Effects of β-hydroxybutyrate on rat embryos grown in culture

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Summary. In order to investigate further the relationship between maternal diabetes and fetal malformation, rat embryos were grown in vitro in the presence of β-hydroxybutyrate, one of the ketone bodies produced by diabetics. At 10 mM, β-hydroxybutyrate produced minor abnormalities and at 20 mM it produced major abnormalities in rat embryos.

Key words. Rat, embryo; embryo, rat; β-hydroxybutyrate; diabetes, maternal; fetal malformation; malformation, fetal; ketone bodies.

Diabetic mothers are more likely to give birth to abnormal infants than non-diabetic mothers[2]. The malformations shown by infants of diabetic mothers include defects of the heart and skeleton, and abnormalities of the central nervous system such as anencephaly and spina bifida[3]. Abnormalities are also found in the fetuses of laboratory rodents with natural diabetes[4] or with diabetes which has been induced by injection with the diabetogenic drugs alloxan or streptozotocin[5]. Thus laboratory rodents have been used as a model for the study of abnormal embryogenesis associated with maternal diabetes in man[6].

A major physiological disturbance of diabetes is hyperglycemia which, since glucose freely crosses the placenta[7], might result in the exposure of the developing fetus to elevated levels of glucose. Rodent embryos exposed to raised glucose while in culture over the period of organogenesis show abnormal development[8,9]. Thus the current view is that hyperglycemia is the factor responsible for the fetal malformations associated with maternal diabetes[10]. However recent experiments in which rat embryos were cultured in human sera in which the concentrations of glucose had been standardized show that such human diabetic serum produces more abnormalitites than human non-diabetic serum with similar glucose levels[11]. This suggests that hyperglycemia is not the only physiological disturbance of diabetes which might contribute to fetal malformation.

Another metabolic disorder of diabetes is the production of ketone bodies. When the capacity of the liver to degrade fatty acids is exceeded acetoacetate is produced and then transformed to acetone and β-hydroxybutyrate. Thus it is possible that these metabolites, as well as hyperglycemia, might contribute to the fetal abnormalities associated with maternal diabetes. β-hydroxybutyrate at 5 mM allows normal growth and morphogenesis of rat embryos[12]. Recently, however, β-hydroxybutyrate at 32 mM has been shown to cause abnormalities of the caudal neural tube in mouse embryos[13]. The aim of the present experiments has been to gain further information about the possible teratogenic effect of diabetic ketosis by determining the concentration at which β-hydroxybutyrate is teratogenic to rat embryos, and to observe details of the morphological abnormalities produced by β-hydroxybutyrate using scanning electron microscopy.

Materials and methods. Embryo culture. Embryos at the head-fold stage were obtained from random-bred Wistar rats at 9.5 days of gestation, timed from midnight preceding the morning on which the vaginal plugs were observed. The embryos were explanted in Hank's balanced saline with 4.2×10^{-3} M sodium bicarbonate (Flow Laboratories Ltd., U.K.) and were cultured for 48 h at 37 °C in rotating glass bottles[14]. Each bottle contained 5 embryos in 5 ml of culture medium. The culture medium was pooled rat serum obtained from blood centrifuged immediately after withdrawal and to which had been added streptomycin and penicillin to final concentrations of 100 mg/ml and 100 IU/ml respectively. The serum was stored at −20 °C. Immediately prior to use, the thawed serum was heat-inactivated at 56 °C for 30 min. β-Hydroxybutyric acid, sodium salt (Sigma Ltd., U.K.) was added to various concentrations. Measurements showed that the pH of the inactivated serum was 7.4 when equilibrated with the gas mixture containing 5% CO_2; addition of sodium β-hydroxybutyrate at the concentrations used did not affect the pH of the serum. For each concentration of β-hydroxybutyrate embryos from the same litters acted as controls.

Initially the culture bottles were equilibrated with a 5% O_2, 5% CO_2, 90% N_2 gas mixture. After 24 h and 45 h the cultures were re-equilibrated (20% O_2, 5% CO_2, 75% N_2 and 40% O_2, 5% CO_2, 55% N_2 respectively)[15]. At the end of the culture period the yolk-sac diameters and crown-rump lengths of embryos were measured, and the embryos were scored for heart-beat, yolk-sac circulation and somite number, and any abnormalities were noted. The embryos were then prepared for scanning electron microscopy.

Scanning electron microscopy. Embryos were removed from culture, rinsed briefly in Hank's saline and then transferred to

Effects on rat embryos of exposure to various concentrations of β-hydroxybutyrate during 48 h culture in vitro from the head-fold stage

Concentration of hydroxybutyrate	Number of embryos	Morphological description				Somite number	Yolk-sac diameter (mm)	Crown-rump length (mm)
		Normal	Abnormal	Abnormalities				
			Total	'Major'	'Minor'	(+ SE)	(+ SE)	(+ SE)
Control	(12)	12	0	0	0	22.4 ± 0.38	3.70 ± 0.13	3.10 ± 0.29
0.5 mg/ml (4 mM)	(13)	12	1	0	1	22.4 ± 0.38	3.40 ± 0.21	3.10 ± 0.11
Control	(17)	17	0	0	0	23.1 ± 0.37	3.76 ± 0.05	3.14 ± 0.05*
1.25 mg/ml (10 mM)	(18)	12	6	1	5	22.2 ± 0.40	3.59 ± 0.09	2.96 ± 0.05
Control	(23)	21	2	0	2	23.0 ± 0.31***	3.76 ± 0.06**	3.11 ± 0.04***
2.5 mg/ml (20 mM)	(24)	4	20	7	13	20.5 ± 0.31	3.46 ± 0.08	2.77 ± 0.06
Control	(16)	13	3	2	1	22.9 ± 0.35***	3.72 ± 0.09***	3.12 ± 0.05
5.0 mg/ml (40 mM)	(17)	0	17	17	0	14.9 ± 0.55	3.25 ± 0.09	–

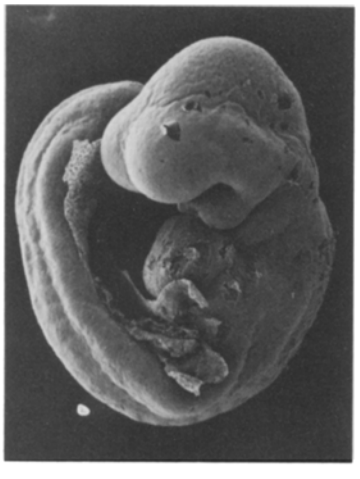
'Minor' abnormalities include abnormal optic vesicles, reduced brain vesicles, slightly abnormal turning and slightly irregular somites. 'Major' abnormalities include open rhombencephalon and/or open neuropores, crooked neural tube and grossly abnormal turning. Asterisks indicate significant differences between controls and experimentals (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3[16]. Following fixation overnight the embryos were washed in changes of cacodylate buffer, and at this stage the embryos were dissected from their embryonic membranes. The embryos were then dehydrated in a graded acetone series, the absolute acetone was replaced with liquid CO_2 and the embryos were dried using the critical point method. The dried specimens were mounted onto stubs. Finally the embryos were coated with a gold-palladium mixture and observed an photographed using a Philips 501B scanning electron microscope.

Results and discussion. By the end of the 48-h culture period control embryos had reached the 21–23 somite stage. The neural folds had fused and the major brain vesicles had formed (fig. 1). The heart was beating and a yolk-sac circulation had been established. In a preliminary experiment embryos were exposed to β-hydroxybutyrate without correcting the final serum osmolarity. Under such conditions embryos exposed to 0.5 mg/ml (4 mM) β-hydroxybutyrate were similar to controls, but all of the embryos exposed to 5.0 mg/ml (40 mM) β-hydroxybutyrate were abnormal. Embryos were stunted, head development was retarded and the embryos had failed to rotate.

In the main experimental series embryos were exposed to a range of concentrations of β-hydroxybutyrate with the osmolarity corrected to the normal value for rat serum (300 mOsm). A range of malformations was observed by scanning electron microscopy and these were classed as 'minor' and 'major' abnormalities. Minor abnormalities included abnormal optic vesicles, reduced brain vesicles, abnormal turning, slightly irregular somites and slight retardation of growth (fig. 2). Major abnormalities included neural tube defects such as open rhombencephalon, open anterior neuropore, open posterior neuropore or a combination of these defects. In such embryos all brain vesicles were severely reduced and optic and otic vesicles were abnormal or underdeveloped. Such abnormalities were generally accompanied by abnormal or incomplete turning, crooked neural tube, irregular or obliterated somites and general growth retardation (fig. 3). The incidence of these abnormalities is summarized in the table. At 0.5 mg/ml (4 mM) β-hydroxybutyrate did not appear to cause abnormal development. At 1.25 mg/ml (10 mM) β-hydroxybutyrate produced some abnormal embryos, and there was an increase in the proportion of abnormal embryos with increasing concentration of β-hydroxybutyrate up to 5.0 mg/ml (40 mM), when all embryos were abnormal. There was also a shift to more major types of abnormalities with increasing concentrations of β-hydroxybutyrate. For the analysis of somite numbers, yolk-sac diameters and crown-rump lengths of embryos the experimental embryos were compared with their respective controls from the same batches of embryos (table). Comparisons of somite numbers suggested that 0.5 mg/ml (4 mM) or 1.25 mg/ml (10 mM) β-hydroxybutyrate did not affect somite number, but that 2.5 mg/ml (20 mM) and 5.0 mg/ml (40 mM) β-hydroxybutyrate reduced somite number ($p < 0.001$ in both cases). Similarly, yolk-sac expansion was not affected by either 0.5 (4 mM) or 1.25 (10 mM) mg/ml β-hydroxybutyrate whereas 2.5 mg/ml (20 mM) and 5.0 (40 mM) mg/ml β-hydroxybutyrate reduced yolk-sac expansion ($p < 0.01$ and $p < 0.001$ respectively). Crown-rump lengths of embryos grown in 1.25 mg/ml (10 mM) and 2.5 mg/ml (20 mM) β-hydroxybutyrate were lower than controls ($p < 0.05$ and $p < 0.001$ respectively). No valid measurements of crown-rump lengths could be made on the abnormal embryos produced by exposure to 5 mg/ml (40 mM) β-hydroxybutyrate since the embryos had failed to rotate, that is assume the characteristic fetal position, normally.

These results indicate that exposure of rat embryos cultured in vitro over the period of organogenesis to a concentration of β-hydroxybutyrate as low as 10 mM can cause developmental abnormalities. Although comparisons of the teratogenicity of compounds between rodents and man must be treated with caution since the relative susceptabilities of rodent and human embryos to teratogenic insult is unknown, the demonstration that β-hydroxybutyrate is teratogenic to rats emphasizes the possibility that this component of the disturbed metabolism of diabetes might contribute to the increased incidence of abnormal infants born to diabetic mothers. Ketone bodies are elevated in diabetics and although mean levels do not approach 10 mM, there is diurnal variation and variation between individuals, and patients in severe diabetic ketosis may have total blood ketone bodies of up to 16 mM. Even when glucose levels have been normalized in diabetic patients the serum concentrations of other metabolites such as ketone bodies may remain



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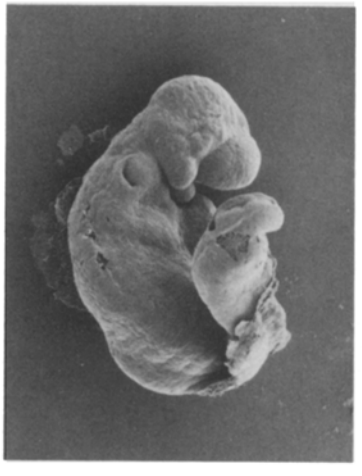

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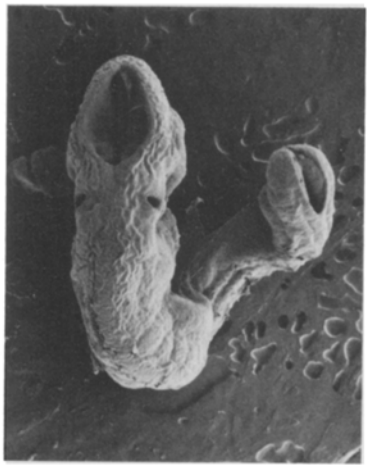

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Figure 1. Scanning electron micrograph of control rat embryo explanted at 9.5 days and cultured for 48 h in vitro. Embryo is fully rotated and shows well developed brain vesicles. × 30.6.

Figure 2. Scanning electron micrograph of rat embryo explanted at 9.5 days and cultured for 48 h in vitro in presence of 2.5 mg/ml (20 mM) β-hydroxybutyrate. Embryo has rotated abnormally and posterior neuropore has failed to close. × 27.3.

Figure 3. Scanning electron micrograph of rat embryo explanted at 9.5 days and cultured for 48 h in vitro in presence of 2.5 mg/ml (20 mM) β-hydroxybutyrate. Embryo is grossly abnormal, rotation is abnormal, anterior and posterior neuropores have failed to close and cerebral hemispheres are absent. × 28.

abnormal[17]. In addition, in the case of diabetes ketone bodies are accompanied by other metabolic disturbances which may act in concert to perturb the normal development of the fetus. The present results indicate that the fetal abnormalities associated with maternal diabetes may be linked not only to hyperglycemia but also to other metabolic disturbances. Thus the possibility is raised that other conditions such as hyperemesis gravidum, which can also cause ketosis, might disturb the normal development of the fetus. It would be interesting to determine whether a correlation exists between the occurrence of hyperemesis during human pregnancy and the incidence of congenital defects.

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The effect of castration, testosterone and estradiol on ^{14}C-serotonin metabolism by organ cultures of male rat pineal glands

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Summary. The pineal gland of the male rat does not appear to rely on prior conversion of testosterone to estradiol for the stimulant effect of testosterone on pineal melatonin and 5-methoxytryptophol synthesis.
Key words. Rat pineal gland; pineal gland, rat; estradiol; testosterone; methoxyindoles; hydroxyindole-O-methyltransferase; castration; serotonin metabolism.

The gonadal sex steroids are known to have a feedback relationship with the pineal gland with regard to synthesis of 5-methoxyindoles[1]. Of these 5-methoxyindoles, melatonin and 5-methoxytryptophol have been shown to possess antigonadotrophic activity. That such feedback exists has been demonstrated in previous studies which show that castration results in a significant decrease in activity of the enzyme responsible for synthesis of 5-methoxyindoles in the pineal gland namely, hydroxyindole-o-methyltransferase (HIOMT), in both male[2] and female[3] rats. Administration of both low doses of testosterone to castrated male[2] rats and estradiol to castrated female[3] rats results in restoration of HIOMT activity to that of control values and higher. Since these 5-methoxyindoles possess antigonadotrophic[4,5] activity, they play an important role in the reproductive cycle. It is therefore also of importance to know how the sex steroids modulate the synthesis of the 5-methoxyindoles by the pineal gland. The pineal gland of the male rat is readily able to convert testosterone to estradiol[6]. However it is not known as to what extent the pineal gland of the male rat relies on this conversion for modulation of 5-methoxyindole synthesis. The modulatory effect of testosterone could therefore be an indirect one. We therefore decided to investigate the extent to which the pineal gland of the male rat depends on the conversion of testosterone to estradiol with regard to the synthesis of 5-methoxyindoles.

Materials and methods. 5-Hydroxy (side-chain-2-^{14}C) tryptamine creatinine sulphate was purchased from Amersham, England; Testosterone propionate, estradiol and the 5-methoxyindoles from Sigma, 0.25 mm GF254 TLC plates from Merck, Germany. BGJb culture medium from Gibco Europe. Male rats of the Wistar strain (230–250 g) were castrated or sham-operated 2 weeks prior to use and were housed under a light cycle of LD 12:12 with food and water ad libitum. The rats were killed by neck fracture at 09.00 h and the pineal

The effect of castration, 10 nM testosterone and 10 nM estradiol on conversion of ^{14}C-serotonin to ^{14}C-5-methoxyindoles by organ cultures of male rat pineal glands (dpm/20 µl medium/gland ± SEM)

5-Methoxyindole	Sham-operated	Castrated	10 nM testosterone	10 nM estradiol
Melatonin	441.2 ± 18[a]	286.7 ± 8[a,c]	454.2 ± 12[c]	205.7 ± 25
5-Methoxytryptophol	1069.7 ± 39[b]	721.7 ± 12[b,d]	981.4 ± 35[d]	840.1 ± 46
5-Methoxyindoleacetic acid	434.0 ± 37	484.6 ± 26[e,f]	545.0 ± 13[e]	653.4 ± 4[f]

[a] $p < 0.005$; [b] $p < 0.001$; [c] $p < 0.005$; [d] $p < 0.02$; [e] $p < 0.05$; [f] $p < 0.001$ (Student's t-test).