IN VITRO STUDIES ON SYNOVIAL MEMBRANE

EFFECT OF SOME THERAPEUTIC AGENTS AND CHEMICALLY RELATED COMPOUNDS

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Considerable knowledge has been gained from the clinical study of various therapeutic agents in rheumatoid arthritis, yet comparatively little information is available on the metabolic action of these substances at the tissue level. A satisfactory *in vitro* approach to this problem could not be made until it could be established that *in vitro* metabolic changes occurred in the presence of rheumatoid disease in the synovium. We have shown that normal synovial membrane was a tissue with an extremely low oxidative metabolism, though its glycolysis was readily measurable. The tissue reaction to the disease involved the appearance of considerable oxidative metabolism, as well as an increase in glycolysis (Dingle and Page Thomas, 1956). It was also noted that the highest rate of metabolism was found in villous tissues, and that a positive correlation existed between the proliferative state of the disease in the knee joint and the metabolism of the excised tissue.

In the cell, the synthesis of energy-rich bonds is achieved mainly by the oxidative breakdown of carbohydrate. The increase in synovial oxidative metabolism in rheumatoid disease is probably associated with the increased energy requirements of the proliferating synovium. Hence, any agent which alters this metabolic state might play a part in modifying the response of the tissue to the disease stimulus.

Preliminary results have indicated that hydrocortisone is a potent inhibitor of synovial metabolism (Page Thomas and Dingle, 1955). The degree of inhibition of oxygen uptake produced *in vitro* by a standard concentration of hydrocortisone on rheumatoid synovia of varying degrees of metabolic activity was greatest in those tissues which exhibited the highest oxygen uptake (Page

Thomas and Dingle, 1957). In this series of experiments, the *in vitro* action of hydrocortisone has been compared with Compound B (corticosterone), Compound S (11-desoxy 17-hydroxycorticosterone), cortisone (11-dehydro 17-hydroxycorticosterone), and DOCA (desoxycorticosterone acetate).

Other compounds which have been investigated are o-hydroxy benzoic acid (salicylic acid), m- and p-hydroxybenzoic acids, phenylbutazone (Butazolidin), and insulin.

Materials

(1) **Steroids.**—Recent work has shown that the method of presentation of steroids to synovial tissue *in vitro* is important (Page Thomas and Dingle, unpublished results). Different methods of solution or presentation of the same steroid have given quantitative and qualitative differences in the metabolic effect on rheumatoid synovium. In the present series of experiments, the steroids have been mainly used as crystalline suspensions in Krebs-Ringer phosphate (Umbreit, Burris, and Stauffer, 1949). The suspensions were prepared by dissolving a weighed quantity of steroid in A.R. acetone, samples of which were pipetted into Warburg flasks, and the acetone was then evaporated off in a warm oven. The film of material on the wall of the flask, when shaken with Krebs-Ringer phosphate gave a suspension containing an accurate amount of material per flask. The control flasks were treated with the same volume of pure acetone.

Two other methods of steroid presentation have been used. In one experiment the steroids were dissolved in 1, 2 propanediol and a comparison of aerobic and anaerobic glucose utilization was made. The standard steroid used for comparative purposes has been a solution of hydrocortisone obtained by autoclaving (Miller, 1954).

(2) **Hydroxybenzoic Acids.**—o-hydroxybenzoic acid (salicylate acid), and m- and p-hydroxybenzoic acids were dissolved in Krebs Ringer phosphate and adjusted to

pH 7·4 with sodium hydroxide. A final concentration of 1 mg./ml. was used.

(3) Phenylbutazone (Butazolidin).—0·015 g. of this substance was dissolved in 0·5 ml. pure ethanol and then added to 99·5 ml. Krebs-Ringer phosphate. The control solution was similarly prepared using ethanol alone.

(4) Insulin.—The crystalline material was dissolved in Krebs-Ringer phosphate to give a final concentration of 10μg./ml.

Method

Rheumatoid synovial tissue was obtained at the time of operation from patients undergoing synovectomy or arthrodesis of the knee. The tissue was dissected free from the underlying fat and fibrous tissue and prepared for *in vitro* experiments as previously described (Dingle and Page Thomas, 1956). All synovia used were shown to have the histological changes characteristic of the disease. Oxygen consumption was measured by conventional Warburg techniques. For aerobic experiments 100 per cent. oxygen was used, and for anaerobic experiments 100 per cent. nitrogen. After incubation the tissue was separated off and glucose, glycogen, lactic acid, and nitrogen were estimated as previously described (Page Thomas and Dingle, 1955). All results were expressed on a tissue nitrogen basis, *i.e.* $Qo_2(N)$ being $\mu l \cdot O_2$ uptake per hr per mg. tissue nitrogen.

Results

Steroids.—Table I shows the effect of six corticosteroids on the metabolism of rheumatoid synovial villi. The Table is divided into two parts showing the metabolic effects of these compounds in the presence and absence of added substrate. The corticosteroids were present at a concentration of 1 mg./ml. Five of the steroids were used as suspensions, the sixth, hydrocortisone (free alcohol), was present as a solution.

(a) In the Presence of Added Substrate.—Compounds B, DOCA, S, and E, in that order, strongly inhibited oxygen uptake, glucose utilization, and lactate production. Compound F acetate had no appreciable effect on metabolism; its soluble form, however, gave almost complete inhibition of metabolic activity. Though in the presence of glucose the control flasks showed no utilization of glycogen, Compounds B, S, and E acetate increased its breakdown to a marked extent. Compounds DOCA and F acetate were not so active in this respect.

(b) In the Absence of Added Substrate.—Again Compounds B, DOCA, and S, in that order, inhibited oxygen uptake, though their inhibitory effects were less marked (Table I). Compounds E and F acetate had little effect, but again the soluble form of F showed complete inhibition of respiration. In the absence of glucose, glycogen and some lactic acid were utilized by the control flasks. The tissue utilization of glycogen was again increased in the

TABLE I

EFFECT OF CORTICOSTEROIDS ON METABOLISM OF RHEUMATOID SYNOVIAL VILLI SHOWING HIGH
METABOLIC ACTIVITY

Substrate	Glucose 10 mM				None		
Steroid	Qo2(N)	Glucose Utilized (μg./hr/mg.N)	Tissue Glycogen Change (μg./hr/mg.N)	Lactate (μg./hr/mg.N)	Qo2(N)	Tissue Glycogen Change (μg./hr/mg.N)	Lactate (μg./hr/mg.N)
Hydrocortisone free alcohol (Compound F)	Trace	−110	—	+45	trace	—	—
Hydrocortisone acetate (Compound F acetate)	22·4	−630	−29	+230	21·8	−43	−10
Cortisone acetate (Compound E acetate)	12·0	−440	−38	+145	22·0	−47	0
Desoxycorticosterone acetate (DOCA)	5·5	−250	−22	+104	10·5	−47	+12
11-desoxy 17-hydroxy cortisterone (Compound S)	9·5	−240	−49	+108	15·5	−43	−5
Corticosterone (Compound B)	2·5	−320	−41	+82	3·5	−38	+16
Control	22·0	−600	−3	+226	23·0	−27	−10

− indicates utilization by tissue
+ indicates production by tissue
Each observation is the mean of duplicate estimations

Steroids present at concentration of 1,000 μg./ml.
Gas phase 100 per cent O₂
Initial glycogen level 67 μg./mg.N

presence of all the steroids, but their action on the formation of lactic acid appeared to be erratic. This may be due to the metabolism of lactic acid by the tissue in the absence of exogenous carbohydrate.

The above results were obtained by the use of rheumatoid villous tissue of relatively high metabolic activity. In order to test the generality of such tissue responses, we also investigated the effect of the steroids on rheumatoid tissues of low metabolic activity. Table II shows that Compound F (soluble form) is still the most potent steroid, though it did not give complete inhibition of activity. With respect to oxygen uptake, Compound B was again the strongest inhibitor of those steroids used in suspension. The steroids in general did not give such a high degree of inhibition as with the more active villous tissue. With the exception of Compound B, glucose utilization was not inhibited, and Compound E may even have stimulated uptake by the tissue. The production of lactic acid by the tissue was not markedly affected by the presence of the steroids in suspension. The initial glycogen levels in this type of tissue were extremely low and the measured changes in tissue glycogen are of doubtful significance.

It would appear that, in synovial tissue of low metabolic activity, the inhibitory power of these steroids is diminished. These findings are substantiated by results reported elsewhere (Page Thomas and Dingle, 1957). It is apparent, therefore, that the increased oxidative metabolism associated with the disease state is markedly inhibited by some of these steroids, the most effective being Compounds F (solubilized), B and S.

Previous experiments have also indicated that an increase in aerobic and anaerobic glycolysis occurs in proliferating synovial tissues (Dingle and Page Thomas, 1956). We have therefore investigated the effect of the more potent *in vitro* corticoids on the utilization of glucose. Compounds B, S, and F were dissolved in 1, 2 propanediol at a concentration of 10 mg./ml. Samples were then added to the Warburg flasks to give a final concentration of 250 µg./ml. The same quantity of 1, 2 propanediol alone was added to the control flasks. The results are shown in Table III. Under these conditions, the steroids appeared to be more effective in inhibiting glucose utilization when the tissue is incubated under anaerobic conditions. The control values confirm the previous findings that the glucose utilization of synovial tissue is markedly reduced in the presence of oxygen.

TABLE III

EFFECT OF CORTICOSTEROIDS ON AEROBIC AND ANAEROBIC GLUCOSE UTILIZATION

Steroid	Glucose Utilization (µg./hr/mg.N)	
	Aerobic	Anaerobic
Corticosterone (Compound B) ..	− 250	−250
11 desoxy 17 hydroxy corticosterone (Compound S)	−250	− 340
Hydrocortisone acetate (Compound F acetate)	− 230	− 340
Control	− 290	− 520

− indicates utilization by tissue
Glucose 10 mM present in all flasks
Each observation is the mean of three estimations
Steroids dissolved in propylene glycol 10 mg./ml.
Final steroid concentration in Krebs-Ringer-Phosphate 250 µg./ml.
Gas phase, aerobic 100 per cent. O_2, anaerobic 100 per cent. N_2

TABLE II

EFFECT OF CORTICOSTEROIDS ON METABOLISM OF RHEUMATOID SYNOVIAL MEMBRANE OF RELATIVELY LOW METABOLIC ACTIVITY

Steroid	$Qo_2(N)$	Utilization of Glucose (µg./hr/mg.N)	Change in Tissue Glycogen (µg./hr/mg.N)	Lactate (µg./hr/mg.N)
Hydrocortisone free alcohol (Compound F)	0·8	− 7	—	0
Hydrocortisone acetate (Compound F acetate)	6·5	·· 61	+ 5	+ 47
Cortisone acetate (Compound E acetate) ..	7·5	−76	+ 3	+ 49
Desoxycorticosterone acetate (DOCA)	7·0	− 50	0	+ 44
11-desoxy 17-hydroxy corticosterone (Compound S) ..	5·2	−65	0	+ 43
Corticosterone (Compound B)	1·5	− 27	0	+ 32
Control	7·8	− 52	0	+ 36

− indicates utilization by the tissue
+ indicates production by the tissue
Glucose 10 mM present in all flasks

Each observation is the mean of duplicate estimations
Steroids present at a concentration of 1,000 µg./ml.
Gas phase 100 per cent. O_2

Hydroxybenzoic Acids.—Table IV shows the effect of o-, m-, and p-hydroxybenzoic acids on glucose utilization, lactate production, and endogenous glycogen content of rheumatoid synovium, under aerobic conditions. The m-hydroxybenzoic acid was found to inhibit glucose utilization and lactate production. Of the other compounds, p-hydroxybenzoic acid may have had a slight inhibitory effect on glucose utilization; the other values given show little change from the control figures. The Figure shows the effects of these three compounds on the rate of oxygen uptake by rheumatoid synovium. The p- and m-hydroxybenzoic acids inhibited the rate of oxygen uptake. This effect was more marked with the meta form. The ortho compound, however, produced an initial stimulation of oxygen uptake in the first 10 minutes, after which period the rate per minute fell off rapidly.

Insulin.—Table V illustrates the effect of insulin on the metabolism of rheumatoid synovium in the presence and absence of hydrocortisone. The presence of insulin alone resulted in an increase in the rate of oxygen uptake, glucose utilization, and lactate production. There was also an increase in the synthesis of glycogen in the flasks containing insulin. In flasks in which both hormones were present, no change in the rate of oxygen uptake took place as compared with the control flasks, but
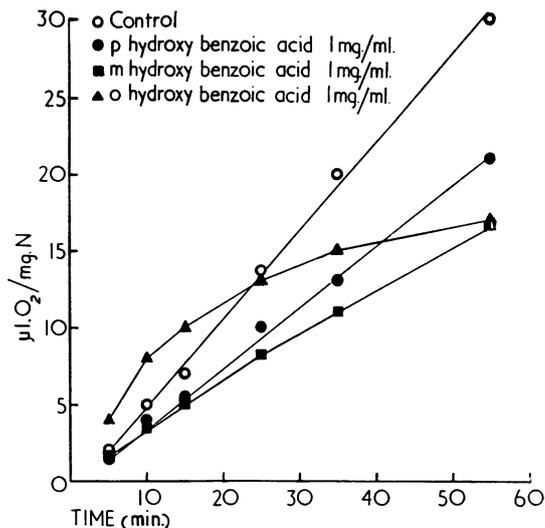
Each point is the mean of three observations. Glucose 10mM present in all flasks. Gas phase 100% oxygen.

Figure.—Effect of hydroxybenzoic acids on the oxygen uptake of rheumatoid synovium.

there was an overall reduction in the amounts of glucose utilized and lactate produced. There was also an inhibition of glycogen synthesis. The presence of hydrocortisone alone resulted in an

TABLE IV

EFFECT OF HYDROXYBENZOIC ACIDS ON METABOLISM OF RHEUMATOID SYNOVIUM

Substance tested	Glucose Utilized (μg./hr/mg.N)	Change in Glycogen Content (μg./hr/mg.N)	Lactate Produced (μg./hr/mg.N)
Control	−430	−1	+180
o-hydroxy benzoic acid	−410	−3	+170
m-hydroxy benzoic acid	−320	0	+130
p-hydroxy benzoic acid	−385	0	+170

− indicates utilization by tissue
+ indicates production by tissue
Glucose 10 mM present in all flasks
Each observation is the mean of three estimations

Gas phase 100 per cent. O_2
Initial glycogen content 24 μg./mg.N
Hydroxybenzoic acids used at final concentration 1 mg./ml.

TABLE V

EFFECT OF INSULIN AND INSULIN+HYDROCORTISONE ON METABOLISM OF RHEUMATOID SYNOVIUM

Experimental Conditions	$Qo_2(N)$	Glucose Utilized (μg./hr/mg.N)	Glycogen Change (μg./hr/mg.N)	Lactate Produced (μg./hr/mg.N)
Control	10·0	−260	+4	+110
Insulin 10 μg./ml.	14·8	−300	+15	+130
Insulin 10 μg./ml.+hydrocortisone free alcohol 220 μg./ml.	9·0	−145	0	+27
Hydrocortisone free alcohol 220 μg./ml.	7·0	−148	+4	+57

− indicates utilization by tissue
+ indicates production by tissue
Glucose 10 mM present in all flasks

Each observation is the mean of three estimations
Gas phase 100 per cent. O_2
Initial glycogen 60 μg./mg.N

inhibition of oxygen uptake, glucose utilization, and lactate production; no effect on the endogenous glycogen level was apparent.

Phenylbutazone.—At the concentration used (150 μg./ml.), no effect on oxygen uptake, glucose utilization, or lactate production was evident, though a slight increase in glycogenolysis may have occurred.

Discussion
Steroid Effects

(a) Glycogen Metabolism.—Tissues are often capable of both glycogen synthesis and breakdown *in vitro*. This complicates the interpretation of the effect of added steroids. Hansen, Rutter, and Samuels (1951), using rat diaphragm, found that, when the initial glycogen values were greater than 1 μM/100 mg., the glycogen content of the tissue after incubation decreased in proportion to the amount originally present. When the glycogen level was less than 1 μM/100 mg., the glycogen content of the tissue increased upon incubation. Similar findings with rat diaphragm have also been reported by Beloff-Chain, Chain, Bovet, Pocchiari, Catanzaro, and Longinotti (1953). Rheumatoid synovial tissue has also given similar results (Page Thomas and Dingle, 1957). To investigate the effect of various steroids upon the glycogen content of rheumatoid synovial tissue, we chose a synovial tissue with an initial glycogen content of 67 μg./mg.N. At this level of tissue glycogen and in the presence of glucose 10 mM. little change in glycogen content occurred in the control flasks during incubation. Under these conditions, the addition of Compounds F, E, DOCA, S, and B caused a marked increase in glycogenolysis. In the absence of substrate, glycogenolysis occurred in the control flasks, and the presence of the steroids again increased the rate of glycogenolysis. Valentine, Follette, and Lawrence (1953) found that the glycogen content of human leucocytes was slightly lowered by Compound E. Verzár and Wenner (1948) investigated the inhibitory effect of DOC, A, and B on glycogen synthesis of rat diaphragm. These results were confirmed by Leupin and Verzár (1950), who showed that glycogenolysis was increased by DOC>B>E and F>A. Pletscher and von Planta (1954) have also shown that Compound E inhibits glycogen synthesis in rat diaphragm. Huisman (1953) was unable to show any effect of Compound E acetate on glycogen synthesis with the same tissue. Chiu (1950), however, using rat liver slices, found considerable glycogen synthesis to occur in the presence of DOC, A, and E. Apart from the results reported by Chiu on liver tissue, which may possibly reflect the response of a specialized organ, the majority of *in vitro* work appears to indicate that the corticosteroids tested all tend to diminish the level of tissue glycogen.

(b) Glucose Metabolism.—Rheumatoid synovial tissues were able to utilize more exogenous glucose than endogenous glycogen. This glucose utilization was increased under anaerobic conditions. In tissues with lower metabolic activity, where much smaller quantities of glucose were utilized (*e.g.* rheumatoid "membrane"), only Compounds B and F showed significant inhibition of glucose utilization. Indeed S and E may have stimulated glucose uptake. However, in more actively metabolizing tissue, only Compound F acetate showed no inhibitory action. Under anaerobic conditions Compounds B, S, and F still showed considerable inhibitory power. Leupin and Verzár (1950) have shown inhibition of glucose utilization of rat diaphragm by DOC. Using the same tissue, Pletscher and von Planta (1954) showed that Compound E had a similar effect. The glucose utilization of human placenta (Villee, 1953) and of human leucocytes (Martin, Chaudhuri, Green, and McKinney, 1954) *in vitro* was found to be again inhibited by Compound E.

(c) Lactate Production.—The inhibition of lactate production of rheumatoid villi and membrane by the various steroids tested, closely paralleled the results found for glucose utilization. Bacila and Barron (1954) found that Compounds F, E, and DOC inhibited the anaerobic glycolysis of mouse diaphragm. Huisman (1953), however, reported little or no effect with Compound E acetate on lactic acid production with the same tissue. Martin and others (1954), with human leucocytes, found that E and F were slightly inhibitory, whereas DOC and S were without effect upon glycolysis. Miller (1954), using rat thymus lymphocytes, found that, although DOC was inhibitory, Compounds E and F both stimulated glycolysis. It would appear, therefore, that *in vitro* experiments on the effect of corticosteroids upon glycolysis may give different results with different tissues. The same steroid may be apparently stimulatory or inhibitory depending upon the tissue in question. It is also possible that the state of the tissue may play a part in determining its metabolic response to the steroid. Thus, in actively metabolizing villous synovial tissues, all the steroids appeared to inhibit glycolysis, whereas in synovial membrane of much lower metabolic activity there is some evidence that stimulation may occur.

(d) Oxygen Consumption.—The importance of the increased oxidative metabolism of rheumatoid

synovia has been discussed elsewhere (Dingle and Page Thomas, 1956). It is evident that the steroids tested were more effective in inhibiting the oxygen uptake of the actively respiring villous tissues than the less active "fibrotic" villous and "membrane". The *in vitro* inhibitory effect of various cortico-steroids has been shown in a variety of tissues. Gordon, Bentinck, and Eisenberg (1951) found that DOC inhibited the respiration of rat brain homo-genates. Hayano and Dorfman (1951) reported that DOC inhibited the oxygen uptake of rat liver, kidney, and brain slices. Brichta and Parzer (1954) found that Compound E inhibited the oxygen uptake in lymphatic leukaemia cells. Compounds E and F were also found by Miller (1954) to inhibit the oxygen uptake of rat thymus lymphocytes. Tipton (1939) found that Compound B inhibited the oxygen uptake of brain slices. Kit and Barron (1953) showed that Compound F inhibited the oxygen consumption of rat and mouse spleen. Chiu (1950), however, was unable to demonstrate any effect of DOC, A, and E on the oxygen uptake of rat liver slices, and Martin (1954) was unable to show an inhibitory effect of E and F on human leucocytes. Huisman (1953) reported that Compound E slightly increased the oxygen consumption of rat diaphragm.

The above results with rheumatoid synovia give a qualitative impression of corticosteroid action upon some aspects of *in vitro* metabolism. It is not possible at this stage to give a quantitative com-parison of their *in vitro* action. There are three main reasons for this difficulty. Firstly the amount of steroid available to the cell is unknown, as the solubility of the various compounds differs widely. Secondly, recent unpublished observations have shown that the mode of presentation of the steroid to the tissue may give qualitatively and quanti-tatively dissimilar results. Thirdly, very little information is available to date on the degradation and/or interconversion of the various cortico-steroids by synovial tissues. Preliminary observa-tions have indicated that this may occur under our experimental conditions.

Hydroxybenzoic Acids

Meade (1954), who measured the effect of various hydroxybenzoic acids in the oxygen consumption of Wistar rats, found that ortho hydroxybenzoic acid stimulated oxygen uptake, and that the meta form was inhibitory, while the para compound had little effect. He postulated that the stimulatory action of o-hydroxybenzoic acid (salicylic acid) upon oxygen uptake might resemble that of the dinitrophenols which interfere with oxidative phosphorylation pro-cesses. This work was extended by Smith and Jeffrey (1956), who studied the effect of salicylate on oxygen consumption and carbohydrate metabolism in the isolated rat diaphragm. They found that salicylate 5×10^{-3}M always produced a marked increase in the rate of oxygen uptake of the diaphragms for the first 30 to 60 min. of the experi-ment, but that the rate subsequently declined, and had almost entirely ceased after 2 hours. Though our time intervals were of shorter duration with rheumatoid synovium, the effect, as shown in the Figure for O-hydroxybenzoic acid, is similar to their findings. The same authors also found that salicylate caused an increased glycogen breakdown and lactate accumulation under both aerobic and anaerobic conditions, as well as a decreased glucose uptake. We have been unable to show any com-parable effect on rheumatoid synovia with salicylate, though both the meta and para hydroxybenzoic acids gave inhibition of glucose utilization.

Insulin

Gemmill (1940) showed that insulin increased glycogen formation and glucose utilization in the isolated rat diaphragm. Verzár and Wenner (1948) showed that hydrocortisone inhibited glycogen formation in the presence of insulin. Our figures for synovial tissue serve to emphasize the antagon-istic effect of these two hormones.

It would appear, therefore, that the more meta-bolically active rheumatoid synovial tissues behave *in vitro* in a similar manner to other active animal and human tissues, and react readily to the presence of various hormones and therapeutic agents.

Summary

(1) The effects of Compounds B, S, E, F, and DOCA on some aspects of the *in vitro* metabolism of rheumatoid synovial tissue have been investigated.

(2) In general, these compounds had an inhibitory action upon both oxidation and glycolysis.

(3) Meta- and para-hydroxybenzoic acids were found to inhibit glucose utilization, lactate produc-tion, and oxygen uptake. Ortho-hydroxybenzoic acid, however, initially stimulated the rate of oxygen uptake which later fell off rapidly.

(4) Insulin was found to stimulate glycogen synthesis, glucose utilization, and oxygen uptake. These stimulatory effects were antagonized by hydrocortisone.

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Etudes IN VITRO sur la membrane synoviale
Effets de quelques agents thérapeutiques et de composés chimiquement apparentés
RÉSUMÉ

(1) Les effets des composés B, S, E, F et DOCA sur quelques aspects *in vitro* du métabolisme du tissu synovial rhumatismal on été étudiés.

(2) En général ces composés avaient une action inhibitrice à la fois sur l'oxydation et la glycolyse.

(3) Les acides méta- et para-hydroxybenzoïque inhibaient l'utilisation de glucose, la production de lactate et l'absorption d'oxygène. L'acide ortho-hydroxybenzoïque cependant, stimulait d'abord le taux d'absorption d'oxygène qui, plus tard, diminuait rapidement.

(4) L'insuline stimulait la synthèse de glycogène, l'utilisation de glucose et l'absorption d'oxygène. Ces effets stimulants étaient inhibés par l'hydrocortisone.

Investigaciones IN VITRO de la membrana sinovial
Efectos de algunos agentes terapéuticos y de compuestos químicamente afines
SUMARIO

(1) Se investigaron los efectos de los compuestos B, S, E, F y DOCA sobre algunos aspectos *in vitro* del metabolismo del tejido sinovial reumático.

(2) En general estos compuestos ejercían una acción inhibitoria tanto sobre la oxidación como sobre la glicolisis.

(3) Los ácidos meta- y para-hidroxibenzoico inhibían la utilización de glucosa, la producción de lactato, y la absorción de oxígeno. El ácido orto-hidroxibenzoico, sin embargo, primero estimulaba la absorción de oxígeno, que luego disminuía rapidamente.

(4) La insulina estimulaba la síntesis de glicógeno, la utilización de glucosa y la absorción de oxígeno. Estos efectos estimulantes se veían inhibidos por la hidrocortisona.