C^{14} activity would be subject to absorption and re-secretion several times before reaching the point of actual fecal excretion. This constant absorption and re-secretion of cholesterol and bile acids may form part of the mechanism controlling hepatic synthesis, esterification, and degradation of cholesterol.

An attempt was made in these studies to determine whether any gross differences in the nature of C^{14} metabolites excreted by the different groups could be demonstrated. It is recognized that the procedure used for separation of bile acids from cholesterol or other sterols does not result in completely separated fractions. Within the limitations imposed by this technic, no statistically significant differences were observed between the various groups. However, slightly greater amounts of petroleum ether extractable C^{14}-substances were found in feces of the groups fed hydrogenated cottonseed oil and corn oil than were found in the group fed coconut oil and these paralleled the amount of digitonin-precipitable C^{14} (cholesterol) in the petroleum ether fraction. Wilson and Siperstein(5) observed an increase in non-digitonin precipitable fecal sterols in rats fed corn oil compared to those receiving lard or a fat free diet.

Although animals of each group have been treated together in this discussion, the rats were killed at intervals from 30 to 76 days to determine if any trend (with time) could be observed in any of the measurements made. The most suggestive of these was the increasing amount of fecal C^{14} and of fecal cholesterol in the group fed hydrogenated cottonseed oil and to some extent in the other 2 groups.

Summary. Rats were maintained for 30 to 76 days on a purified diet containing 20% fat as hydrogenated cottonseed oil, corn oil, or coconut oil and then injected intravenously with a tracer dose of cholesterol-4-C^{14}. In the 6 days following injection higher excretion of C^{14} was observed in animals maintained on hydrogenated cottonseed oil. Of the activity extracted by petroleum ether after saponification, a lower percentage was present in the digitonin-precipitable fraction in animals fed coconut oil. Smaller amounts of cholesterol were excreted by the coconut oil-fed rats. No consistent differences were observed in total C^{14} activity of various organs of the different dietary groups at any time period. Neither serum cholesterol nor serum C^{14} activity was affected by variation of the type of dietary fat given these rats.

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Multiple Forms of Glutamic-Oxalacetic Transaminase in Tissues. (27975)

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Glutamic-oxalacetic transaminase (GOT) is an enzyme which is widely distributed in both animal and plant tissues(1) and is of considerable significance due to its role in glutamate oxidation(2). In addition, the elevation in serum levels of this transaminase in certain disease states(1) makes the study of this enzyme of particular importance. Recently,

interest in multiple molecular forms of enzymes has become widespread and more than 30 enzymes have been shown to exist in these multiple forms within individual organisms (3).

It has been reported that 2 forms of GOT could be separated by electrophoresis of liver and heart extracts on paper(4), of rat liver and serum in agar gel(5) and from sera by column chromatography(6). In general, starch gel electrophoresis has a greater ability to resolve proteins than these other methods. We decided to employ this technic to investigate the possible existence of additional isozymes of GOT and to obtain information concerning the specificity of the isozymes for a particular organ. Detection of GOT activity on starch gel by available methods was unsatisfactory and an improved staining procedure for detecting enzymatic activity has been developed.

Methods. Male Sprague-Dawley rats (Holzman Co., Madison, Wis.) weighing 300-330 g were sacrificed, the tissues were removed and used immediately or quickly frozen and stored at −20°C. The weighed tissues were homogenized in a Duall homogenizer (Kontes Glass Co., Vineland, N. J.) in 9 volumes of 0.1 M Tris buffer pH 7.4 and the whole homogenate was designated as Fraction I. Further fractionation was obtained by centrifuging the whole homogenate for 30 minutes at 15,500 × *g* in the cold and reserving the supernatant (Fraction II). In some experiments a portion of the whole homogenate (Fraction I) was subjected to sonic degradation in a Raytheon sonicator for 30 minutes. This portion is referred to as sonicated homogenate (Fraction III). A portion of the sonicated homogenate was centrifuged at 15,500 × *g* and the sonicated supernatant designated as Fraction IV.

To protect the enzyme during electrophoresis, the fractions were diluted with bovine albumin* to a final concentration of 50 mg albumin/ml of extract. The gel was prepared with hydrolyzed potato starch (Connaught Med. Research Laboratories, Toronto) and a 0.025 M boric-acid-0.010 M NaOH buffer, pH 8.9. Vertical electrophoresis was employed at 12°C for 22 hours, voltage 4.8 V/cm with the 0.30 M boric acid-0.06 M NaOH, pH 8.2, bridge buffer described by Smithies(7). The slits were placed about one-third of the distance from the end of the gel and the electrodes were reversed in order to permit better visualization of the cathodic GOT enzymes which travel faster than the anodic portions in the buffer system described.

Stain technic. Reagents. Prepare the following aqueous solutions: (1) phosphate buffer, 0.2 M pH 7.4, (2) pyridoxal-5-phosphate, 500 μg/ml, (3) bovine albumin,* 30 mg/ml, (4) L-aspartic acid, 0.2 M adjusted to pH 7.4 with KOH, and (5) alpha-ketoglutaric acid, 0.1 M, adjusted to pH 7.4 with KOH. Pyridoxal-5-phosphate solution is stored frozen, the other solutions are kept in the refrigerator. Prepare the following within one hour of use: Solution A. *Diazonium salt solution.* Dissolve 50 mg of the diazonium salt, 6-benzamido-4-methoxy m-toluidine diazonium chloride† in 1.7 ml water. Solution B. *Substrate mixture.* Combine and mix thoroughly: 0.75 g polyvinylpyrrolidone,‡ 5.3 ml phosphate buffer, 0.2 ml pyridoxal-5-phosphate, 0.4 ml bovine albumin, 1.7 ml L-aspartic acid and 0.7 ml alpha-ketoglutaric acid. Solution C. *Blank mixture.* The mixture is prepared with the same ingredients as solution B, except that L-aspartic acid is omitted and 1.7 ml water is substituted.

Slice the starch gel horizontally in the usual manner and cut a piece of chromatography grade Whatman #1 paper the same size as the gel slice. Place the paper carefully over the cut gel surface so that no air spaces are trapped between the gel and the paper. Combine diazo solution A and substrate solution B and apply the mixture immediately to the paper. This volume of solution (approximately 10 ml) is sufficient to stain 2 gels, each 13 × 24 cm. The same procedure is followed for the blank gel, except that solution A is

* Bovine albumin powder (Fraction V from bovine plasma), Armour Pharmaceutical Co., Kankakee, Ill.

† Generous quantities of the 50 mg tablets were obtained through the courtesy of Dr. Arthur L. Babson, Warner-Lambert Research Institute, Morris Plains, N. J. The diazonium salt is also available from Alliance Color and Chemical Co., Newark, N. J.

‡ PVP, M. W. 40,000, Mann Research Laboratories, New York.

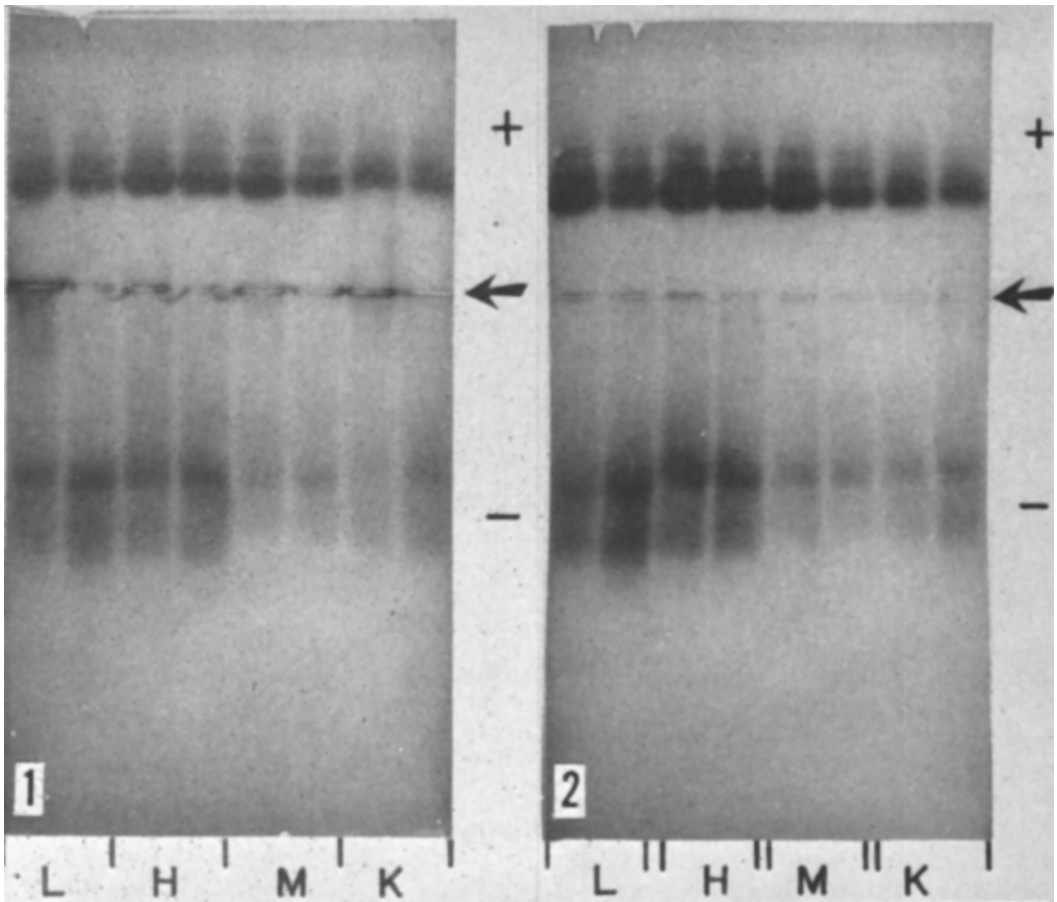

FIG. 1. Electrophoretic patterns of GOT in homogenate. From left to right in each set, first channel contains homogenate (Fraction I) and second channel contains sonicated-homogenate (Fraction III) of liver, heart, muscle and kidney.

FIG. 2. Electrophoretic patterns of GOT in supernatant. From left to right, in each set, first channel contains supernatant (Fraction II) and second channel contains sonicated-supernatant (Fraction IV) of liver, heart, muscle and kidney.

mixed with solution C (no aspartic acid is present). The gels are then incubated at 37° C. In the presence of the substrate mixture, pinkish-red color appears at the site of GOT activity; this color is usually visible within 10 minutes and the bands become most distinct at about 20 minutes, depending on enzyme concentration. As soon as the bands are clearly developed, discontinue the incubation and carefully strip the paper from the gel. Since colored bands develop on both paper and gel, the paper can be dried (50°C oven) thus providing a permanent record. After incubation, one may fix the stained zones by immersing the gel in a 5% acetic acid solution for a few minutes. If the stained gel is not fixed, it must be photographed immediately since the color tends to diffuse rapidly in the developed gel.

Results and discussion. A new staining method was devised utilizing a diazonium salt which couples directly with oxalacetate(8), a product of the transaminase reaction. This procedure is a direct, one-step stain, the areas of enzymatic activity are pink-red color, readily visible, and the progress of the reaction may be observed during the incubation period. Starch gels shown in Fig. 1, 2 and 3 were stained with the diazonium-substrate mixture. The other half of the starch gel was incubated with the blank mixture (solutions A and C), and, in no case was there any portion which

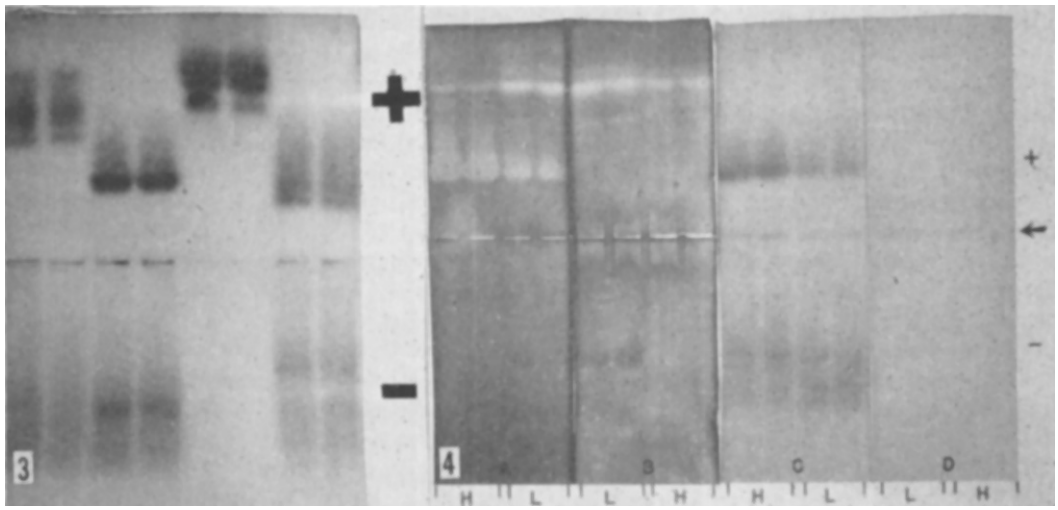

FIG. 3. Rate of migration of GOT from heart extracts of various species. From left to right (in duplicate) are homogenates of human heart, rat heart, purified enzyme from pig heart and homogenate of dog heart.

FIG. 4. Comparison of starch gels stained for GOT enzyme activity. Section A is stained according to Boyde's tetrazolium method; B is the blank. C is stained by the newly described diazonium method and D is the blank. Duplicate channels were inoculated with supernatants (Fraction II) of rat heart (H) and liver (L).

stained in the absence of aspartic acid, indicating that the stain method is specific for GOT activity.

Bovine albumin was routinely added to the extracts to stabilize the enzymes during electrophoresis. The added albumin also prevented the curving of the pattern which is sometimes observed when one is dealing with tissue extracts of varying low protein concentrations. The added albumin did not influence the number or mobility of the isozymes since enzyme activity was found in the same location when albumin was omitted. However, in the absence of albumin, the bands were less intense particularly on the cathode side, indicating loss or denaturation of the enzyme.

After electrophoresis, the enzyme bands were found in the same location whether Tris homogenates of fresh or frozen tissues were used. Therefore, only the results obtained with fresh tissues are presented in the first two figures. Rat liver, heart and muscle extracts show 3 distinct GOT bands migrating toward the anode and one or more bands migrating toward the cathode (Fig. 1). It would appear that the third anode band (the band furthest from the slit) is missing in rat kidney although the possibility that its apparent absence is due to a low concentration of that particular isozyme in kidney has not been eliminated.

There was some enzymatic activity in the whole homogenate (Fraction I) which did not move away from the slit (Fig. 1). Sonicated whole homogenate (Fraction III) shows little or no enzymatic activity at point of application and the cathodic portion of the Fraction III bands are more intense than the corresponding bands for the non-sonicated homogenate. In addition, spectrophotometric assay by the Karmen assay(9) showed that the sonicated fractions had a greater GOT activity than the non-sonicated material. It would appear that sonication releases additional enzyme, probably particle-bound GOT (10,11).

Electrophoresis of the supernatant (Fig. 2) resulted in the same bands as the whole homogenate except that there was no enzyme remaining at the slit. It should be noted that no attempt was made to maintain the integrity of the mitochondria during the extraction procedure used, and the supernatant fraction undoubtedly contained material released by disruption of the mitochondria. Experiments in progress on cellular fractionation by conventional methods should clarify this point.

Fig. 3 demonstrates the differences in rate of migration of GOT obtained from several species. The gel was inoculated with Tris supernatants of dog, rat and human (autopsy material) heart and commercially available purified transaminase. Purified GOT from pig heart§ has the fastest rate of migration in the anode direction and 4 anode bands can be seen; no cathodic portion was detectable. However, the cathode-GOT is considerably more labile than the anode-GOT and cathodic-GOT may have been denatured or eliminated in the process of enzyme purification. GOT from human heart migrates slightly slower than the pig enzyme; there are 3 anode bands, the cathode region contains at least one band. Dog heart has at least 2 anodic bands and one cathode band which migrate at the slowest rate of the 4 species reported here. The enzyme from rat heart migrates at a distance intermediate between dog and human GOT.

Boyd(5) proposed a photographic technic for detection of GOT in agar gel. Boyde and Latner(12) devised a 2-step tetrazolium method for starch gel. After an incubation of up to 4 hours in the Karmen assay reagents, the gel was incubated in a solution containing the tetrazolium salt MTT and N-methylphenazonium methosulfate. Transaminase zones are indicated by pale yellow areas due to the absence of the formazan and the background is violet.

Fig. 4 demonstrates the differences obtained with the tetrazolium method(12) and the diazonium method described here. The 4 sections are from the same starch gel. Section A was stained with the tetrazolium method; the anode GOT bands were not well differentiated and no GOT bands could be seen in the cathode area. A second section, B, served as a blank and was incubated in the same reagents as section A except that aspartic acid was omitted. There were several stained areas in the anode portion of the blank B probably due to reaction with glutamic dehydrogenase or "Nothing dehydrogenase"(13). These stained areas were also present on the substrate-incubated gel A. The use of the diazonium stain on section C permitted visualization of distinct bands of enzyme activity in the anode and cathode areas and there were no stained areas on the blank D. Eluates of serial sections of the gel were analyzed for enzyme activity by the Karmen spectrophotometric method(9). GOT activity was found only in eluates of areas corresponding to the diazo stained bands.

A greater number of GOT isozymes have been demonstrated by use of starch gel electrophoresis and an improved staining technic than had been reported by other investigators. The diversity in electrophoretic mobility of these enzyme proteins from several species suggests molecular variations similar to those reported for isozymes of alkaline phosphatase, peroxidase and esterases(14). It remains to be determined whether the heterogeneity may be due to differences in content of sialic acid or other residues attached to the proteins(15), to differences in amino acid composition, or to other factors.

Summary. Three anionic and one or more cationic isozymes exhibiting glutamic-oxalacetic transaminase activity have been separated by starch gel electrophoresis of rat liver, heart and muscle extracts. The fastest migrating, third, anodic band does not appear to be present in rat kidney. Four anodic bands were found in a purified enzyme preparation from pig heart. There are species differences in rate of migration of the isozymes which suggest structural differences in the enzyme proteins from the various sources. A direct, one-step, stain procedure, using a diazonium salt which couples specifically with oxalacetate, is described for detection of the transaminase on starch gel.

§ Glutamic oxalacetic transaminase from pig heart, analytical reagent grade, suspended in 3.0 M ammonium sulfate-ketoglutarate-maleate solution, C. F. Boehringer and Soehne, G.m.b.H., Mannheim, Germany.

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Agglutinogens and Isoagglutinins in Caries-Immune and Susceptible Human Salivas.* (27976)

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Putkonen(1) reported the presence of isoagglutinins and isoagglutinogens in human saliva. Many authors have since reported the presence of blood group substances (agglutinogens) in human saliva and other body fluids. Schiff and Sasaki(2) found that ability to secrete blood group substances is dependent on the Mendelian Law. More recently, Moharram(3) reported the presence of group specific agglutinins in human saliva but did not discuss it in detail.

The purpose of this study was to compare dental caries-immune and susceptible individuals (as classified by Weisenstein and Green)(4) according to blood type, ability to secrete agglutinogens, and ability to secrete isoagglutinins. It was considered that this work might indicate if immunity to caries is linked to any blood group characteristics.

Materials and methods. Whole paraffin-stimulated saliva was collected from 12 caries-immune and 16 caries-susceptible individuals and parotid saliva from some of each group. Blood types were determined by the slide test technic. Whole saliva was placed in the refrigerator after collection allowing the mucin to precipitate. The saliva was then centrifuged and the supernatant divided into 2 portions, one of which was boiled, and both were frozen. Parotid saliva was collected with a modified Carlson-Crittenden device(5) and immediately frozen.

Agglutinogens. Secretors of agglutinogens were separated from non-secretors by the *Ulex europeus* seed extract technic described by Boyd and Shapleigh(6). One-tenth ml of whole stimulated boiled saliva of various dilutions was mixed with 0.1 ml (2 units) of *Ulex* extract in 75 × 8 mm test tubes. (A 1:16 dilution of *Ulex* extract usually caused agglutination of type O RBC's; a 1:8 was considered 2 agglutinating units.) The tubes were incubated for 30 min at room temperature and 0.1 ml of washed 1% type O RBC's was added and mixed. After 1 hr the tubes were examined for hemagglutination inhibition. All hemagglutination inhibition tests were read by the Salk pattern method and then gently tapped to detect very strong agglutination which forms a button resembling a negative Salk pattern. Inhibition was considered to have occurred only when both methods were negative.

Secretion of A and B agglutinogens was checked in a similar manner using anti-A and anti-B blood grouping sera. Those individuals found to be secretors by the *Ulex* method and showing no A or B agglutinogens were considered to be type O secretors.

Isoagglutinins. A combination of hemagglutination and hemagglutination inhibition technics was used to detect isoagglutinins in saliva. One-tenth ml of saliva (not boiled)

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