blood urea formation and decreased non-protein nitrogen excretion in the urine. It also produces an early and significant rise in blood lactic acid. From studies made on surviving slices of guinea pig liver, it is evident that PEDG increases lactic acid formation and increases glycogen depletion, but it does not affect glucose output. PEDG also exerts an effect on isolated rat diaphragm, increasing glucose uptake, increasing lactic acid production, decreasing glycogen formation, and decreasing oxygen uptake.

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Calcification XVIII. Lack of Correlation between Calcification *in vitro* and Glycolytic Enzymes.* (23387)

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Harris(1) reported that the glycogen present in preosseous cartilage played a role in calcification. Robison and Rosenheim(2) found that certain enzyme inhibitors such as iodoacetate, cyanide and fluoride caused inhibition of calcification *in vitro*. From these observations it was suggested that a phosphatase enzyme system in preosseous cartilage was responsible for calcification. Later, Gutman(3,4) stated that one of the major difficulties of the Robison postulate was its failure adequately to demonstrate the presence of significant amounts of phosphoric ester substrate at site of phosphatase activity in the hypertrophic cartilage. Gutman then presented the hypothesis that a phosphorylative enzyme initiated the degradation of glycogen to glucose-l-phosphate which then passed through the glycolytic cycle of hexose and triose phosphates to phosphopyruvate and that one of these phosphoric esters acted as substrate for the phosphatase present in the preosseous cartilage. In a study of the effect of glycolytic enzyme inhibitors on calcification *in vitro* it was concluded that calcification was directly dependent, in some way, on glycogenolysis. More recently, Albaum, Hirshfeld and Sobel(5) reported that most of the enzymes of glycolysis present in muscle and yeast appeared to be present in epiphyseal cartilage.

The aim of this investigation was to explore the relationship that may exist between calcification *in vitro* and the glycolytic enzymes. Our previous studies, utilizing an *in vitro* calcification technic, revealed that freezing(6), heating(7) and demineralizing(8) rachitic rat tibial slices evoked inactivation of the calcifying mechanism in preosseous cartilage which could be reactivated following appropriate treatment with $CaCl_2$.

In the case of the demineralized bone it was necessary to treat first with chondroitin

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sulfate or basal salt solution at a pH of 8.2 then with $CaCl_2$ to adequately reactivate the calcifying mechanism. In the present study, enzyme activity of the preosseous cartilage and *in vitro* calcification studies on similarly treated bones are reported.

Materials and methods. Twenty-five to 30 rats of an inbred Wistar strain were used in each experiment. The animals were weaned after 3 weeks and placed on a rachitogenic diet consisting of 75% degerminated corn meal, 19% wheat gluten, 2% brewer's yeast, 3% calcium carbonate and 1% sodium chloride. After 3 to 4 weeks on the diet, the animals were sacrificed, the tibiae excised and freed of soft tissue. The proximal ends (except in the case of the whole tibiae) were sliced longitudinally, in a frontal plane, into sections approximately 1 mm in thickness. A section from each animal was immediately tested to determine if healing of the rickets had taken place. Where the silver line test showed the slightest trace of healing, the animal was discarded. In addition, a daily check of weights and appearance of the animals was made and if an animal lost weight on 2 successive days, it was discarded.

In the *first experiment,* tibial slices from the left leg were placed without further treatment into a deep freeze chest at –25°C for 2 weeks. The slices from the right tibiae were shaken 1 hour in 150 meq/l of $CaCl_2$ solution at pH 7.2. The slices were rinsed with distilled water to remove excess Ca ions and then placed in the deep freeze chest for 2 weeks at –25°C. After this period of storage the cartilages were dissected out in the cold room and used for enzyme activity studies. Several of the untreated and Ca ion treated slices, both before and after deep freeze storage, were subjected to *in vitro* calcification studies.

In the *second experiment,* the whole tibiae from the left legs were placed in deep freeze chest for 24 days. The cartilages from the right tibiae were excised and immediately tested for enzyme activity. Two of the unfrozen tibiae were sliced and subjected to *in vitro* calcification. After 24 days the cartilages of the deep freeze stored bones were excised and studied for their enzyme activity, and simultaneously, 2 of the tibiae were sliced and used for *in vitro* calcification.

In the *third experiment,* slices for the left tibiae were shaken 1 hour in 150 meq/l of $CaCl_2$ at pH 7.2. The right tibial slices were shaken 1 hour in 150 meq/l of NaCl at pH 7.3. After rinsing with distilled water to remove excess of reagent, the slices were stored for 2 weeks in deep freeze at –25°C. The cartilages were removed and used for enzyme studies. Several slices, before and after deep freeze, were subjected to *in vitro* calcification.

In the *fourth experiment,* the cartilages of fresh, untreated tibial slices from the left legs were excised in the cold room. The slices from the right tibiae were shaken 1 hour in 150 meq/l of $CaCl_2$ at pH 7.2, rinsed in distilled water and the cartilages excised in the cold room. Both sets of cartilages were used for enzyme activity studies. Simultaneously, several treated and untreated slices were used for *in vitro* calcification.

In the *fifth experiment,* tibial slices were heated for 30 or 60 minutes in distilled water at 65°C in a thermostatically controlled water bath. One slice/10 ml of water in a 15 ml capacity pyrex test tube was used. Following this, the cartilages were excised and studied for enzyme activity. Several slices were used for *in vitro* calcification and fresh, unheated slices were used for controls.

In the *sixth experiment,* disodium ethylenediamine tetraacetic acid (EDTA) at pH 4 was used to demineralize tibial slices. Three, fresh portions of EDTA were added over a 48-hour period with intermittent agitation. Chemical analysis showed that all of the calcium had been removed from the bones. The slices were thoroughly washed with 0.04% acetic acid to remove the EDTA and divided into 4 portions. One portion, "A", was shaken slowly for 20 hours at 24-26°C in 1:10 basal salt solution (described in the second footnote) at pH 8.2. Portion "B" was shaken 20 hours in the basal salt solution, rinsed with distilled water at pH 7.4 and shaken 1 hour in 500 meq/l of $CaCl_2$ adjusted to pH 9 with dilute NaOH. Portion "C" was treated 18 hours at 5°C in 1% chondroitin sulfate solu-

tion† at pH 4, rinsed with distilled water at pH 7.4 and shaken 20 hours in the basal salt solution. Portion "D" received the same treatment as "C" and in addition was shaken 1 hour in 500 meq/l of $CaCl_2$ at pH 9. All slices were given a final rinse with distilled water. Several slices from each portion were used for *in vitro* calcification. Enzyme activity was determined on both the excised cartilages and the decalcified true bone tissues (epiphyseal trabeculae and diaphysis) of the "A" and "D" portions.

All *in vitro* calcification studies were carried out in an inorganic medium which was prepared by adding to a one liter volumetric flask, 100 ml of a stock basal salt solution,‡ 50 ml of a NaH_2PO_4 - Na_2HPO_4 solution that had a phosphorus concentration of 1 mg/ml and 700 ml of double, all glass, distilled water. The pH was lowered to about 6.3 by aerating with 100% CO_2. To this solution were added 6 ml of specially purified calcium chloride solution that had a Ca ion concentration of 20 mg/ml (1000 meq/l). Thus, the *in vitro* calcifying medium had a Ca x P product of 60, *i.e.*, 12 mg% Ca and 5 mg% P. Distilled water was added to the liter mark. The pH of final solution was adjusted to 7.3 by aerating with 100% nitrogen. A model G Beckman pH meter was used.

For the *in vitro* calcification process, a tibial slice was placed in a 50 ml Erlenmeyer flask filled with the calcifying medium at pH 7.3 $\pm$ 0.5. The flask was tightly stoppered with a paraffined cork and a rubber band that was stretched doubly over the cork and under the bottom of the flask. After incubation for 18 hours in a thermostatically controlled 37°C incubator, the slice was removed with forceps and rinsed in a stream of distilled water. It was placed in a concavity of a glass spot plate, covered with 3% silver nitrate solution, exposed to light from a 100 watt tungsten filament lamp for 5 minutes, and examined with a Leitz wide field stereobinocular microscope at a magnification of 32 X. Degree of calcification *in vitro* was evaluated by height and length of the silver stain obtained in the hypertrophic, provisional zone of calcification of the epiphyseal cartilage. The grading or evaluation has been described by Sobel(9).

Activity of the different glycolytic enzymes was measured on aqueous extracts of cartilage slices or on cartilage removed from whole bones. In a few instances shafts were employed. Approximately 250 mg of tissue was ground in cold water with a glass homogenizer. The extract so obtained was filtered through fine cheese cloth to remove material that could not be homogenized and made to final volume so that each ml of final extract contained 10 mg of tissue. The different enzymes were measured by adding their specific substrates to a system in which oxidation or reduction of DPN was used as indicator of activity. For example, for measurement of lactic dehydrogenase the test system contained 0.2 ml of 0.54 M sodium pyruvate, 0.1 ml of .002 M reduced DPN, 0.3 ml of tissue extract, 0.2 ml of 0.1 M ammonium phosphate buffer pH 7.6 and water to 3 ml. On addition of the pyruvate, which was added last, the cuvette was placed in the spectrophotometer previously set at 3400 Å and decrease in density due to oxidation of DPN was measured at 15 second intervals. Since pyruvate and DPN are present in excess, rate of oxidation of DPN becomes an index of lactic dehydrogenase present in the extract. By similar procedures, using the same buffer and a final volume of 3.0 ml, 0.1 ml of 0.25 M glucose in the presence of 0.2 ml of .0007 M ATP was used to measure hexokinase activity; 0.1 ml of .0058 M glucose-6-phosphate in place of the glucose was used to measure phosphohexoisomerase activity. The substitution of 0.1 ml of .0058 M fructose-6-phosphate in place of the glucose was used as an index of phosphofructokinase activity. Two-tenths of .00016 M hexose diphosphate in place of both ATP and glucose was used to measure aldolase activity.

In this last group of determinations changes in density were recorded at 5-minute inter-

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‡ The stock basal salt solution contained 0.7 M NaCl, 0.05 M KCl and 0.22 M $NaHCO_3$/liter. It was Berkefeld filtered before use and stored in a pyrex bottle.

TABLE I. Influence of Deep Freeze and Calcium Ions on Calcification *In Vitro* of the Epiphyseal Cartilage.

Treatment prior to calcification *in vitro*	Degree of calcification *in vitro* (Ca × P = 60) Mean
a. Tibial slices stored at −25°C for 2 wk	0 (0)
b. *Idem*; shaken 1 hr in 150 meq/l $CaCl_2$	1.0 (++++)
c. Tibial slices shaken 1 hr in 150 meq/l $CaCl_2$; deep freeze stored at −25°C for 2 wk	1.0 (+++)
d. Whole tibiae stored at −25°C for 24 days	0 (0)
e. *Idem*; shaken 1 hr in 150 meq/l $CaCl_2$	1.7 (+++)
f. Whole tibiae prior to deep freeze storage	2.0 (++++)
g. Tibial slices shaken 1 hr in 150 meq/l of NaCl	0 (0)
h. *Idem*; deep freeze stored at −25°C for 2 wk	0 (0)
i. Tibial slices after NaCl and deep freeze treatments; shaken 1 hr in 150 meq/l of $CaCl_2$	1.0 (++++)
j. Fresh tibial slices (not frozen)	1.5 (++++)
k. *Idem*, shaken 1 hr in 150 meq/l of $CaCl_2$	2.0 (++++)
l. Fresh tibial slices, shaken 1 hr in 150 meq/l of NaCl; shaken 1 hr in 150 meq/l of $CaCl_2$	1.3 (++++)

vals; the tables record calculated change in density occurring/10 minute interval. Operation of this test system is based on the assumption that the system contains a glycerophosphate dehydrogenase. That such a system occurs was demonstrated previously(5). To verify the presence of this latter enzyme a glycerophosphate itself was used as a substrate.

One final enzyme assay was carried out: phosphoglyceric acid was employed to determine enolase activity. Each cuvette contained .3 ml of tissue extract, .2 ml of .00085 M ADP, .2 ml of .016 M phosphoglyceric acid, .1 ml of .002 M reduced DPN, .2 ml of .1 M ammonium phosphate buffer pH 7.6 and water to 3 ml. In one control cuvette the substrate was eliminated. In a second control cuvette .3 ml of .1 M sodium fluoride was substituted for an equivalent quantity of water. In this test system the phosphoglyceric acid is converted into phosphopyruvic acid which then contributes its phosphate to ADP, forming pyruvate. In the presence of excess lactic dehydrogenase, known to be present in cartilage extracts, the pyruvate is converted to lactate with a consequent oxidation of DPN, measured as a decrease in density at 3400 Å. Enolase activity is presumed to be present only when the change in density in presence of substrate is in excess of that occurring in its absence, and when this increase may be abolished in the presence of fluoride. Changes in density were recorded at 5-minute intervals and the rate calculated on a 10-minute basis.

Results. From Table I it is apparent that deep freeze storage inactivates the calcifying mechanism in the epiphyseal cartilage as measured by the silver line test. However, this inactivation can be reversed or prevented in the presence of Ca ions(6). Metachromasia studies provided further evidence that inactivation took place during deep freeze storage. The procedural details and the method of evaluating the degree of metachromasia have been described(10). Briefly, the deep freeze stored slices showed practically no metachromasia when soaked for 18 hours at 25-26°C in a solution of toluidine blue O containing 39 mM of the dye/l of solution. When the toluidine blue O contained Ca ion, metachromasia became evident and reached a maximum at 10 meq/l and declined to 0 at 100 meq/l. Fresh untreated controls or $CaCl_2$ treated fresh or frozen sections showed some metachromasia even in the absence of Ca ion. In all cases, the metachromasia increased with Ca ion reaching the maximum at 10 meq/l and declining to 0 at 100 meq/l. The intensity of these at the maximum was more than that of the frozen bone.

TABLE II. Glycolytic Enzymes following Various Treatments That Affect Calcifiability of Hypertrophic Preosseous Cartilage.

	Change in density, 10 min. Preliminary treatment								
	Fresh untreated		Frozen§ control						
Substrate	Mean†	Range	Mean‡	Range	Fresh $CaCl_2$ treated	$CaCl_2$ treated then frozen	30 min. at 65°	EDTA‖	EDTA‖ + C.S.¶ + Ca ions
No substrate	3.3	1.5- 5.5	3.2	3.0- 3.4	.5, 1.2	.0, .0	.0	.0	.0
" " ATP	3.8	2.5- 5.5	3.8	3.3- 4.0	.5, 1.2	.0, .0	.0	.0	.0
Glucose	4.2	3.0- 6.2	4.5	4.0- 5.2	.5, 1.2	.0, 1.0	1.0	.0	.0
Glucose-6-P "	5.3	3.7- 7.0	4.9	4.2- 6.0	.5, 1.0	.0, .0	.0	.0	.0
Fructose-6-P "	6.5	4.5- 10.0	6.0	5.0- 7.0	1.0, 1.2	.0, .0	.0	.0	.0
Hexose diphosphate	33.2	10.0- 69.0	27.7	20.0- 42.0	.0, 1.0	.0, .0	.0	.0	.0
Glycerophosphate	19.8	7.5- 42.0	28.2	21.0- 36.0	.0, 1.0	.0, .0	.0	.0	.0
Phosphoglyceric acid	9.7	6.0- 16.0	9.3	7.0- 12.0	5.8, 1.8	2.0, 5.0	.0	.0	.0
Pyruvic acid*	158.3	120.0-187.0	161.3	140.0-180.0	63.0, 62.0	41.0, 57.0	.0	.0	.0

* 30 sec. change. † Mean—6 experiments. ‡ Mean—3 experiments. § "Frozen" refers to 2 wk in deep freeze at −25° ± 1.0°C.
‖ EDTA demineralized bone. ¶ "C.S." refers to chondroitin sulfate.

The absence of metachromasia without Ca ion in the frozen bone may be an index of the inactive form of the molecule responsible for initiating calcification *in vitro*.

As seen in Table II, deep freeze storage *per se* does not destroy the enzymes of the glycolytic system. Such sections (Table I (a)) nevertheless lose their ability to calcify *in vitro*. Those sections removed from the animal and shaken with calcium chloride prior to freezing have lost practically all of their enzymes but nonetheless retain the ability to calcify *in vitro* (Table I(c)). As shown in Table I(b) frozen sections which do not calcify *in vitro* and which appear to have an intact enzyme complement can be induced to calcify if they are shaken in calcium chloride solution after freezing. Control specimens, Table I(j,k), which were not frozen show a 1.5 (++++) *in vitro* calcification while the calcium chloride treated control slices show *2* (++++).

There is little change in enzyme activity after deep freeze storage (Table II). Yet the frozen tissue has lost the ability to calcify *in vitro* (Table I(d)). Prior to deep freeze storage, degree of *in vitro* calcification was *2* (++++). That the system retains calcifiability is shown by the observation (Table I (e)) that when frozen tissue is shaken for 1 hour in calcium chloride solution the mean degree of calcification is 1.7 (+++).

Most of the glycolytic enzymes were either destroyed or removed where bones were treated with $CaCl_2$ (Table II) or an equivalent amount of NaCl. However, a marked difference in the degree of *in vitro* calcification of these slices is apparent from data in Table I(c,h). In addition, the data indicate that calcium chloride was able to reactivate the sodium chloride inactivated slices. For example, the sections that were shaken in NaCl gave zero calcifications either before or after deep freeze treatment (Table I(g,h)). However, similarly treated sections when shaken for 1 hour in calcium chloride gave 1.3 (++++) and 1 (++++) *in vitro* calcifications (Table I(l,i)).

Table II shows the effect of calcium chloride shaking on enzyme activity of fresh carti-

TABLE III. Influence of Heating at 65°C on Calcification *In Vitro*.

Treatment	Degree of calcification *in vitro* (Ca × P = 60) Mean
Tibial slices heated 30 min. at 65°C	0 (0)
" " " 60 " " "	0 (0)
" " " 30 " ; shaken 1 hr in 150 meq/l of $CaCl_2$	1.0 (++)
" " " " " ; " " " 300 " " "	2.0 (+++)
" " " 60 " ; " " " 150 " " "	0 (0)
" " " " " ; " " " 300 " " "	1.7 (++)
Fresh, unheated slices	1.5 (++++)

lage. It is clear that most of the enzyme activity is lost by shaking in calcium chloride, yet ability to calcify *in vitro* is retained(7).

In Table III it may be seen that heating tibial slices in distilled water at 65°C for 30 or 60 minutes inactivates the calcifying mechanism of the preosseous cartilage. Robison *et al.*(2) had observed complete inactivation in 10 minutes at 60°C. However, this inactivation could be reversed to some extent with 150 milliequivalents of Ca^{++}/l or to a greater extent with 300 milliequivalents of Ca^{++}/l, and though the resulting *in vitro* calcification was not as marked as in the controls, it had the typical honeycombed structure precisely situated in the matrix of the hypertrophic epiphyseal cartilage. This reconfirmed the earlier work of Sobel and Hanok(7) on the reversible inactivation of calcification *in vitro*.

The effect of heating at 65° for 30 minutes on the enzymes of the cartilage tissue is shown in Table II along with data from fresh, untreated controls. It is obvious that heating even for 30 minutes destroys all traces of enzyme activity. Yet it is apparent from the data shown in Table II that such slices after shaking in calcium chloride still have some ability to calcify *in vitro*.

From Table IV it is apparent that EDTA treatment alone (portion "A") inactivated the calcifying mechanism. However, after $CaCl_2$ treatment (portion "B") a grayish, silver deposit was observed in the provisional zone of the epiphyseal cartilage which, though atypical, was situated in the calcifiable provisional zone of the tissue. Some stained areas in the epiphyseal trabeculae, in the metaphyseal osteoid and traces in the diaphysis were observed. The chondroitin sulfate treated slices (portion "C") showed inactivation while the $CaCl_2$ treated counterpart (portion "D") showed good reactivation with typical, honeycombed structure in the epiphyseal cartilage and positive stains in the osteoid, diaphysis and epiphyseal trabeculae. The enzyme studies, on the other hand, showed no activity in either the "A" or "D" portions.

Of interest is the recent *in vivo* experiment in which EDTA decalcified cortical bone of rats was broken down in a Waring blendor

TABLE IV. Influence of Chondroitin Sulfate and Calcium Chloride on Calcification *In Vitro* of Hypertrophic Epiphyseal Cartilage of EDTA Demineralized Tibial Slices.

Portion	Preliminary treatment	Degree of calcification *in vitro* (Ca × P = 60) Mean
"A"	20 hr in basal salt solution	0 (0)
"B"	*Idem*; shaken 1 hr in 500 meq/l $CaCl_2$	1.0 (+++)*
"C"	18 hr in 1% chondroitin sulfate; 20 hr in basal salt solution	0 (0)
"D"	*Idem*; shaken 1 hr in 500 meq/l of $CaCl_2$	2.5 (+++)†
	Fresh untreated controls	2.0 (++++)

* Grayish, atypical silver stain.

† Dense, dark gray silver stain; positive stains in diaphysis, epiphyseal trabeculae and metaphyseal osteoid.

and implanted in a trephined hole in the skull of a rat. A portion of the "blendorized" material was treated with chondroitin sulfate and then implanted in another trephined hole in the skull. After 6 weeks, the hole that received the chondroitin sulfate treated material showed excellent healing of the trephined area as compared to the decalcified, non-chondroitin sulfate treated material. Gross and microscopic examination showed no foreign body or other deleterious effects. X-ray shadow photographs confirmed the gross findings.

These experiments confirm the previous observations(8,10) that led to the postulate that a special structure of collagen which can be produced by chondroitin sulfate may be the essential part of the calcifying mechanism.

The results of EDTA treatment on the glycolytic enzymes in the cartilage and in the shafts and epiphyseal trabeculae are summarized in Table II. It is clear that treatment with EDTA and chondroitin sulfate has led to the destruction of all enzyme activity in both types of tissue under investigation. Nevertheless, calcifiability *in vitro* is retained.

Although the precise roles of phosphatase and the phosphorylative enzymes of the glycolytic cycle are still obscure, there is some histochemical evidence that, *in vivo,* they may be related in some way to preosseous cellular metabolism(11) and to the elaboration of the components of the matrix(12,13) that is calcifiable and that will, indeed, calcify provided sufficient amounts of calcium and phosphate ions are available in the extracellular fluids that bathe the matrix. It is also possible that the glycolytic cycle, in producing ATP, activates the mechanism responsible for the initiation of calcification(14).

From the present study it appears that, at least those enzymes investigated and therefore the glycolytic system as a whole, are not essential for *in vitro* calcification but that calcium ions are required to activate, reactivate or preserve the calcifying mechanism, which resides in collagen of special structure or the collagen-mucopolysaccharide moiety in the preosseous cartilage and in such configuration as to receive and react with the mineralizing ions to produce the bone salt, apatite. Several reports have appeared that are in harmony with this concept(8,15-22).

Summary. (1) Glycolytic enzymes and their relation to *in vitro* calcification of the preosseous cartilage of rachitic rat bone tissue have been studied. The findings indicate that although glycolytic enzymes are present in deep frozen tissue, ability to calcify *in vitro* is lost. However, if the tissue is treated first with calcium chloride and then stored in the deep freeze, the glycolytic enzymes are either absent or present only in trace amounts while the calcifying mechanism remains virtually intact. (2) Bone sections heated at 65°C and then treated with calcium chloride retain their ability to calcify *in vitro* despite the destruction of the glycolytic enzymes during the heating process. (3) Demineralization of bones results in a loss of glycolytic enzyme activity. However, when the demineralized bones are treated with chondroitin sulfate and then with calcium chloride, the calcifying mechanism is restored but not the glycolytic enzyme activity. (4) It is concluded that glycolytic enzymes are not part of the minimal system required for calcification.

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Stimulation of Vit. B_{12} Uptake in Tissue Slices by Intrinsic Factor Concentrate.* (23388)

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For several years we have held the view that intrinsic factor may influence absorption of vit. B_{12} by tissues other than the intestinal mucosa. Currently it is held that intrinsic factor is a mucopolypeptide of about 5,000 molecular weight(1). This size would not preclude the possibility of its being absorbed into the blood stream and hence becoming available for possible action on all tissues. However, no reports have yet appeared that demonstrate the effect of intrinsic factor on peripheral tissues.

Data are presented that demonstrate the stimulatory action of intrinsic factor on uptake of vit. B_{12} by slices of rat liver† or kidney *in vitro*. It appears that these observations may form the basis for an *in vitro* assay of intrinsic factor.

Materials and methods. Wistar strain, weanling rats were fed a diet of the following percentage composition: vitamin-free casein, 22; sucrose, 68.5; salts mixture (U.S.P. No. 2), 4.15; crisco, 4; iodinated casein, 0.04 or 0.05; L-cystine, 0.2; choline, 0.1. Each 100 g of diet contained α-tocopherol, 2.5 mg; menadione, 50 μg; vit. A. 75 I.U.; vit. D, 25 I.U.; riboflavin, 600 μg; thiamin, 400 μg; pyridoxine hydrochloride, 400 μg; calcium pantothenate, 1600 μg; niacin amide, 2000 μg; paraaminobenzoic acid, 10 mg; inositol, 20 mg; biotin, 10 μg; and folic acid 20 μg. In diets *B* and *D* vit. B_{12}, 2 μg/100 g diet was added. Iodinated casein was not added to diets *C* and *D*, (Table II). After 4 weeks on the diets the animals in groups A and C showed depressed growth rates and animals from all groups were ready for experiments. Rats were sacrificed by decapitation and the liver quickly removed and cooled on cracked ice. Slices were prepared 0.5 mm thick, blotted on filter paper, weighed on torsion balance before being placed in 7 ml Warburg flasks, and incubated at 37°C. Each flask contained a total volume of 3 ml of buffer pH 7.4 described by Hastings *et al.*(2). The radioactive vit. B_{12}, intrinsic factor concentrate‡ and other additives were made up in buffer solution. The system was equilibrated with gas (5% CO_2, 95% O_2) and vit. B_{12} tipped-in from the side arm after temperature equilibrium had been reached. After incubation, the slices were removed from the flask,

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† Preliminary report, *Fed. Proc.*, 1957, v16, 393.

‡ Intrinsic factor concentrate was prepared from hog mucosa and was supplied by Abbott Laboratories, North Chicago, Ill. This preparation had a biological oral dose potency of about 5 mg/day.