account for the very definite facts presented here. For the theory suggested above... namely, that the precipitation is due to the lowering of solubility by neutralization of polar groups of antibody and hapten (or antigen), and concommitant steric hindrance of other polar groups of neighborning molecules in the complex, I wish to propose the name, occlusion theory.'

PAULING never referred to the occlusion theory; he and co-workers[12] later found that 20 haptens, each containing two or more combining groups per molecule, always precipitated with each of his 4 antisera. PAULING el al.[12] dismissed BOYD's contrary results by saying, '...we consider it likely that his experiments were carried out under conditions unfavorable to precipitation – his antisera may have been too weak, or his antigens may have contained monohaptenic impurities.' They evidently did not compare the reported strength of BOYD's sera with their own, or they would have noticed that BOYD's strongest serum contained more antibody per ml than did the weakest of their sera, which they nevertheless found to precipitate all their haptens. As to the other objections; I stick to my guns; my compounds were pure, and my conditions ideal for precipitation.

As already said, the lattice theory was eventually virtually universally accepted. But there are indications that opinion is beginning to change. MARRACK[13], always one of the clearest and most objective thinkers in this field, expressed himself in 1961 as no longer entirely entirely satisfied with his own lattice theory, and stated that '...we are now back to BOYD's occlusion theory.'

The there are some recent experiments that bear importantly on the question. It was always obvious that if specific precipitation should ever be observed with a *univalent* hapten, the lattice theory would have to be modified or abandoned. For it is impossible to imagine lattice formation with antibody and a molecularly dispersed univalent hapten. When the theory was proposed, however, this had never been observed, even in the extensive inhibition studies carried out by LANDSTEINER and his many followers, including myself. But at last the unexpected seems to have happened. SPRINGER and DESAI[14] report that the 7S globulin of eel serum that possesses specific blood group anti-H (0) activity precipitates specifically with either of 2 monohaptenic monosaccharides, viz., 3-*O*-Methyl-D-fucose and 3-*O*-methyl-D-galactose. The possible objection that these monosaccharides might be aggregated in solution was disposed of by vapor pressure osmometry and freezing point depression measurements. With admirable restraint SPRINGER and DESAI remark, 'It is difficult to reconcile these findings with the lattice theory of immune precipitation...'.

It seems possible that the whole question of the mechanism of specific precipitation ought to be reconsidered.

Zusammenfassung. Neue Beweise für eine Theorie der Präzipitationsreaktion (BOYD's «occlusion-theory»).

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STUDIORUM PROGRESSUS

Morphological and Enzymehistochemical Changes in the Interscapular Adipose Tissue of Adult Guinea-Pigs During Prolonged Exposure to Cold

In mammals the function of brown aidpose tissue has been described as thermogenic, both in the infant or when the animal is exposed to cold[1].

In the newborn guinea-pig, the interscapular adipose tissue is typical brown fat with multilocular cells and has remarkable thermogenic ability. However, after 3 or 4 weeks, thermogenesis becomes impaired as the multilocular fat cells change to unilocular[2]. This cytological change is associated with a disappearance of the histochemical reactions of cytochrome oxidase, β-OH butyrate and succinate dehydrogenase, and monoamino oxidase from the fat cells. Thus, after 3 weeks, the morphology and the enzyme pattern of interscapular fat cells resembles more that of the white fat cell than of the brown fat cell[3]. Thus, the guinea-pig differs from the rat which retains some brown adipose tissue throughout life, irrespective of age or ambient temperature.

However, thermogenesis in the unilocular interscapular adipose tissue can be reactivated by exposing the guinea-pigs to prolonged cold. This effect is more pronounced in young animals than in older ones. When thermogenesis is increased, at least part of the fat cells in the interscapular fat pad are multilocular, being scattered among the unilocular fat cells[4].

Based on the above observation, adult guinea-pigs raised at room temperature were exposed to prolonged cold stress and the histochemical reactions of some oxidative enzyme were studied in the interscapular fat in association with morphological changes. Further, the approach was expanded to include electrophoretic characterizations of two of the enzymes which display strong histochemical reactions in both types of fat cells. We hypothesized that if the reappearing multilocular fat cells were indeed brown fat, then some of the enzymes of brown adipose tissue that are normally associated with a high potential for oxidative metabolism should be directly verifiable by histochemistry.

Material and methods. Before the main experiments, a pilot test was made with 2 guinea-pigs at 5 °C $\pm$ 2 °C for 1 month and 1 control at 24 °C $\pm$ 2 °C. After acclimatization, samples from the interscapular adipose tissue (IAT) were excised and the wounds were left to heal for 1 month. These guinea-pigs were then included in the first longer acclimatization test. The samples showed no changes in morphology during the 1 month's cold acclimatization

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when compared to the control; all 3 animals had ordinary unilocular adipose tissue. The enzyme reactions were slightly more intense in the central fat cells of the cold exposed guinea-pigs. On the basis of these findings, the acclimatization time was extended to 2 months.

The first longer experiment (2 months) was carried out with adult male guinea-pigs, including both young adults weighing 400–600 g and older ones from 700–950 g, because in older guinea-pigs, the interscapular fat has been said to be less apt to respond to the cold[4]. 11 animals were placed in separate cages at 5 °C ± 2 °C and their weight matched controls (8 animals) were kept at 24 °C ± 2 °C. Both groups received carrots, cabbage, pelleted food and fresh tap water. One of the control animals died 1 week after the beginning of the test. Of the cold-acclimatizing animals, 3 died after 4 weeks. No illnesses were discovered in these animals, but they were emaciated. In spite of the emaciation, a small amount of brown adipose tissue was found in the interscapular fossa. These animals were not included in the final analysis.

Because no difference was observed between the younger and older guinea-pigs in the first experiment, the second 2 month's experiment was carried out with male guinea-pigs, weighing 520–760 g. 12 animals were moved into the cold chamber (+ 5 °C ± 2 °C) and the 8 controls were kept at 24 °C. A sample of IAT was excised from 3 animals before the experiment to characterize the fat cells of the animals living at room temperature. All had unilocular IAT, whose cells were histochemically, enzymatically inactive. The 3 guinea-pigs were then included in the cold-exposure group. One guinea-pig of the cold-exposure group died after 5 days. After 1 month, IAT samples were taken from 3 other of the cold-exposed animals to check the expected change. The animals were returned to cold. The samples were processed as those obtained at the end of the experiment.

After the exposure period, the animals were weighed, killed, and the interscapular fat pad was excised. 1 sample from each was immediately frozen in liquid nitrogen and mounted on a cryostat chuck for enzyme histochemistry, a second was fixed in 10% neutral formalin, the rest of the tissue was frozen in liquid nitrogen and preserved for the electrophoretic enzyme studies.

Histology and histochemistry. The formalin-fixed tissue was embedded in paraffin by usual methods and sections stained with hematoxylin-eosin. 15 µm thick sections were cut from the piece of frozen tissue in the cryostat and mounted on slides. The sections were then placed in cold (4 °C) acetone for 15 min to remove triglycerides. No other type of fixation was utilized. Using the methods presented in the textbook by BARKA and ANDERSON[5], the following enzyme reactions were run:

Cytochrome oxidase (CYO): The NADI-method of BURSTONE[5]. Incubation times 1 h and 2 h. Monoamino oxidase (MAO): The method of GLENNER et al.[5]. Incubation times 1 h and 2 h. Succinate dehydrogenase (SDH): Method by NACHLAS et al.[5]. Incubation times 1 h and 2 h. β-hydroxybutyrate dehydrogenase (β-OHBDH): Method of HESS et al.[5] with NAD as cofactor. Incubation times 1 h and 2 h. Lactate (LDH) and α-glycerophosphate (GPDH) dehydrogenases: Methods of HESS et al.[5] with NAD as cofactor. Incubation times $^1/_2$ h and 1 h.

Control sections were incubated without substrate, or for CYO in the presence of 0.001 *M* KCN and for MAO in the presence of L-isonicotinyl-2-isopropyl-hydrazine phosphate. Sections from cold adapted and control animals were incubated simultaneously in the same jar in every enzyme reaction.

Electrophoresis. Samples were prepared for starch gel electrophoresis by the homogenization of 2 g of interscapular fat in 4 ml of 0.1 *M* phosphate buffer, pH 7.4, with a Vir-Tis blade homogenizer for 3 min at medium speed. The homogenizing vessels were then rinsed with 2 ml of the 0.1 M phosphate buffer and the samples plus washing were centrifuged at 10,000 *g* for 20 min.

The supernatant from each sample was concentrated 20 times by vacuum dialysis[6], and electrophoresed following the method of FINE and COSTELLO[7], except that 12% gels and Whatman No. 3 filter paper were used. Separation was carried out horizontally for $4^1/_2$ h at 4 °C with an applied voltage across the gel of 8 V/cm.

After electrophoresis each gel was sliced into 3 horizontal sections with a No. 32 gauge stainless steel wire. Each section was then stained for 1 of 3 enzymes. The results were recorded by photographing the gels with a Polaroid MS-3 Land camera.

The enzymes investigated were LDH and GPDH. The method used for the demonstration of LDH was that of FINE and COSTELLO[7], for GPDH that of HESS et al., except that phenazine methosulfate (1.2 mg in 50 ml staining solution) was added.

Results. Morphology. After the 2 months exposure 13 of the animals kept in the cold had lost weight (80–170 g) and 6 had gained (25–62 g). Only 5 of the warm-acclimatized animals had lost weight. Each guinea-pig had a large interscapular and epididymal adipose tissue pad. The colour of the interscapular fat of the control guinea-pigs was yellow. In the cold-exposed animals, the fat pad was evenly light brown, no separate masses of white and brown fat was recognizable macroscopically.

Microscopically the interscapular fat cells of all the control guinea-pigs were unilocular in appearance. In the adipose tissue of all animals – even the oldest ones – cold-exposed for 2 months, several patches of multilocular cells were found among the unilocular cells (Figure 1). The multilocular cells were generally located in the central part of the fat lobules. The borderline between the areas accupied by the 2 different fat cell types was not distinct. The proportion of multilocular cells varied from 30–90% of all fat cells. The amount of red blood cells was increased in sections from 3 cold adapted guinea-pigs, other cold adapted animals did not differ from the controls in this respect. Of the IAT samples taken from the 3 cold animals at 1 month, 2 contained about 10% multilocular cells, 1 had only unilocular cells. At the end of the experiment, IAT from these animals contained 30% multilocular cells.

Histochemistry. Qualitatively, but not quantitatively, the enzyme reactions for the samples taken after 1 month of cold exposure were the same as those described below for 2 months exposure.

Cytochrome oxidase (CYO). The reaction of CYO was absent or very weak in the unilocular fat cells of the control animals. Without exception, in the samples from the cold-acclimatized animals, CYO reaction was moderate or even strong in the cytoplasm of multilocular cells and distinct in some unilocular cells in the central part of the lobules. The reaction appeared in small granules concentrated around lipid droplets, the rest of the cytoplasm was pale (Figure 2). Amongst these positive cells there were numerous unilocular fat cells which did not display any

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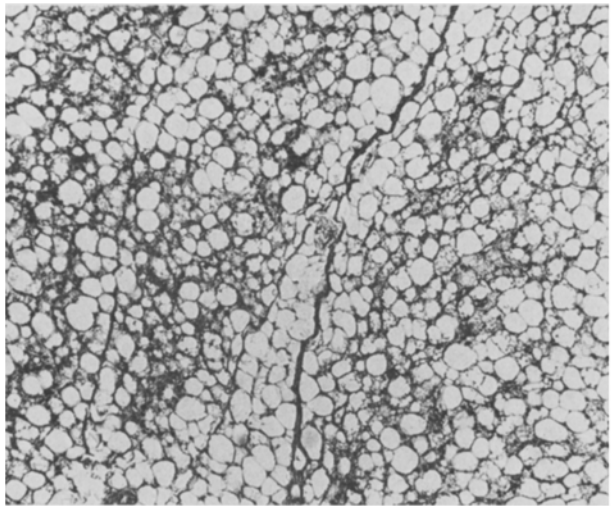

Fig. 1. Interscapular adipose tissue (IAT) from a cold-exposed guinea-pig. Hematoxylin and Eosin. Several multilocular fat cells among unilocular cells. The multilocular cells show different amounts of cytoplasm. The fat cells of control animals were all unilocular resembling ordinary white fat such as seen on the right side of the photograph. ×60.

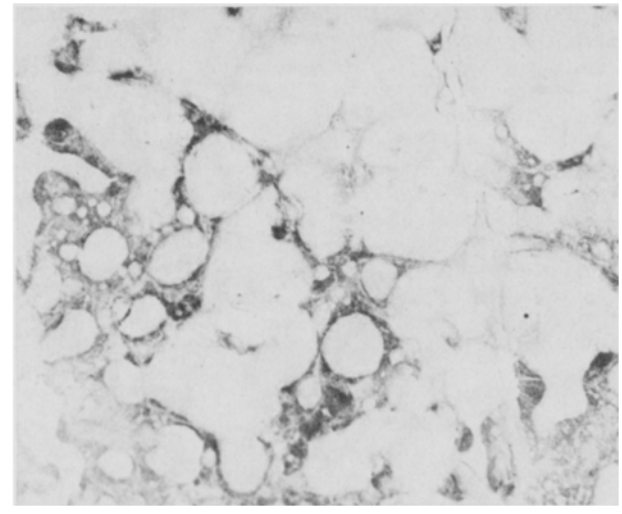

Fig. 2. Cytochrome oxidase reaction from the IAT of a cold-acclimatized animal. Multilocular and some unilocular fat cells display distinct granular reaction. Some unilocular fat cells stain weakly or are negative. These cells are in the periphery of the lobules. ×250.

CYO reaction despite the long incubation (2 h) (Table). The sections incubated with KCN showed no reaction.

Monoamino oxidase (MAO). The reaction of MAO was absent in the adipose cells of the control animals as well as in the large unilocular fat cells of the cold-adapted guinea-pigs. In the multilocular fat cells of the cold-adapted animals MAO reaction became distinct after 1 h incubation and became more intense when incubated 2 h. The reaction product was confined in small cytoplasmic granules analogously to the CYO reaction (Figure 3). No reaction appeared in the sections incubated with inhibitor.

Succinate and β-OH butyrate dehydrogenase. These 2 dehydrogenase reactions were absent or very weak in the unilocular fat cells of the control animals (Table).

In the sections of all the cold-exposed animals both SDH and β-OHBDH reactions became distinctly visible in the multilocular fat cells, as well as in some unilocular centrally located fat cells with 1 h incubation time (Figure 4, Table). In each sample from these animals, there still were unilocular fat cells which displayed a very weak or no reaction resembling the fat cells from the warm-acclimatized animals. 2 h incubation did not enhance the reaction.

Both SDH and β-OHBDH reactions were granular in character. The granules (presumably mitochondria) were heavily concentrated around the lipid droplets. In regard to the number of these enzyme positive granules, 2 types of unilocular cells were discernible. One had a dense zone of intensely stained granules around the fat vacuole

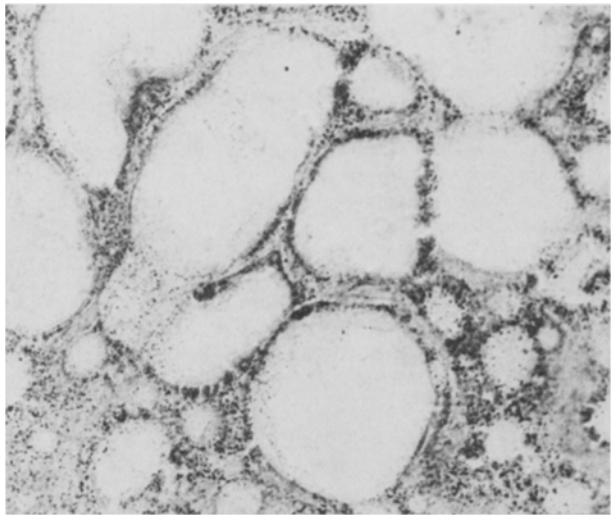

Fig. 3. Monoamino oxidase reaction in the IAT from a cold-acclimatized animal. Reaction product is confined in mitochondria that surround the fat lobules. In control animals MAO reaction was absent. ×400.

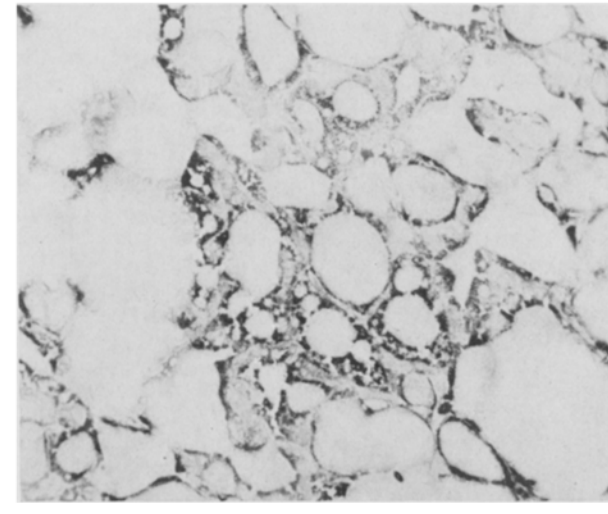

Fig. 4. β-hydroxy butyrate dehydrogenase reaction in the IAT of a cold-exposed guinea-pig. Strongly positive multilocular fat cells and unilocular fat cells. Besides these an area of negative unilocular cells is seen. In the multilocular cells intensely stained granules (obviously mitochondria) fill the cytoplasm around fat droplets. ×250.

The approximate proportion of unilocular and multilocular fat cells and the intensity of reaction of oxidative enzymes in the fat cells in the group of the warm- and cold-acclimatized guinea-pigs

	Unilocular fat cells (%)	Multilocular fat cells (%)	Cytochrome oxidase	Succinate dehydrogenase	β-OH-butyrate dehydrogenase	Monoamino oxidase	Lactate dehydrogenase	α-glycero PO_4 dehydrogenase
Cold-acclimatized	10–70	30–90	0–±/++	0–±/+++	0–±/++	0/++	++/+++	++/+++
Warm-acclimatized	100	0	0	0–±	0	0	++	++

The reaction intensity in the peripheral unilocular fat cells is given above; that in the multilocular cells beneath the line.
0, no reaction; ±, traces; +, weak; ++, moderate; +++, strong reaction.

(unilocular brown fat cells), the second had a few very weakly stained granules scattered in the cytoplasm (white fat cell).

Succinate dehydrogenase reaction did not develop in the sections incubated without substrate. Incubation in the dehydrogenase control solution for β-OHBDH without the specific substrate yielded a weak reaction in the multilocular fat cells but no colour in the unilocular fat cells.

Lactate (LDH) *and α-glycerophosphate dehydrogenase* (GPDH). The reaction of LDH was distinct in the unilocular fat cells from the control animals as well as in the unilocular and multilocular cells of the cold-exposed animals. In the areas of multilocular cells in the cold animals, the reactions were more intense than in the adjacent white fat cell areas in the same section, but the difference was not as striking as with SDH (Table). The reaction of the enzyme was both evenly distributed and granular in the fat cells' cytoplasm.

The GPDH reaction was very weak or sometimes negative in the unilocular (white) fat cells of the control animals. In the cold-adapted animals the reaction became very intense in the multilocular fat cells with 30 min incubation (Figure 5). The longer, 1 h incubation did not increase the colour. The reaction product was both evenly distributed in the cytoplasm and appeared in small granules (mitochondria?).

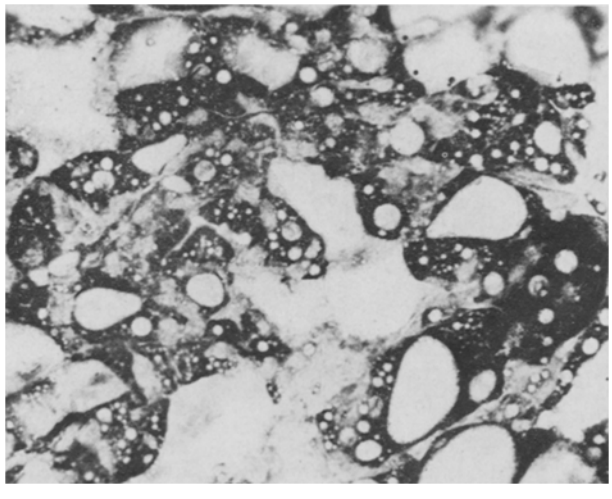

Fig. 5. α-glycerophosphate dehydrogenase reaction in the IAT of a cold-exposed guinea-pig. An area with strongly positive multilocular cells is seen). A less intense reaction is seen in some unilocular cells amongst the multilocular ones. Similar patterns were observed in the lactate dehydogenase reaction. ×250.

The dehydrogenase control solution without lactate and α-glycerophosphate gave a weak reaction in the multilocular cells, but no reaction in the unilocular cells.

Starch Gel electrophoresis. An interesting finding of the LDH electrophoretic studies on guinea-pig interscapular adipose tissue is the prominence of all 5 isozymes in both the experimental and control animals (Figure 6, *1*). This is particularly evident in the control animals (*b* and *d* of Figure 6, *1*). However, in the experimental animals (*a* and *c*), although all 5 bands are well defined, there is a definite graduation toward a higher concentration of LDH-1.

In the 2 experimental animals shown, as well as the other cold-exposed animals, the predominate LDH isozyme was LDH-1 while the least was always LDH-5. In these animals the overall activity of LDH appeared to be greater than that found in the control animals.

In the control group, the LDH isozymes did not fall into any definite pattern. 2 samples had predominantly LDH-2; 2 others, LDH-2 and LDH-5, both showing equal activity and greater than LDH-1; the 5th animal had predominantly LDH-5 and the last animal had a pattern similar to the cold-exposed animals.

In the erythrocyte LDH patterns, LDH-1 was found to be the predominant isozyme, while LDH-5 activity was very low, considerably less than that found in LDH from fat. There was no LDH serum activity.

The other enzyme characterized, GPDH, showed no consistent differences in the electrophoretic patterns between the experimental and control animals. Nor were there any consistent differences in the relative activities in these 2 groups. Typical patterns are seen in Figure 6, 2 for GPDH. The enzyme had one major and several minor bands of activity after electrophoresis. The major band was composed of multiple sub-bands; a maximum of 5 were found. The patterns of the minor bands showed no consistent differences in the 2 groups of animals.

Discussion. The reappearance of multilocular fat cells, coupled with our histochemical evidence, confirm that a longer exposure to cold will reactivate the quiescent metabolic potential of the interscapular fat cells in the adult, warm-acclimatized guinea-pig. These findings extend the earlier observations on the reappearance of multilocular fat cells in this species under similar conditions, concomitant with an increased capacity for nonshivering thermogenesis[4].

In the cold-acclimatized guinea-pigs there were 2 different kinds of fat cells in regard to their oxidative enzymes, but only 1 type in the controls. The most marked difference was the appearance or intensification of cytochrome oxidase (CYO) monoamino oxidase (MAO), succinic dehydrogenase (SDH), β-OH butyrate dehydro-

genase (β-OHBDH) and α-glycerophosphate dehydrogenase (GPDH) reaction. These enzyme reactions were absent or gave very weak colour in the unilocular fat cells of the control guinea-pigs and in some of the unilocular cells of the cold-acclimatized animals. In the last-mentioned animals, there were also other unilocular fat cells that displayed intense enzyme reactions. These results suggest that histochemical techniques are more discriminating than multilocular appearance in distinguishing brown fat cells.

The intense reactions of CyO and SDH in the multilocular and some unilocular fat cells of the cold-acclimatized guinea-pigs indicates enhanced oxidative capacity. In brown fat, oxidation is connected with the generation of heat during cold exposure. Thus, the enzymatic changes are a further proof of the adaptative mechanisms having occurred in the guinea pigs kept at 4 °C for 2 months.

The apparent activation of the β-OHBD and GPDH reactions further supports the view that portions of the interscapular adipose tissue regain its heat generating capacity. These enzymes are associated with lipid metabolism and it is known that fat is the primary energy source for thermogenesis in brown fat[8]. The presence of MAO reaction in fat cells fits well with the importance of adrenergic innervation in cold expoxure[9] and the lipolytic effect of noradrenaline. Studies concerning the development of a more dense adrenergic innervation during cold-acclimatization of guinea-pigs are in progress.

The shift toward LDH-1 in the cold exposed animals could be a de novo change in either or both cell types. It could also reflect an increased contribution of LDH-1 from erythrocytes since there is known to be an increase in the vascularity of brown adipose tissue in cold-exposed animals[1]. Results in the control animals suggest there are two or more separate sources of LDH. Erythrocytes were found in this study, as has been shown by VESELL and BEARN[10], to contain mainly LDH-1 and very little LDH-5. One study[11] of subcutaneous inguinal and pericardial adipose tissue in dogs showed the greatest fractional activity to the LDH-5 in the former and LDH-1 in the latter. In interscapular brown adipose tissue of hamsters[12], a shift toward LDH-5 was observed when the animals were exposed to cold (4 °C ± 1 °C) for 54 days.

The GPDH isozyme patterns from liver, kidney and several other organs from various animals have been reported by AGRELL and KJELLBERG[13]. The isozymes of GPDH reported here are similar to those of these investigators in that there are 3 bands of activity. In this study, the slower migrating band was always the most intense band and possessed up to 5 sub-bands. These were found in both the control and the cold-exposed animals.

The enhanced enzyme reactions were associated with an increase in the number of mitochondria in the fat cells. The numerous mitochondria both in multilocular and unilocular brown fat cells were evident as granular cytoplasm in hematoxylin-eosin staining and as enzyme-positive granules, particularly in CYO, MAO, β-OHBDH and SDH reactions. These enzymes are known to be located in mitochondria. The great number of mitochondria indicate new synthesis induced by the cold stress, as suggested in earlier studies on guinea pigs[14] and on rats[15,16].

Although the cold-acclimatization changes in the IAT of rat and guinea-pig resemble each other, in some ways there are also differences. The rat retains multilocular brown fat regardless of ambient temperature and adaptive changes to cold can be readily followed in this species[17,18]. On the other hand, the guinea-pig appears to lose multilocular brown fat from the interscapular region, which is replaced by white fat[2,3]. Similar involution of brown fat occurs also in man between 10 and 20 years[19]. There is no information on possible changes in the human adipose tissue after prolonged cold exposure. Based on the present results and those on man[19] the guinea-pig is probably a more suitable model than the rat in cold-exposure studies for man.

Zusammenfassung. Dauereinwirkung von Kälte (2 Monate +4 °C) führte beim Meerschweinchen zum Auftreten multilokularer Fettzellen, welche intensive histochemische Reaktion für mehrere oxydative Enzyme zeigten. Die Zahl der Mitochondrien und LDH-1 war im Kälteversuch vermehrt.

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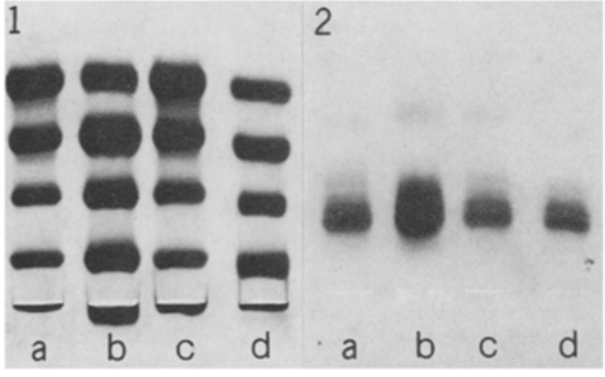

Fig. 6. Starch gel electrophoresis of guinea-pig brown fat LDH and GPDH: lactate dehydrogenase (1) and α-glycerophosphate dehydrogenase (2). In both, *a* and *c* are samples from cold-acclimatized animals while *b* and *d* are samples from control animals. Each was stained by methods listed in the text.

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[20] Acknowledgment. This study was initiated when Dr. J. HIRVONEN worked as a post-doctoral fellow of NIH, U.S. Public Health Service (No. F 05 TW 1546) at Arctic Health Research Center, Fairbanks (Alaska).
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