONS IN RAT LIVER[a]

Metabolite	Metabolite concentration (nmoles/mg DNA)				
	Absolute tranquility[b]	Tranquility[c]	Slight irritation[d]	Maximal irritation[e]	Neck fraction[f]
ATP	1360 ± 170	1330 ± 120	1030 ± 70	980 ± 40	1070 ± 94
ADP	250 ± 20	270 ± 20	320 ± 20	370 ± 20	339 ± 20
AMP	8 ± 1	9 ± 1	26 ± 4	42 ± 5	45 ± 7
Glucose-6-*P*	69 ± 6	67 ± 4	67 ± 6	75 ± 4	55 ± 8
Fructose-6-*P*	16 ± 2	16 ± 2	16 ± 2	17 ± 1	12 ± 1
Dihydroxyacetone-*P*	20 ± 2	24 ± 2	22 ± 2	22 ± 4	10 ± 2
Glycerol-1-*P*	99 ± 14	71 ± 14	73 ± 15	94 ± 10	76 ± 8
Pyruvate	80 ± 6	82 ± 7	78 ± 4	77 ± 6	43 ± 4
Lactate	490 ± 30	370 ± 60	430 ± 70	520 ± 110	387 ± 30

[a] Unanaesthetized animals were killed by the double hatchet method and liver samples were freeze stopped 3–5 sec later. All values were extrapolated to zero time using the linearity of anoxia induced alterations of metabolite concentrations, demonstrated in Table I. The results are given as means ± SD ($n = 6$).

[b] *Absolute tranquility:* All procedures were done in the morning in a very silent, slightly darkened room. The rats were carefully transferred from their cages to the chopping block and killed 4–6 min later while they were dozing.

[c] *Tranquility:* In this and the following groups all procedures were done in a nondarkened room with normal background noise. The rats, dozing in their cages, were carefully transferred to the block and immediately killed.

[d] *Slight irritation:* The animals were transferred from one cage into another five times within 2 min and then immediately killed.

[e] *Maximal irritation:* The rats were frightened for 2 min by sudden, short, shrill noises, reproduced by a tape recorder. Maximal irritation by this method was obvious.

[f] *Neck fraction:* The rats were killed by dislocation of the neck; a portion of the liver was rapidly removed and immediately frozen between aluminum blocks precooled in liquid N_2 ; the average time between cervical dislocation and deep-freezing the tissue was 8–10 sec.

have a characteristic effect on substrate levels, should no longer be used in experiments, in which substrate levels are studied.

Stunning and neck fraction. Several authors avoided narcosis by stunning (12, 23, 17, 20) or neck fraction (24–35). Those, who prefer the latter method, assume that cervical dislocation has the advantage to maintain circulation and therefore to prevent anoxia (25). This assumption is supported by our results: glucose-6-*P*—a very sensible parameter to anoxia—remained practically constant, and the lactate/pyruvate- and the glycerol-1-*P*/dihydroxyacetone-*P*-ratio increased only slightly (Table II). From this fact as well as from the similarity between the alterations of adenine nucleotide concentrations induced by "maximal irritation" and neck fraction we conclude, that cervical dislocation causes a severe irritation of the nervous system. This is obvious, too, from the violent muscular convulsions observed after neck fraction (and stunning). They have never been observed when rats were cut by the double hatchet. Using our method we measured the lowest hepatic AMP concentrations, described so far (30 nmoles/g liver). On the other hand authors, using cervical dislocation, have obtained AMP values as high as 570 nmoles/g liver (32).

Anoxia. Using the double hatchet method the unwanted influences of narcosis and stress on substrate concentrations can be avoided. On the other hand an anoxia time up to 4.5 sec has to be accepted. Yet, this disadvantage of the method is compensated for by the possibility to extrapolate to zero time with considerable accuracy (Table I). Since the characteristics of anoxia curves depend on the glycogen content of the liver (36) as well as on its enzyme activities, it is

evident that for each metabolic condition separate anoxia curves have to be obtained, if extrapolation is planned.

Apart from the initial phase of anoxia, which has not been measured so far, the characteristics of our anoxia curves are similar to those published recently by Brosnan *et al.* (25) for malate, pyruvate/lactate, ATP, ADP and AMP and by Hems and Brosnan (26) for glycolytic intermediates and adenine nucleotides. Yet, our AMP values differ strikingly from those results as

TABLE III

CONCENTRATION OF GLYCOLYTIC INTERMEDIATES AND RELATED COMPOUNDS IN RAT LIVER AND THE INFLUENCE OF NEMBUTAL NARCOSIS

Metabolite	Measured values (nmoles/g liver wet wt) [nmoles/mg DNA]		Extrapolated values[b] (nmoles/g liver wet wt) [nmoles/mg DNA]		P
	Unanaesthetized[a]	Anaesthetized[c]	Unanaesthetized[a]	Anaesthetized[c]	
ATP	3400 ± 96	3260 ± 122	3440 ± 77	3310 ± 120	
	[1390 ± 90]	[1360 ± 80]	[1410 ± 72]	[1380 ± 82]	ns
ADP	921 ± 54	747 ± 41	758 ± 61	623 ± 38	
	[380 ± 30]	[310 ± 20]	[312 ± 26]	[258 ± 16]	<0.0005
AMP	30 ± 5	72 ± 8	15 ± 2	37 ± 6	
	[12 ± 2]	[30 ± 5]	[6 ± 1]	[16 ± 2]	<0.0005
UDP-glucose	453 ± 11	445 ± 26	451 ± 11	449 ± 39	
	[186 ± 12]	[186 ± 16]	[182 ± 9]	[184 ± 13]	ns
Glucose-1-*P*	14 ± 2	13 ± 2	13 ± 2	13 ± 2	
	[6 ± 1]	[5 ± 1]	[5 ± 1]	[5 ± 1]	ns
Glucose-6-*P*	278 ± 39	93 ± 10	212 ± 29	74 ± 8	
	[112 ± 24]	[39 ± 5]	[84 ± 9]	[30 ± 3]	<0.0005
Fructose-6-*P*	76 ± 10	41 ± 4	58 ± 7	32 ± 3	
	[31 ± 6]	[17 ± 2]	[23 ± 2]	[14 ± 1]	<0.0005
Fructose-1,6-di*P*	27 ± 3	14 ± 2	23 ± 2	12 ± 2	
	[11 ± 2]	[6 ± 1]	[9 ± 1]	[5 ± 1]	<0.0005
Dihydroxyacetone-*P*	62 ± 8	46 ± 4	69 ± 11	53 ± 4	
	[27 ± 5]	[19 ± 1]	[30 ± 3]	[22 ± 1]	<0.025
Glycerol-1-*P*	150 ± 15	225 ± 35	128 ± 8	183 ± 18	
	[62 ± 19]	[94 ± 15]	[53 ± 3]	[74 ± 6]	<0.0025
3-*P*-Glycerate	278 ± 31	185 ± 36	289 ± 32	193 ± 26	
	[113 ± 22]	[76 ± 17]	[116 ± 14]	[79 ± 7]	<0.0025
P-Enolpyruvate	153 ± 11	64 ± 5	161 ± 11	67 ± 5	
	[63 ± 6]	[27 ± 2]	[66 ± 5]	[28 ± 2]	<0.0005
Pyruvate	132 ± 10	166 ± 18	153 ± 10	187 ± 18	
	[54 ± 6]	[69 ± 8]	[63 ± 6]	[77 ± 8]	<0.0025
Lactate	1080 ± 210	1970 ± 193	918 ± 101	1590 ± 158	
	[440 ± 110]	[780 ± 150]	[374 ± 43]	[668 ± 56]	<0.0005
Acetyl-CoA	38 ± 3	41 ± 5	37 ± 3	40 ± 5	
	[15 ± 2]	[17 ± 1]	[15 ± 1]	[16 ± 1]	ns
Citrate	275 ± 31	152 ± 23	281 ± 30	160 ± 22	
	[108 ± 8]	[62 ± 12]	[112 ± 6]	[65 ± 9]	<0.0025
Oxaloacetate	5.5 ± 0.8	13.0 ± 2.5	7.1 ± 1.2	15.3 ± 2.1	
	[2.4 ± 0.8]	[4.8 ± 1.4]	[3.1 ± 0.3]	[5.7 ± 0.9]	<0.01
Malate	212 ± 22	107 ± 13	173 ± 22	86 ± 9	
	[86 ± 13]	[45 ± 7]	[71 ± 8]	[36 ± 3]	<0.0005

[a] Unanaesthetized animals were handled like group: tranquility, described in Table II. All rats were killed by the double hatchet method. Values are given as means ± SD (n = 8). For further experimental details: see Material and Methods.

[b] Extrapolation to zero time by using the linearity of anoxia induced alterations of metabolite concentrations, demonstrated in Table I.

[c] Nembutal anaesthesia (6 mg/100 g body wt, i.p.) for 30 min.

TABLE IV

CONCENTRATION OF GLYCOLYTIC INTERMEDIATES AND RELATED COMPOUNDS IN THE LIVER OF NORMAL, WELL-FED ALBINO RATS (150–260 g) AS GIVEN IN THE LITERATURE[a]

Substrate	Substrate concentration (nmoles/g liver wet wt)					
	Double hatchet method		Neck fraction[a]	Ether[a]	Nembutal[a]	Other methods[a]: St. = Stunning; Dec. = Decapitation; Hal. = Halothan; Ev. = Evipan; Lu. = Luminal; Ur. = Urethan; Thiobarb. = Thiobarbiturate
	Measured[b]	Extrapolated[c]				
ATP	3,370 ± 109	3,490 ± 101	1,920–2,740 (30–32, 31, 25–26)	2,450–3,650 (37–42, 6, 22, 40–8)	2,450–3,020 (19–36–39)	Hal.: 3,030 (22); Ev.: 3,438 (22); Lu.: 3,188 (18); Ur.: 2,940–3,419 (41–22)
ADP	902 ± 41	722 ± 31	710–1,170 (31–32–25)	672–1,195 (8–37, 22, 42, 6–40)	—	Hal.: 779 (22); Ev.: 659 (22); Lu.: 907 (18); Ur.: 669–770 (22–41)
AMP	42 ± 7	21 ± 3	234–575 (30, 35–31, 26, 25–32)	130–315 (37–8–42)	—	Ur.: 230 (41)
UDP-glucose	483 ± 44	485 ± 44	—	—	400 (36)	St.: 230 (12); Thiobarb.: 320 (43)
Glucose-1-*P*	13 ± 3	11 ± 2	17 (35)	—	—	Thiobarb.: 60 (43)
Glucose-6-*P*	275 ± 28	208 ± 20	192–217 (31–35)	195–390 (21–22, 37, 6, 42–12)	80–322 (36–12–39)	St.: 360 (12); Dec.: 266 (44); Ev.: 63 (22); Ur.: 101 (22)
Fructose-6-*P*	74 ± 7	56 ± 6	43–60 (31–26–30, 35)	60–112 (37–6)	100 (39)	Dec.: 70 (44)
Fructose-1,6-di*P*	27 ± 4	22 ± 4	9–12 (31–26–30, 35)	13–38 (20, 40–6, 42, 20–21)	14–23 (20–39)	St.: 24 (20); Dec.: 16–27 (20–44)
Dihydroxy-acetone-*P*	56 ± 9	61 ± 12	17–28 (26, 35–32–31)	23–53 (20–46, 6, 42, 45, 21, 40–20)	23–38 (20–39)	St.: 29 (20); Dec.: 35–37 (20–44)
Glycerol-1-*P*	160 ± 25	133 ± 20	134–318 (35–26, 32–31)	153–460 (45–6, 46, 42, 21–12)	256–640 (39–12–12)	St.: 420–690 (12–23–47); Dec.: 744 (44)
3-*P*-Glycerate	273 ± 29	285 ± 26	175–280 (31–35–26)	296–380 (21–42–6)	—	—
P-Enolpyruvate	145 ± 12	153 ± 12	32–99 (31–35–26)	111–117 (42–21–6)	84 (19)	—
Pyruvate	129 ± 18	151 ± 19	34–220 (26–35, 24, 25, 31, 32, 34, 29, 27–28)	79–256 (22–20, 45, 6, 46, 42, 40, 37, 20–21)	51–138 (20–19–39)	St.: 50 (20); Dec.: 84–207 (44–20); Hal.: 51 (22); Ev.: 73 (22); Lu.: 53 (18); Ur.: 67 (22)
Lactate	1,010 ± 192	880 ± 89	450–2,520 (26–32, 31, 34, 35, 25, 29, 27–28)	1,050–2,084 (22–20, 37, 46, 6, 45–40)	772–2,640 (19–39–17)	Dec.: 1,480 (20); Hal.: 246 (22); Ev.: 338 (22); Ur.: 334 (22)
Acetyl-CoA	38 ± 3	37 ± 3	14–30 (31–28–35)	14–29 (13–8–37)	41 (39, 48)	St.: 16 (47); Dec.: 22 (44)
Citrate	264 ± 52	278 ± 52	294–338 (34–30, 35–31)	196–262 (21–8–37)	231–300 (39–49)	Dec.: 113 (44)
Oxaloacetate	11 ± 4	15 ± 5	12 (31)	6–8 (45–46–6)	8–28 (39–19)	—
Malate	211 ± 42	164 ± 31	307–428 (34–35, 25–31)	294–498 (45–21, 6–46)	374–423 (19–39)	Dec.: 340 (44); Lu.: 138 (18)

[a] The literature is given according to the reported substrate concentration beginning with the lowest one. Values were only considered, if freeze clamp or immersion techniques were applied.

[b] Values are given as means ± SD (n = 38)

[c] Extrapolation according to Table I. These values represent the physiological substrate concentration.

well as from all data published so far in the literature for normal, well-fed Albino rats (Table III). Since most of them were obtained after killing rats by cervical dislocation, we believe the difference to be caused by the maximal stress. In fact, the AMP-concentration in freeze clamped livers of rats, anaesthetized with ether (8, 37) and nembutal (Table III) were significantly lower, although still elevated. Since AMP has proved to be sensitive not only to anoxia (compare also: Chance *et al.* (38)) but to stress as well (Table II), we propose that this parameter be studied in any experiments, concerned with substrate concentrations in animal tissue.

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APPENDIX

The calculation of the cooling curve of Fig. 3, which applies to kidneys of different sizes, has been obtained under the following assumptions: (1) The geometry of the kidney can be treated as a sphere with the radius R. (2) At the time $t = 0$ the entire organ is at a constant temperature $\theta_1 = 37°$. (3) The loss of heat from the surface of the sphere to the surrounding medium is proportional to the temperature difference between the two media. The proportionality constant h is very large i.e., the heat loss ought to be calculated at maximum heat transfer. (4) The constants of heat conduction λ for water and ice amount to 1.5×10^{-3} and 4×10^{-3} cal $\times$ cm^{-1} $\times$ sec^{-1} $\times$ degree^{-1}, respectively. Since in our experiments the heat is mainly conducted by ice a mean value of 3.0^{-3} cal $\times$ cm^{-1} $\times$ sec^{-1} $\times$ degree^{-1} is used. (5) Based on a water content of 80 % of the tissue wet weight the heat of melting has been calculated to 65 cal $\times$ g^{-1}. Neglecting the effect of freezing we have to raise the actual specific heat c of tissue from 0.85 cal $\times$ g^{-1} to 1.15 cal $\times$ g^{-1}.

Due to spherical symmetry the differential equation of heat conduction: $\partial\theta/\partial t = \frac{\partial\theta}{\partial t} = \frac{\lambda}{c \cdot p} \cdot \Delta\theta = a^2 \cdot \Delta\theta$ (ρ being the specific weight of tissue) simplifies to the form: $\frac{\partial\theta}{\partial t} = a^2 \cdot \frac{1}{r^2} \cdot \frac{\partial}{\partial r}\left(r \cdot \frac{6\theta}{\partial r}\right)$. The solution is also expected to be found in spherical coordinates: $\theta = \theta(r, t)$.

We may formulate the initial value from (2) as follows: $\theta \mid_{t=0} = \theta_1$, for $r \leqq R$. According to (3) the boundary condition obtains: $\partial\theta/\partial r + h(\theta - \theta_2) = 0$, for $r = R$. A shift of the zero point of the temperature scale has been assumed in order to have the temperature of the refrigerant at zero ($\theta_2 = 0$).

The above simplifications of the partial differential equation suggest to seek for a solution of the form:

$$\theta = A \cdot \exp(-\omega^2 t) \cdot U(r),$$

leading to the function of $U(r)$ by integration:

$$U(r) = c_1 \cdot \frac{\cos Kr}{r} + c_2 \cdot \frac{\sin Kr}{r}$$

$$K^2 = \frac{\omega^2}{a^2}$$

As the solution ought to be finite at the center of the sphere ($r = 0$) we have to choose $c_1 = 0$. From the boundary condition the value for K can be obtained by inserting $U(r)$. We find:

$$\tan(KR) = KR/(1 - hR).$$

If we calculate the limit for h to infinity we obtain:

$$\lim_{h \to \infty} \tan KR = 0 \text{ i.e.,}$$

$$K_n = n \cdot \pi/R \qquad (n = 1, 2, \cdots).$$

Thus we get the complete solution of the differential equation:

$$\theta(r, t) = \sum_{n=1}^{\infty} c_n \cdot \exp(-n^2 \cdot a^2\pi^2 t/R^2) \times \sin(n\pi r/R)/r.$$

The values of c_n are extracted from the initial value:

$$c_n = \theta_1 \cdot (-1)^{n+1} \cdot 2 \cdot R/n \cdot \pi.$$

In the present case of our experiments we are interested in the time history of the temperature in the center of the sphere thus:

$$\theta(0, t) = \theta_1 \cdot \sum_{n=1}^{\infty} 2 \cdot (-1)^{n+1} \times \{\exp(-a^2\pi^2 t/R^2)\}.^{n^2}$$

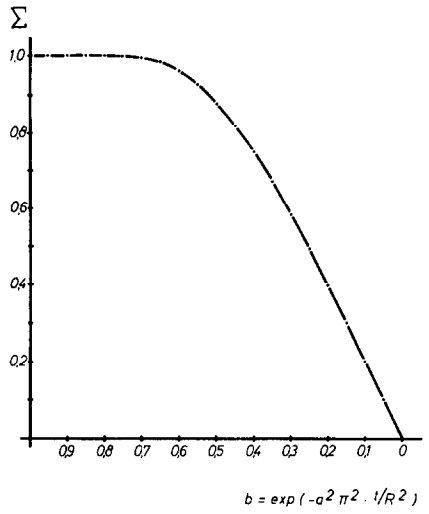

FIG. 6.

For all values of $t > 0$ this series converges. In Fig. 6 we have calculated the sum $\sum$ for the values of $b = \exp(-a^2\pi^2 t/R^2)$, $b \in (0, 1)$.

If we cool the kidney from 37 to 0° with a refrigerant of the temperature of boiling N_2 (−196°) we find a quotient $\theta(0, t)/\theta_1$ of 0.84. From the curve of Fig. 6 we find a value of $b = 0.47$. In 4 and 5 we defined values for λ and c. Inserting these we find: $0.47 = \exp(-a^2\pi^2 t/R^2)$. We use a value of $a^2 = 2.5 \times 10^{-3}\ \mathrm{cm}^2 \times \mathrm{sec}^{-1}$ (this value has also been measured by Wollenberger *et al.* (1)) and calculate the functional relationship between zero-cooling time t and radius R of the sphere: $t = 30\ R^2$. The weight of the kidney is found by multiplying the volume of the sphere with the kidneys specific weight $G = 4/3 \cdot R^3 \cdot \rho$.

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